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Dinaciclib Induces Anaphase Catastrophe in Lung Cancer Cells via Inhibition of Cyclin Dependent Kinases 1 and 2

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Abstract

Despite advances in targeted therapy, lung cancer remains the most common cause of cancer-related mortality in the United States. Chromosomal instability is a prominent feature in lung cancer and because it rarely occurs in normal cells, it represents a potential therapeutic target. Our prior work discovered that lung cancer cells undergo anaphase catastrophe in response to inhibition of cyclin-dependent kinase 2 (CDK2), followed by apoptosis and reduced growth. In this study, the effects and mechanisms of the multi-CDK inhibitor dinaciclib on lung cancer cells were investigated. We sought to determine the specificity of CDK-dependent induction of anaphase catastrophe. Live cell imaging provided direct evidence that dinaciclib caused multipolar cell divisions resulting in extensive chromosome missegregation. Genetic knockdown of dinaciclib CDK targets revealed that repression of CDK2 and CDK1, but not CDK5 or CDK9 triggered anaphase catastrophe in lung cancer cells. Overexpression of CP110, which is a mediator of CDK2 inhibitor-induced anaphase catastrophe (and a CDK1 and 2 phosphorylation substrate),

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antagonized anaphase catastrophe and apoptosis following dinaciclib treatment. Consistent with our previous findings, acquisition of activated *KRAS* sensitized lung cancer cells to dinaciclib-mediated anaphase catastrophe and cell death. Combining dinaciclib with the mitotic inhibitor Taxol augmented anaphase catastrophe induction and reduced cell viability of lung cancer cells. Thus, the multi-CDK inhibitor dinaciclib causes anaphase catastrophe in lung cancer cells and should be investigated as a potential therapeutic for wild-type and *KRAS* mutant lung cancer, individually or in combination with taxanes.

Keywords

dinaciclib; cyclin-dependent kinase; anaphase catastrophe; lung cancer; *KRAS*

Introduction

Cyclin-dependent kinases (CDK) regulate the cell cycle and are responsible for its orderly progression (1). To become catalytically active, CDKs associate with specific cyclins during the different phases of the cell cycle. CDK4/6-cyclin D and CDK2/cyclin E complexes sequentially phosphorylate the retinoblastoma (Rb) protein leading to its inactivation, thus facilitating progression through the G₁/S checkpoint (1). CDK1 and its partners cyclins A and B then ensure G₂/M phase transition (1). In cancer cells, diverse genetic and epigenetic events result in over-expression of cyclins, constitutive activation of CDKs, loss of CDK inhibitors (such as p27 and p16), and mutations of the retinoblastoma protein (1, 2). These events lead to cell cycle deregulation and confer a selective growth advantage to cancer cells. CDK activity is not restricted to cell cycle proteins. CDK7/cyclin H and CDK9/cyclin T promote phosphorylation of the carboxy-terminal domain of RNA polymerase II, facilitating initiation and elongation of RNA transcription, respectively (3, 4). Inhibitors of CDK activity have been developed and are undergoing evaluation as anticancer treatments. CDK inhibitors that act on CDK1, 2 and 9 (5–7) or CDK4/6 (8) have shown preclinical antitumor activities. In mouse knockout studies, there is functional redundancy between CDK2, 4 and 6, but not CDK1 (9–12). It is not clear with current CDK inhibitors what is or are the dominant targets of these multi-CDK inhibitors.

CDK2 and cyclin E are aberrantly expressed and confer unfavorable prognosis in non-small cell lung cancer (NSCLC) (13, 14). Our prior work provided direct evidence for the importance of cyclin E in lung carcinogenesis (15). Transgenic mouse models were engineered with surfactant C–targeted cyclin E expression in the lung (15). This conferred chromosomal instability and caused lung cancers to form in mice with tumors recapitulating key features of human lung carcinogenesis (15). Furthermore, using lung cancer cells derived from this model, CDK2 inhibition was found to trigger anaphase catastrophe, a lethal event where cells with supernumerary centrosomes segregate chromosomes into more than two daughter cells, resulting in non-viable daughter cells (16–18). In this study, the mechanism of anaphase catastrophe induced by CDK inhibition was further investigated using the novel multi-CDK inhibitor dinaciclib.

It has been postulated that by simultaneously targeting multiple CDKs involved in both the cell cycle and transcription, the drug potency would become enhanced (5, 19). Dinaciclib inhibits CDK1, 2, 5 and 9 with an IC_{50} of 3, 1, 1 and 4 nM, respectively (20). Dinaciclib has exhibited pre-clinical activity in multiple tumors, including pancreatic cancer (6), melanoma (21), and B-cell malignancies (22). In this current study, it is shown that dinaciclib induces anaphase catastrophe in lung cancer cells via inhibition of CDK1 and CDK2, but not of CDK5 or CDK9. Activated *KRAS* mutations sensitized lung cancer cells to dinaciclib-mediated anaphase catastrophe and cell death. Activated *KRAS* mutations are known to enhance resistance to chemotherapy including tyrosine kinase inhibitors (23), however, dinaciclib was similarly effective as an anti-proliferative agent in wild-type and mutant *KRAS* lung cancer cell lines. Finally, when combined with the anti-microtubule agent and mitotic inhibitor Taxol, the effects of dinaciclib were enhanced in NSCLC cell lines. In summary, these data provide a strong rationale to study further multi-CDK inhibitors, including dinaciclib, as potential therapeutic agents for lung cancers. This is especially proposed for NSCLC cases harboring activated *KRAS* mutations.

Materials and Methods

Cell culture and drugs

ED1 murine lung cancer cell line was derived from transgenic mice harboring lung cancers expressing human surfactant C-driven wild-type cyclin E in 2007 (15). Human lung cancer cell lines (HOP62, H522, H23, H1299 and H1703) were obtained from American Type Culture Collection (Manassas, VA) in 2010. Each cell line was briefly cultured and frozen in liquid nitrogen and only early passages (less than two months of passage) of each cell line were used in these experiments. Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in 5% CO₂ in a humidified incubator. Dinaciclib (SCH727965) was provided by Merck Research Laboratories (Palo Alto, CA). Taxol was obtained from LC Laboratories (Woburn, MA). Dimethyl sulfoxide 10 mmol stock solutions were prepared for each agent and stored at -20°C.

Cell viability and proliferation assay

Apoptosis was measured in duplicate using the ApoScreen Annexin V apoptosis kit, as previously described (24). Briefly, cells were trypsinized, washed in PBS and resuspended in 150 μ l of Annexin V binding buffer; 1 μ l of Annexin V-PE and 1 μ l of 7-AAD (Southern Biotech, Birmingham, AL) were added. Cells were incubated for 15 minutes protected from light on ice, followed by flow cytometry analysis on FACSCalibur (Becton Dickinson, Palo Alto, CA). To determine cell proliferative activity, cells were plated in 96-well plates (3000/well in 100 μ l, 6 wells per sample) and treated with drugs the next day. After 48 hours of culture, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added at a final concentration of 0.55 mg/ml. Acid isopropanol was added after 4 hours and absorbance at 570 nm was measured using an EMax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA).

Immunoblot analyses

Cells were lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Na₃PO₄, 1 mM NaVO₃, 1 mM EDTA, 1 mM EGTA, supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mM PMSF). Proteins were analyzed by immunoblotting, as described (24). The following antibodies were used: cleaved PARP, survivin, CDK1, CDK2, phospho-Rb^{S780} (Cell Signaling, Danvers, MA), phospho-Rb^{T821} (Life Technologies, Carlsbad, CA), Rb (C-15), CP110 (N-14), K-Ras (F234; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-RNA Polymerase II ser-2 (H5) and ser-5 (H14), total RNA polymerase (8WG16, all from Covance, Dedham, MA), β -actin (Sigma, St. Louis, MO), and horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies (Bio-Rad, Hercules, CA).

siRNA-mediated gene silencing and transfections

The siRNA-mediated gene silencing in lung cancer cells was performed using Lipofectamine 2000 Plus (Life Technologies). The siRNA oligonucleotides targeting CDK1 (CDK1.1, sense strand 5'-GGAACUUCGUCAUCCAAUAGTC-3', CDK1.2 sense strand 5'-GACUAACUAUGGAAGAUUACCAA-3'), CDK2 (CDK2.1, sense strand 5'-ACAAGAGCGAGAGGUAUACUGCGTT-3', CDK2.2 sense strand 5'-GCCACAAUGUUUAAAGGCCAAAT-3'), CDK5 (CDK5.1, sense strand 5'-GCCAGACUAUAAGCCCUAUCCGATG-3' and CDK5.2, sense strand 5'-GCGUAUCUCAGCAGAAGAGGCCCTG-3') were synthesized by Integrated DNA technologies (Coralville, IA); CDK9 (CDK9.1 sense strand 5'-GGUGCUGAUGGAAAACGAG-3', CDK9.2 sense strand 5'-GGAGAAUUUACUGUGUUU-3') were from Ambion/Life Technologies. For enforced expression, pCDEF3-CP110 plasmid was obtained from Dr. Brian David Dynlacht (25), pcDNA-CDK1 and pcDNA-CDK2 plasmids and vector control (pcDNA) were purchased from Addgene (Cambridge, MA). Lung cancer cells were transfected using Xtreme Gene 9 transfection reagent according to the manufacturer's protocol (Roche, Indianapolis, IN).

Reverse transcription polymerase chain reaction (RT-PCR) assays

Total RNA from cells was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized from 500 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative real time PCR (qRT-PCR) assays were performed in a C1000 Thermal Cycler (Bio-Rad) using Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems, Foster City, CA), with template cDNA and gene specific probes. The following Taqman probes were used: *CDK1*: Hs00938777_m1; *CDK2*: Hs01548894_m1; *CDK9*: Hs00977896_g1. Amplification of the sequence of interest was compared to a reference probe (*RPS18*, #4308329; all from Life Technologies). All samples were analyzed in duplicate. We used the comparative Ct method for relative quantitation (2^{-Ct} , where $Ct = C_{tp} - C_{tk}$; P=Probe and K=reference sample). For CDK5 mRNA quantification, total RNA was isolated from cells using the RNA Easy Kit (Invitrogen). Reverse transcription (RT) was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with a Peltier Thermal Cycler (MJ Research). Quantitative real-time PCR assays were done using SYBR Green PCR Master Mix (Applied Biosystems) and

the 7500 Fast Real time PCR System (Applied Biosystems) for quantitation. RT-PCR assays were conducted following the manufacturer's protocol (Applied Biosystems). Three replicate experiments were done. Primers sequences were human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-ACGTGTCAGTCAGTGGTGGACCT-3' and reverse 5'-GTCCACCACCCTGTTGCTG-3' and human CDK5 forward 5'-TCTGTGACCAGGACCTGA-3' and reverse 5'-GCACATTGCGGCTATGAC-3' sequences.

Chromosome stability assay

Cells were fixed in 10% formalin, stained with anti- α -tubulin-specific antibody (Sigma Aldrich) and independently mounted with Pro-Long Gold antifade reagent supplemented with 4',6-diamidino-2-phenylindole (Life Technologies). Fluorescent images were captured with an F-view II monochrome camera (Olympus, U-CMAD3) mounted on an Olympus BX51 microscope. Total anaphase cells were counted and those that contained three or more spindle poles were scored as multipolar.

Live cell imaging

HOP62 and H1299 cells were plated on glass coverslips in complete RPMI media and treated with 25–50 nM dinaciclib or vehicle control for 24 hours prior to live cell imaging. For live cell imaging, cells were placed in a modified chamber and maintained at 37°C in complete RPMI media and 25–50nM dinaciclib. DIC images were acquired with a Nikon Eclipse Ti microscope and an Andor cooled CCD camera using a 60 $\times$ 1.4NA oil immersion objective. 11 z-axis optical sections of 1 μ m were acquired at 10min intervals for a total of 25 hours (H1299) and 13hr 40min (HOP62). Images were processed using Nikon Elements and Adobe Photoshop software. DIC images represent the most in focus single focal plane at each time point.

Generation of stable KRAS expressing transfectants

Either pCGN KRAS^{12V,188L} expressing (Addgene) or an empty vector were transfected into ED1 cells concomitantly with pPUR plasmid (Clontech) using Lipofectamine 2000 as per the manufacturer's protocol. KRAS-transfected pools were selected with puromycin beginning 24 hours after transfection. Engineered expression of the KRAS was confirmed by immunoblotting.

High throughput proliferation assays

Logarithmically-growing cells were seeded at optimized densities for each line into 384 well tissue culture plates in triplicate. Proliferation assays were performed using the CellTiter-Glo Luminescent Assay (Promega) following 72 hours exposure to serial-fold drug dilutions. IC50 and IC70 values were estimated using the drexplorer R package.

Statistical analysis

Results of individual experiments were analyzed using paired and unpaired Student's *t*-test, Fisher Exact test, non-parametric Mann-Whitney test and Spearman *r*. Statistical analyses were completed using the GraphPad Prism 6.07 software package (GraphPad Software, Inc.,

La Jolla, CA). All tests were two-sided, and data were considered to be statistically significant when $P < 0.05$

Results

Dinaciclib induces multipolar cell division in lung cancer cells

Our previous work reported that pharmacologic inhibition of CDK2 with seliciclib led to anaphase catastrophe in murine and human lung cancer cell lines (16, 26, 27). The effect of the multi-CDK inhibitor dinaciclib in inducing apoptosis and anaphase catastrophe in lung cancer cells was investigated. Apoptosis was induced in a dose-dependent manner in ED-1 cells as compared to vehicle-treated cells (Fig. 1A). Apoptosis induction was accompanied by anaphase catastrophe induction (Fig. 1B; Supplementary Fig. 1). At dinaciclib doses of 100 nM or higher, the mitotic index was lower likely due to inhibition of transcription via CDK9 inhibition (data not shown).

Using real-time live cell imaging techniques, we followed the fate of individual human lung cancer cells undergoing chromosome missegregation induced by dinaciclib treatment. As shown in Fig. 1C (videos are provided in Supplementary Fig. 2), H1299 and HOP62 lung cancer cell lines underwent multipolar anaphase followed by multipolar cell divisions when treated with dinaciclib (25 and 50 nM). While this event was shown to be incompatible with survival (18), apoptosis of daughter cells of multipolar cell divisions may be delayed by 12–72 hours upon completion of a multipolar division or occur following one additional mitotic division (18). Decreased cell metabolic activity as measured by the MTT assay was detected in lung cancer cell lines following 24 hours of dinaciclib treatment (Fig. 1D).

Anaphase catastrophe is mediated via CDK1 and CDK2 inhibition and CP110

Since dinaciclib potently inhibits CDK1, CDK5 and CDK9, in addition to CDK2, *in vitro* (20), the consequences of ablation of the individual CDKs on anaphase catastrophe induction were assessed in lung cancer cells. Knockdown of individual CDKs was confirmed by RT-PCR assays (Fig. 2A). Knockdown of CDK2 resulted in multipolar anaphases in H1299 and HOP62 cells, confirming our earlier observations (Fig. 2B). Notably, knockdown of CDK1 also led to an increase in multipolar anaphases in both cell lines (Fig. 2B). Finally, knockdown of CDK5 or CDK9 did not have an appreciable effect on anaphase catastrophe induction in lung cancer cells (Fig. 2B). We postulated from these findings that forced expression of CDK1 or CDK2 would protect lung cancer cells from inducing anaphase catastrophe after dinaciclib induction. However, increased levels of CDKs *in vitro* alone led to an increased frequency of multipolar anaphases and subsequent addition of dinaciclib had no appreciable effect, complicating experimental interpretation (Supplementary Fig. 3).

Next, expression and phosphorylation of known targets of CDK1, 2, 5 and 9 were investigated after dinaciclib treatments at varying concentrations in lung cancer cells. As expected, decreased phosphorylation of RNA polymerase II in serine 2 position in H1299 cells upon exposure to dinaciclib was detected likely due to CDK9 inhibition (Fig. 2C). Downregulation of survivin and decreased Rb phosphorylation at residue T821 (Fig. 2C)

provide evidence for CDK1 and CDK2 inhibition, respectively (28, 29). At the same time, Rb^{S780} phosphorylation was unchanged, indicating that cyclin D and its partners CDK4 and CDK6 were not inhibited by dinaciclib. Dinaciclib also inhibits CDK5, but since CDK5 function is most prominent in neuronal tissue (30) and knockdown of CDK5 did not induce anaphase catastrophe in lung cancer cells (Fig. 2B), we did not investigate its downstream substrates phosphorylation status.

Our recent work reported that CDK2 inhibition-induced anaphase catastrophe is mediated through the centrosomal protein CP110 (26, 27). CP110 is a substrate for both CDK1 and CDK2 kinases and is involved in centrosome duplication and separation (25, 31). Engineered expression of CP110 reduced the frequency of multipolar anaphases in H1299 and HOP62 cells treated with dinaciclib (Fig. 2D). In contrast, CP110 did not prevent induction of aberrant anaphases by Taxol, a microtubule-targeting agent that is not known to inhibit CDK activity (Fig. 2D). Together, these data indicate that dinaciclib induces anaphase catastrophe through inhibition of both CDK1 and CDK2 and that this is mediated at least in part by CP110.

Activated *KRAS* mutations can sensitize lung cancer cells to dinaciclib

Our previous studies reported that activated *KRAS* mutations sensitized lung cancer cells to the CDK2 inhibitor seliciclib (16, 27). This provided a rationale to study whether activated *KRAS* mutations also sensitized lung cancer cells to dinaciclib. Three lung cancer cell lines with *KRAS* mutations (H1299, HOP62 and H23) and two without (H522 and H1703) were screened for *NRAS*, *HRAS* or *KRAS* mutations within codons 12, 13 and 61 using Sanger sequencing, as previously described (32). In aggregate, activated *KRAS* mutant lung cancer cell lines exhibited a higher susceptibility to dinaciclib-induced apoptosis than wild-type *KRAS* expressing lines (Fig. 3A). Concurrently, such cell lines demonstrated higher frequency of multipolar anaphases (Fig. 3B). To further study the biologic effects of activated *KRAS* mutations with minimal cell line genetic background differences *in vitro*, lentiviral-mediated mutant *KRAS* expression in ED-1 cells was achieved. Expression of oncogenic *KRAS* was confirmed by immunoblot analyses (Fig. 3C, bottom panel). ED1 cells that expressed mutant *KRAS* exhibited a significant increase in apoptosis induction when treated with dinaciclib compared to mock-transduced cells (Fig. 3C). Increased apoptosis rate was accompanied by significantly higher frequency of multipolar anaphases in these cells (Fig. 3D). Therefore, the acquisition of activated *KRAS* increases susceptibility to dinaciclib-induced anaphase catastrophe and apoptosis in lung cancer cells.

To extend the analysis of lung cancer cell lines, a high-throughput system was used to test the dinaciclib sensitivity of 108 lung cancer cell lines with known *KRAS* mutation status for cell growth using the CellTiterGlo assay. IC₅₀ and IC₇₀ concentrations were calculated for each cell line. IC₅₀ values ranged from 0.05–1.4 μM and these data are shown in Supplemental Fig. 4. From our previous study the average IC₅₀ values for seliciclib were closer to 15 μM for lung cancer cell lines (16). This indicates the increased potency of dinaciclib as compared to seliciclib. While acquisition of mutant *KRAS* sensitized cells to dinaciclib-mediated apoptosis, the cell line analysis indicated wild-type and mutant *KRAS* cell lines were similarly sensitive to dinaciclib treatment.

Dinaciclib cooperates with taxol to induce anaphase catastrophe

Taxanes are microtubule-targeting agents that induce apoptosis through multiple mechanisms including mitotic catastrophe (33). Our prior work indicated that combinations of taxanes with seliciclib, a CDK2 inhibitor, enhanced effects of apoptosis and anaphase catastrophe-induction in lung cancer cells as compared to single agent treatment (16). Given this, effects of combining dinaciclib with Taxol in inducing anaphase catastrophe were investigated in a panel of lung cancer cells.

As shown in Fig. 4A, treatment of Taxol alone at the 1 and 5 nM dosages did not induce a substantial reduction in cell growth in H23 and HOP62 lung cancer cell lines. Similarly, dinaciclib had no appreciable effect on this outcome when used at low dosages (5 and 10 nM). In contrast, concurrent treatment with the low dosages of Taxol and dinaciclib resulted in a significant ($P < 0.05$) decrease in lung cancer cell metabolic activity (Fig. 4A).

The effect of combining Taxol with dinaciclib in anaphase catastrophe-induction was investigated. As shown in Fig. 4B, H23 cells showed at least a 2-fold increase in multipolar anaphases when treated with Taxol combined with dinaciclib as compared with single agent treatments. A less marked, but statistically significant increase in anaphase catastrophe-induction was also observed in the Hop62 cells. Analysis of these data revealed that the combination of dinaciclib with taxol was additive for both H23 and Hop62 cells. Taken together, dinaciclib cooperates with a microtubule-stabilizing agent to deregulate anaphase and restrict growth of lung cancer cells.

Discussion

It was estimated over 225,000 were diagnosed with and nearly 160,000 patients succumbed to lung cancer in 2015, accounting for the most cancer-related deaths in both men and women (34). The 5 year survival rate for lung cancer is less than 17%, and this drives the development of innovative therapeutic approaches for lung cancer (34).

Lung cancer cells exhibit substantial chromosomal instability (35). Many cases exhibit aneuploidy and also have structural cytogenetic abnormalities, such as translocations, deletions and gene copy number gains, leading to decreased function of tumor suppressors and enhanced activity of oncogenes. Chromosomal instability correlates with poor prognosis in lung cancer and other solid tumors, indicating that increasing genetic diversity contributes to enhanced tumor survival and chemoresistance (36). However, because of the propensity for lung cancers to be genetically unstable, this represents a potential tumor-specific therapeutic target.

We previously demonstrated that either genetic or pharmacologic (seliciclib) inhibition of cyclin E-driven CDK2 activity induces anaphase catastrophe and cell death (16, 27). In this current work it is demonstrated that dinaciclib, a novel inhibitor of CDK1/2/5/9, like seliciclib, induces multipolar anaphases in lung cancer cells, leading to apoptosis. It is established here that treatment with dinaciclib leads to multipolar cell divisions in lung cancer cells, which is incompatible with viable progeny (18) and accounts for lung cancer apoptosis observed in these studies.

Dinaciclib inhibits both CDK1 and CDK2 with high affinity in *in vitro* kinase assays (1 and 3 nM, respectively) (20). Hence, the individual contribution of these kinases to the induction of anaphase catastrophe may not be elucidated with dinaciclib treatment alone. In an effort to confirm the role of CDK2 in dinaciclib-mediated induction of anaphase catastrophe, genetic knockdown of individual CDKs was achieved in human lung cancer cells. Surprisingly, it was found that, like CDK2 antagonism, siRNA-mediated knockdown of CDK1 resulted in induction of anaphase catastrophe in the studied lung cancer cells. In fact, incidence of multipolar anaphases occurred with similar or higher frequency in response to CDK1 manipulation, compared with that of CDK2. This finding is in contrast to our previous studies where anaphase catastrophe was not observed in response to genetic knockdown of CDK1 (16). The potential reasons are: (a) higher efficiency of CDK1 suppression was achieved in the current work and (b) earlier experiments were performed in ED1 cells, which are addicted to cyclin E expression and may be therefore less responsive to manipulations of CDK1.

CDK1 is a mitotic kinase active during G₂-M transition, and has multiple phosphorylation targets, many of which are also targets of CDK2/cyclin complexes (37). We previously reported that the centrosomal protein CP110 mediates CDK2 inhibition-induced anaphase catastrophe, as CP110 overexpression partially compensates for loss of CDK2 activity (26, 27). Since CP110 is also regulated by CDK1 (25), it is possible that loss of CDK1-mediated phosphorylation of CP110 results in enhanced susceptibility to anaphase catastrophe. Consistent with this, engineered overexpression of CP110 was found to confer at least partial protection from dinaciclib-induced anaphase catastrophe. Of the unique CDK1 targets, survivin is an important mitotic regulator with a distinct anti-apoptotic function in cancer (29). CDK1-mediated phosphorylation prevents survivin degradation and thus inhibition of CDK1 rapidly downregulates its expression (30). Interestingly, interference with survivin function results in formation of abnormal mitotic spindle and multipolar mitoses, similar to those observed in our study (38). Here we demonstrated that dinaciclib treatment results in downregulation of survivin in lung cancer cells, and this could account for the development of multipolar anaphases. Thus, it is likely that anaphase catastrophe is induced through several mechanisms downstream of CDK1 and 2 inhibition.

A phase II trial with dinaciclib as a single agent in patients with NSCLC did not report objective responses (39). Yet, as for other targeted agents, the distinct genetic characteristics of the tumor should be used to guide therapeutic decision-making. Mutations in the *RAS* oncogene are present in up to 30% of lung cancers, typically occurring in smokers, and this confers poor prognosis (40). Point mutations in codon 12 of *KRAS* with G->T transversion are among the most common *KRAS* alterations. Here we demonstrate that acquisition of mutant *KRAS* enhanced sensitivity towards dinaciclib treatments. Human lung cancer cells with mutations in *KRAS*, and murine lung cancer cells genetically modified to express activated *KRAS*, demonstrated increased dinaciclib-mediated anaphase catastrophe and apoptosis. High throughput analysis of 108 human lung cancer cells did not indicate a significant difference in sensitivity between *KRAS* wild-type or mutant cells. However, *KRAS* mutant cancers are frequently resistant to chemotherapeutic agents that are effective for *KRAS* wild-type cancers (23).

Finally, we observed that dinaciclib cooperates with Taxol, a microtubule-stabilizing agent, to restrict growth of the lung cancer cells. While Taxol alone did not appreciably affect cell growth (at the studied concentrations), a marked enhancement in the frequency of multipolar anaphases was detected in its combination with dinaciclib, and was accompanied by a reduction in cell viability. We demonstrated a similar additive effect previously with seliciclib (16), suggesting that sensitization to anaphase catastrophe may be one of the underlying mechanisms accounting for cooperation between the CDK inhibitors and taxanes, two distinct classes of drugs, which affect cellular progression through mitosis.

In summary, here we report here that dinaciclib disrupts anaphase progression and restricts growth of lung cancer cells. Our study indicates a need to further investigate dinaciclib and other CDK inhibitors as a potential therapeutic approach in lung cancer with activating *RAS* mutations, either alone or in combination with taxanes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Dinaciclib induces anaphase catastrophe in lung cancer cells

A. ED1 cells were incubated with the indicated concentrations of dinaciclib or vehicle control for a period of 10 or 24 hours. Cells were trypsinized and the proportion of Annexin V+ cells was measured by flow cytometry (in duplicate). Data are the mean $\pm$ standard error (SE) of three independent experiments. **B.** ED1 cells were incubated with the indicated concentrations of dinaciclib or vehicle control for 24 hours. Cells were immunostained with α -tubulin and counterstained with DAPI. Total anaphases were counted (100) and the proportion of multipolar anaphases is shown. Data are the mean $\pm$ SE of three independent

experiments. (** $P < 0.01$ as compared to vehicle-treated control). *C.* H1299 (top panels) and HOP62 (lower panels) cells were treated with 25 or 50 nM dinaciclib and imaged at the indicated time points as described in the Methods. White asterisks represent three daughter cells generated from dinaciclib-induced multipolar mitoses. *D.* Cells were incubated with the indicated concentrations of dinaciclib or vehicle control for 24 hours, followed by a washout. Cellular proliferation was determined using an MTT assay after 72 hours. Data are the mean $\pm$ SE of three independent experiments performed in quadruplicate.

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Fig. 2. Dinaciclib mediates anaphase catastrophe via CDK1/2 inhibition

A–B. H1299 and HOP62 cells were transfected with CDK1, CDK2, CDK5 or CDK9-specific siRNA or control siRNA. The mRNA knockdown was confirmed by RT-PCR assays **A.** in H1299 cells and the proportion of multipolar anaphases was analyzed 48 hours later **B.** **C.** H1299 cells were incubated with or without the indicated concentrations of dinaciclib for 10 or 24 hours. Whole-cell lysates were subjected to immunoblotting. A representative immunoblot of three independent experiments is shown. Data are the mean $\pm$ SE of three independent experiments performed in duplicate. **D.** H1299 and HOP62 cells were

transfected with CP110-expressing vector or insert-less vector control for 24 hours and incubated with the indicated concentrations of dinaciclib or vehicle control for 10 hours. CP110 overexpression was confirmed by immunoblotting (right panel, H1299 cells shown). Cells were immunostained with α -tubulin and counterstained with DAPI. Total anaphases were counted and the proportion of multipolar anaphases is displayed in this figure. Data are the mean $\pm$ SE of three independent experiments performed in duplicates. (** $P < 0.01$ compared to vehicle treated control).

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Figure 3. Ras mutations sensitize lung cancer cells to dinaciclib

A, Ras^{WT} (H522, H1703) and Ras^{MUT} (H1299, HOP62, H23) human lung cancer cells were incubated with the indicated concentrations of dinaciclib or vehicle control for 12 hours. Cells were trypsinized and the proportion of Annexin V⁺ cells was measured by flow cytometry in duplicate. *B*, Cells were scored for multipolar anaphases after a 6 hour incubation with dinaciclib. *C*, Activated mutant *KRAS* or vector control-transduced ED1 cells were incubated with the indicated concentrations of dinaciclib or vehicle control for 24 hours. Cells were trypsinized and the proportion of Annexin V⁺ cells was measured by flow

cytometry (in duplicates). *KRAS* protein expression was confirmed by immunoblotting while these experiments were conducted (bottom panel). *D*, Activated mutant *KRAS* or vector control-transduced ED1 cells were scored for multipolar anaphases after 10-hour incubation with dinaciclib. *A–D*, Data are the mean $\pm$ SE of at least three independent experiments. (** $P < 0.01$).

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Fig. 4. Dinaciclib cooperates with Taxol to inhibit growth and induce anaphase catastrophe in lung cancer cells

A and B, H23 and HOP62 cells were incubated with the indicated concentrations of dinaciclib, Taxol, and combination or vehicle control. Cell proliferation was determined using an MTT assay after 48 hours of incubation with the indicated drugs, in quadruplicate *A*. Also, H23 cells were subjected to Annexin V⁺ cells measurements by flow cytometry after 10 hours of incubation with the indicated drugs *B*. H23 and HOP62 cells were incubated with Taxol and dinaciclib alone or the drugs combined for 10 hours. Cells were fixed, total anaphases were counted and the proportion of multipolar anaphases is presented in this figure. Data are the mean $\pm$ SE of three independent experiments. (* $P < 0.05$ compared with either drug alone)