Abemaciclib, a Selective CDK4/6 Inhibitor, Enhances the Radiosensitivity of Non-Small Cell Lung Cancer In Vitro and In Vivo

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Abstract

Purpose: To characterize the ionizing radiation (IR) enhancing effects and underlying mechanisms of the CDK4/6 inhibitor abemaciclib in non–small cell lung cancer (NSCLC) cells in vitro and in vivo.

Experimental Design: IR enhancement by abemaciclib in a variety of NSCLC cell lines was assessed by in vitro clonogenic assay, flow cytometry, and target inhibition verified by immunoblotting. IR-induced DNA damage repair was evaluated by γH2AX analysis. Global metabolic alterations by abemaciclib and IR combination were evaluated by LC/MS mass spectrometry and YSI bioanalyzer. Effects of abemaciclib and IR combination in vivo were studied by xenograft tumor regrowth delay, xenograft lysate immunoblotting, and tissue section immunohistochemistry.

Results: Abemaciclib enhanced the radiosensitivity of NSCLC cells independent of RAS or EGFR status. Enhancement of radiosensitivity was lost in cell lines deficient for functional p53 and RB protein. After IR, abemaciclib treatment inhibited DNA damage repair as measured by γH2AX. Mechanistically, abemaciclib inhibited RB phosphorylation, leading to cell-cycle arrest. It also inhibited mTOR signaling and reduced intracellular amino acid pools, causing nutrient stress. In vitro, abemaciclib, when administered in an adjuvant setting for the second week after fractionated IR, further inhibited vasculogenesis and tumor regrowth, with sustained inhibition of RB/E2F activity, mTOR pathway, and HIF-1 expression. In summary, our study signified inhibiting the CDK4/6 pathway by abemaciclib in combination with IR as a promising therapeutic strategy to treat NSCLC.

Conclusions: Abemaciclib in combination with IR enhances NSCLC radiosensitivity in preclinical models, potentially providing a novel biomarker-driven combination therapeutic strategy for patients with NSCLC. Clin Cancer Res; 24(16); 3994–4005. ©2018 AACR.

Introduction

Over the last decade, advances in molecular translational research have heralded major breakthroughs in the understanding, diagnosis, and management of lung cancer, particularly for the more common (~80%) non–small cell lung cancer (NSCLC). NSCLC is subclassified by histology and driver mutations such as mutated KRAS and activating mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) domain (1–4). The two most common EGFR-TK domain mutations are exon 19 deletions (60%) and L858R missense substitutions resulting in constitutive activation of the receptor without ligand binding (3, 5, 6). Constitutive activation of receptors or protein kinases stimulates a complex cascade of cross-signaling pathways leading to uncontrolled growth, proliferation, and survival (2, 3). Successful targeted therapies in NSCLC involve the identification and inhibition of these upregulated pathways by small molecule tyrosine kinase inhibitors (TKI) or receptor monoclonal antibodies (7–9). Although EGFR-TKIs have been useful in the treatment of EGFR-mutant NSCLC, most responses have not proved to be durable with many patients progressing after 7 to 12 months (4, 8, 10). The most frequent mechanism (~50%) is concurrent acquisition of novel mutation in exon 20 of EGFR, encoding for T790M making tumors refractory to the existing EGFR-TKI therapy (8, 11). Apart from EGFR-TKI, radiotherapy either alone or in combination with chemotherapy, remains the primary modality of treatment for patients with stage III NSCLC. For stage I and II NSCLC, radiotherapy is an alternative curative option to surgery for patients who are medically inoperable or refuse surgery. Overall radiotherapy is an important palliative treatment modality to treat symptoms from the primary or bone or brain metastases and improve patients’ quality of life (12). Despite these medical interventions, 5-year survival rates of NSCLC patients are less than 5% (4, 13). Hence, there is an urgent need to target other signaling pathways or design combination therapy that is more effective than first-line single agents while balancing toxicity and costs. Other than the EGFR or MEK/ERK pathway, cyclin D kinase 4/6 (CDK4/6) activity is typically deregulated and overactive in various cancers including NSCLC (14, 15).

CDK4 and CDK6 are cyclin-dependent kinases that control the transition between the G1- and S-phase of the cell cycle. A major target of CDK4 and CDK6 during cell-cycle progression is the retinoblastoma protein (RB). When RB is phosphorylated, its growth-suppressive properties are inactivated. Selective CDK4/6...
Translational Relevance
Radiotherapy plays a significant role in the management of non–small cell lung cancer (NSCLC). It is useful for all stages of disease with or without additional treatment modalities, such as surgery and chemotherapy. However, the response rate of some tumors remains problematic. The present study evaluates the combination of a highly specific CDK4/6 inhibitor, abemaciclib, with ionizing radiation (IR) to enhance radiation sensitivity of NSCLC in vitro and in vivo. Our data indicate that this novel combination efficiently radiosensitized proliferative and plateau-phase tumor cells and tumor xenografts with minimal normal cell radiosensitization. Abemaciclib inhibited IR-induced DNA damage repair and caused RB-dependent cell-cycle arrest. Further, the study identified possible predictive biomarkers (p53, RB, and SDF-1) to guide treatment response and efficacy of the combination. Collectively, this study highlights the CDK4/6 axis as a potential radiation target for NSCLC and warrants assessment of abemaciclib in clinical trials as a radiation modifier.

Materials and Methods
Cell survival studies
Human lung cancer cell lines (H1975, H820, H1299, H1650, H82, H460, A549) and MCF-10A were purchased from ATCC.

All cell lines were authenticated within the past 6 months by IDEXX BioResearch using Cell Check 9 [9 allele marker STR (short tandem repeat) profile and interspecies contamination test; Supplementary File S1]. All cells were cultured in RPMI-1640 (Invitrogen), supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO2 atmosphere. All CDK4/6 inhibitors were purchased from Selleck Chemicals. A stock solution of drug was prepared in 100% dimethyl sulfoxide (Sigma-Aldrich) and stored at −70°C. The drug was diluted in fresh media prior to each experiment. Abemaciclib was added to cells for 24 hours after IR treatment at a concentration of 10 μmol/L. Clonogenic cell survival studies were performed as described before (24, 25). Experiments were repeated three independent times. Radiation dose-modifying factors (DMFs) were determined at 10% survival levels by dividing the radiation dose for control by the radiation dose for drug treated. DMFs > 1.0 indicate enhancement of radiosensitivity.

Immunoblotting
Exponentially growing cells were washed twice with chilled PBS and cell pellet were collected as a function of time following irradiation. Total protein extraction from cultured cells and xenograft tumors were performed as previously described (25). Acid soluble histone proteins for γH2AX were extracted in 0.2 mol/L sulfuric acid. Protein concentrations were determined using BCA reagent (Thermo Fisher Scientific). Protein samples were resolved on SDS-PAGE and immunoblotting was performed as described before (24, 25). Details of antibodies used for immunoblotting are described in Supplementary Experimental Procedures.

ATP and NAD quantification
ATP (catalog no. K354-100) and NAD (catalog no. K337) colorimetric assays were performed using BioVision kit. Briefly, 1 × 10^6 cells were treated with abemaciclib after IR exposure. Samples were collected 24 hours post-IR, and the assays were performed according to the manufacturer’s instructions. Experiments were performed in triplicate.

Metabolite extraction and mass spectrometry analysis
Cells treated with abemaciclib post-IR for 24 hours were collected by centrifugation and washed with 5% mannitol solution, treated with methanol and water containing internal standards (H3304-1002, Human Metabolome Technologies). Samples were incubated on ice for 5 minutes with occasional mixing. The extract was centrifuged at 2,300 × g and 4°C for 5 minutes and centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 × g and 4°C for 120 minutes to remove proteins. The filtrate was centrifugally concentrated, dried, and resuspended in 50 μL of Milli-Q water for capillary electrophoresis mass spectrometry (CE-MS) analysis. A detailed explanation of CE-TOF and QqQMS methods can be found in Supplementary Experimental Procedures.

Xenograft studies
All animal experiments were carried out in accordance with protocols approved by the National Cancer Institute’s Animal Care and Use Committee (ACUC). For radiation regrowth delay studies, tumor cells (1 × 10^6) were injected into the subcutaneous space of the right hind leg of athymic nude mice, 5 to 6 weeks of age, bred at the National Cancer Institute’s Animal Production...
Abemaciclib significantly inhibits radiation-induced DNA repair with post-IR treatment

We further investigated the influence of abemaciclib on IR-induced DNA damage repair by assessment of γH2AX (at Ser139) induction and the time-dependent resolution which is indicative of DNA double-strand break repair (28). Fig. 2A shows γH2AX kinetics following 7.5 Gy and abemaciclib treatment in H460 and H1299 cells. Following 7.5 Gy γH2AX rapidly increased in H460 (7.9-fold at 0.5 hour) and H1299 (6.7-fold at 0.5 hour) cells, but resolved to unirradiated control levels (1.1- and 1.2-fold, respectively) 24 hours post-IR. With post-IR abemaciclib treatment incubation, γH2AX remained elevated in H460 (3.92-fold at 24 hours) and H1299 cells, consistent with lack of abemaciclib radiosensitization in this cell line (Fig. 2A). To further evaluate the impact of abemaciclib on DNA damage repair, expression of key DNA repair proteins such as Rad51, phosphorylated Chk2, and ATM following abemaciclib exposure alone (27). H82 cells, lacking functional RB protein, showed no radiosensitization by abemaciclib (Fig. 1B). Importantly, the normal mammary epithelial cell line MCF-10A was not radiosensitized by abemaciclib with a DMF of 1.03 ± 0.12 (Fig. 1C). All data described to this point apply to exponentially growing cells. Because tumors often have a large fraction of nonproliferating cells, studies combining abemaciclib and IR exposure were evaluated using quiescent/platau phase H460 and H460Dnp53 cells. Figure 1D shows that plateau phase H460 cells were still sensitive to abemaciclib and IR although with a reduced DMF of 1.35 ± 0.15, whereas H460Dnp53 plateau phase cells did not respond to abemaciclib chemoradiation (DMF, 1.04 ± 0.01; Fig. 1D). Collectively, our data indicate abemaciclib as a novel radiation modifier for NSCLC cells, independent of mutated EGFR and KRAS, but dependent on a functional RB and possibly on a functional p53 protein status.

### Results

Abemaciclib enhances radiosensitivity of NSCLC cells in vitro

To determine the radiation enhancement ability of three novel CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib; 24-hour exposure to 1 μmol/L and 10 μmol/L with single IR dose), pilot studies were performed in H460 cells both with pre- and post-IR treatment protocols (Supplementary Fig. S1A–S1C). Abemaciclib at a concentration of 10 μmol/L showed enhanced radiation sensitivity of H460 cells with single IR dose (Supplementary Fig. S1C). Abemaciclib failed to enhance IR cell killing with DMF of 1.03 (±0.03; Supplementary Fig. S1D) when administered 24 hours prior to IR treatment. Palbociclib and ribociclib at 1 μmol/L or 10 μmol/L concentration failed to exhibit enhancement of radiation sensitivity in H460 cells when added either 24-hour pre- or post-IR treatment (Supplementary Fig. S1A and S1B). We further extended our pilot observation of combining IR and abemaciclib (24-hour post-IR treatment) across a panel of lung cancer cell lines with varied genetic background (Table 1; ref. 26). Abemaciclib (10 μmol/L) when added for 24-hour post-IR enhanced radiosensitivity for majority of NSCLC cell lines (Fig. 1A) with DMF ranging between 1.3 and 1.71. Abemaciclib toxicity and DMF for each cell line are shown in Table 1. Interestingly, cells deficient for functional p53 (H460Dnp53, H1299, and H1650) were non-responsive to abemaciclib + IR combination (Fig. 1B). However, H1975 cells, which does have a mutated p53, was highly sensitive to abemaciclib incubation (Fig. 1A), and likewise to IR exposure alone (27). H82 cells, lacking functional RB protein, showed no radiosensitization by abemaciclib (Fig. 1B). Importantly, the normal mammary epithelial cell line MCF-10A was not radiosensitized by abemaciclib with a DMF of 1.03 ± 0.12 (Fig. 1C). All data described to this point apply to exponentially growing cells. Because tumors often have a large fraction of nonproliferating cells, studies combining abemaciclib and IR exposure were evaluated using quiescent/platau phase H460 and H460Dnp53 cells. Figure 1D shows that plateau phase H460 cells were still sensitive to abemaciclib and IR although with a reduced DMF of 1.35 ± 0.15, whereas H460Dnp53 plateau phase cells did not respond to abemaciclib chemoradiation (DMF, 1.04 ± 0.01; Fig. 1D). Collectively, our data indicate abemaciclib as a novel radiation modifier for NSCLC cells, independent of mutated EGFR and KRAS, but dependent on a functional RB and possibly on a functional p53 protein status.
Importantly, no significant changes in the expression of the above DNA-DSB repair proteins were observed for the p53-deficient cell line, H1299 (Fig. 2D and Supplementary Fig. S2A). We confirmed the inhibition of RB phosphorylation at Ser780 in H460 (Fig. 2E) and H1975 cells (Supplementary Fig. S2B) as well as at Ser811/807 (Supplementary Fig. S2C) in H460 cells after 10 µmol/L of abemaciclib treatment for 24 hours as compared with a 4-hour drug exposure. Reduced RB phosphorylation post abemaciclib was followed by a concomitant increase in p21 expression (Fig. 2E and Supplementary Fig. S2B) leading to an increased G1 cell-cycle arrest (Table 1). Abemaciclib incubation also reduced the expression of RB/E2F target proteins such as TopoIIα (marker for G1–S transition; Fig. 2E and Supplementary Fig. S2B) and showed
a significant decrease in p-HH3 (histone H3) expression at Ser 10 (marker for mitosis) as observed by flow cytometry (Supplementary Fig. S2D). Significantly, the G1 block after abemaciclib incubation was not observed in the RB-deficient H82 cell line (Table 1). There was also an accumulation of late apoptotic cells (annexin V positive + PI positive) after 24 hours of abemaciclib and IR treatment in H460, but not in H1299 and H460DNp53 (Supplementary Fig. S2E) cells. Taken together, our results suggest that inhibition of CDK4/6 activity by abemaciclib in NSCLC cells causes enhanced G1 arrest and inhibits DNA–DSBs repair pathways leading to enhanced IR sensitization.

Abemaciclib inhibits mTOR signaling in vitro

Another signaling pathway known to influence IR sensitivity is the mTOR pathway that also regulates tumor growth, tumor cell metabolism, and cell-stress responses (29, 30). Multiple marker proteins of mTOR activation were clearly reduced by abemaciclib incubation in H460 cells (Fig. 3A). A reduction in expression of p-Akt (Ser 473), p-S6, p-4EBP1, and p-p70S6K (Fig. 3A) was observed. Similar observations were made in H1975 cells (data not shown). mTOR also regulates ATP and total NAD levels (29, 30) as shown in Fig. 3B and C, respectively. ATP and total NAD levels in H460 cells were reduced by 35% and 29% by abemaciclib and IR exposure compared with
IR only, respectively (Fig. 3B and C). Reduction in ATP levels by abemaciclib is important as chromatin-remodeling complexes, which are ATP dependent play an important role in all three major DNA repair pathways: nucleotide excision repair, homologous recombination, and nonhomologous end joining (31). Reduced intracellular ATP by abemaciclib may play a role in inhibition of IR-induced DNA damage repair thereby causing enhanced radiosensitivity. Collectively, these results suggest that abemaciclib-mediated inhibition of mTOR could possibly lead to reduced intracellular ATP and total NAD levels thereby rendering NSCLC cells vulnerable to enhanced radiosensitization.

Abemaciclib alters NSCLC metabolism by disrupting key nutrient sensing pathways

In recent years, it has been well appreciated that cancer cells have the ability to rewire their metabolism and energy production networks in response to altered microenvironment conditions to support rapid proliferation, invasion, metastasis, and resistance to cancer treatment (32, 33). mTOR remains central in regulating

Figure 4. Abemaciclib triggers nutrient stress in H460 cells by altering metabolism. A, LC/MS mass spectrometry analysis showing fold expression of steady-state levels of total NEAA (nonessential amino acid) and EAA (essential amino acid) in H460 cells treated in the presence and absence of abemaciclib or IR (5 Gy). The concentrations of metabolites are expressed as fold change. B and C, Levels of glucose and glutamine consumption and glutamine and lactate secretion were measured by bioanalyzer. The levels of indicated metabolites in the supernatants were normalized to concentration (µg) of protein in the sample. Experiments were done in triplicate; error bar, mean ± SD. **P<0.01; ***P<0.001; and ****P<0.0001. D, Immunofluorescence analysis of H460 cells treated with DMSO or abemaciclib for 24 hours, showing expression of Glut-1 (glucose transporter, shown in green plots A and B, respectively) and ASCT2 (amino acid transporter, shown in red plots C and D, respectively). DAPI was used to stain nucleus (shown in blue). Images were taken at 60× using Zeiss 780 confocal microscope. Scale bar, 10 µm.
were delivered immediately after the abemaciclib doses (immediately after radiation and 6 hours later). Regrowth in combination with fractionated IR exposure leads to sustained mTOR inhibition, limits access to energy and stress. These results indicate that abemaciclib altered the activity of glyceroldehyde 6 phosphate dehydrogenase (G6PD). With exception of reduced levels of citrate, most TCA cycle metabolites did not show any significant change after abemaciclib treatment (Supplementary Fig. S3A). Interestingly, abemaciclib caused a significant decrease in steady-state levels of both nonessential amino acid (NEAA) and essential amino acids (EAA; \*\*\*, \( P < 0.005 \)), suggesting a possible amino acid deficiency (nutrient stress) in H460 cells (Fig. 4A and Supplementary Fig. S3B). One of the best-studied ways by which cells maintain their amino acid levels during starvation or nutrient stress in vitro is by elevating a general amino acid control (GAAC) pathway (34). Studies have indicated that glutamine depletion can also trigger the GAAC pathway, by elevating expression of amino acid transporters such as ASCT2, thereby increasing amino acid uptake and elevating intracellular amino acid levels, such as that of leucine (Leu). Increased Leu has been shown to reactivate mTOR (35, 36). Interestingly, in our metabolic analysis, abemaciclib significantly downregulated levels of Leu (Supplementary Fig. S3B). In addition to changes in steady-state levels of glycolytic and TCA-cycle metabolites, we also observed the kinetics of glucose and glutamine uptake by H460 cells using a YSI-Bioanalyzer. In the presence of abemaciclib, both glucose and glutamine uptakes were decreased (Fig. 4B and C). In agreement with the observed decrease in Leu, glutamine, and glucose uptake, abemaciclib treatment altered the surface distribution of both ASCT2 (shown in red, plot D) and glucose transporter (GLUT1, shown in green panel, plot B; Fig. 4D). Other surface proteins, such as CD98 (also known as 4F2HC or SLC3A2, neutral amino acid transporter) and CD147, a chaperone protein that mediates transport of MCT1 and MCT4 to the plasma membrane, were not affected. Collectively, abemaciclib treatment reduced ATP levels and restricted access to key metabolic substrates mainly amino acids, glutamine, and glucose possibly leading to nutrient and energy stress. These results indicate that abemaciclib exposure leads to sustained mTOR inhibition, limits access to extracellular amino acid pools, and decreases intracellular ATP levels thereby facilitating NSCLC to become sensitive to IR.

**Abemaciclib inhibits IR-induced vasculogenesis and tumor regrowth in combination with fractionated IR**

A pilot H460 xenograft study was conducted combining fractionated IR (three 2 Gy fractions) with daily abemaciclib (100 mg/kg) treatment through oral gavage. Initially, two daily abemaciclib doses (immediately after radiation and 6 hours later) were delivered immediately after the first IR fraction and the second day; however, this was not well tolerated and subsequently abemaciclib was given as daily single dose for the remainder of the week (Wednesday–Friday). This regimen of abemaciclib treatment resulted in modest radiosensitization (Supplementary Fig. S4A) and an increased delay in the tumor regrowth kinetics (\( P = 0.02 \)) as compared with radiation only control, followed with rapid tumor regrowth. In an attempt to further enhance tumor regrowth delay, we considered the tumor vasculature. Targeting tumor vasculature has long been considered for both radiation and chemoradiation as a means of tumor destruction (37). The novel finding of Brown and colleagues who demonstrated using a brain tumor xenograft model, that tumor vasculature can recover post-IR by colonization of circulating BMDC (bone marrow derived cells) primarily CD11b+ myeloid cells. This pathway of blood vessel formation, secondary to tumor angiogenesis which is inhibited by IR (38), known as vasculogenesis, restores tumor vasculature facilitating recurrence of tumor following radiation (39, 40). They further showed that the stimulus for the influx of CD11b+ cells into tumors following IR is increased levels of hypoxia (and HIF-1 expression) that drives secretion of the chemokine stromal cell-derived factor-1 (SDF-1). The kinetics of vasculogenesis in the brain tumor model was observed to be as early as 2 weeks postradiation leading to enhanced tumor regrowth (39). Hence, we hypothesized that should fractionated IR during the 1st week of treatment initiate vasculogenesis, then an additional week of adjuvant abemaciclib treatment might provide additional tumor regrowth delay.

To test this hypothesis, we conducted a second in vivo study where mice with H460 xenografts were exposed to fractionated IR (2 Gy) on Monday, Wednesday, and Friday in addition to daily gavage of the abemaciclib (100 mg/kg) immediately after IR for 5 days in a week (1st week) followed by 3 additional treatments of abemaciclib only within IR (second week). In the absence of radiation, abemaciclib displayed a modest effect on H460 xenograft regrowth by 2 days (\( P = 0.14 \)) versus control untreated tumors (Fig. 5A). However, abemaciclib in combination with IR showed synergistic delay in tumor regrowth of 8 and 9 days, respectively as compared with IR only and control tumor (\( P = 0.006 \) and \( P = 0.001 \), respectively). Adding a second week of adjuvant abemaciclib treatment enhanced tumor delay compared with 1 week of treatment (Supplementary Fig. S4A). This study was repeated with similar findings as shown in (Supplementary Fig. S4C). We next evaluated tumor tissue at the end of the second week of adjuvant treatment to determine if IR-induced vasculogenesis occurred and if abemaciclib inhibited the induction. Reduced immunohistochemical staining of fluorescently labeled CD11b+ monocytes, SDF-1, CD45+, and F4/80-positive macrophages were observed in week 2 tumors following abemaciclib and IR + abemaciclib combination treatment. In good agreement with Brown and colleagues, week 1 tumor post-IR treatment did not show enhanced SDF-1 (40) and CD11b+ BMDC infiltration (data not shown). Abemaciclib alone inhibited RB phosphorylation and tumor cell proliferation as seen by reduced p-HH3 and Ki-67 staining (Fig. 5B and C). Inhibition of vasculogenesis in week 2 tumors by abemaciclib and IR treatment was quantified and shown in Fig. 5C. Western analysis of tumor lysates confirmed modest RB inhibition as seen by cyclin A expression in abemaciclib alone and combination with IR in week 1 tumors (Supplementary Fig. S4B). In good agreement with reduced p-RB expression in week 2 tumor sections (Fig. 5C), we also observed a sustained inhibition of cyclin A expression upon abemaciclib and abemaciclib + IR combination in week 2 tumors (Supplementary Fig. S4B). These results clearly show that inhibition of the CDK4/6 pathway leads to inhibition of IR-induced tumor vasculogenesis.
Furthermore, in H460 xenografts, abemaciclib treatment led to a sustained inhibition of p-S6 levels indicating reduced mTOR activation (Supplementary Fig. S4B). IR treatment enhanced expression of HIF-1 at week 1, was dramatically inhibited by abemaciclib (Fig. 5D). Further, abemaciclib in an adjuvant setting also inhibited IR-induced induction of HIF-1 at week 2, but not completely (Fig. 5D). Abemaciclib inhibited HIF-1 expression independent of IR in untreated tumors (week 2) as tumor growth enhanced the hypoxic fraction (Fig. 5D). The mechanism of how abemaciclib inhibits induction of HIF-1 in vivo remains to be established and will be a topic of further studies.

Discussion

With recent development and FDA approval of highly specific CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) for advanced metastatic breast cancer, there has been growing interest in design of multiple clinical trials with these agents in various solid tumors (https://clinicaltrials.gov/). Successful phase I trial of abemaciclib in patients with advanced stage NSCLC and other solid tumors is very encouraging regarding the design of future combination therapy (20). However, recent reports of abemaciclib failure as monotherapy in phase III trial for KRAS-mutant solid tumors have been discouraging (http://www.ascopost.com/News/58135). There are, nonetheless, potential combinatorial therapies where CDK4/6 inhibitors may be bene-

Radiosensitization of H460 xenografts using fractionated radiation (Fig. 5), combined with abemaciclib, indicated that CDK4/6 inhibition may be an excellent target for clinical radiotherapy. Our data attribute the radiosensitization effect of abemaciclib to several parallel mechanisms in vitro and in vivo. In vitro, abemaciclib and IR combination resulted in significant inhibition of IR-induced DNA damage repair as assessed by γH2AX levels and compromised activation of key DNA damage response proteins to repair DNA (Fig. 2). In the present study, abemaciclib at 10 µmol/L for 24 hours led to sustained inhibition of mTOR both in vitro and in vivo (Fig. 3A and Supplementary Fig. S4B). mTOR inhibition by abemaciclib further leads to reduced amino acid pools as seen by global metabolic profiling (Fig. 4A). These alterations in the nutrient and energy status of cells possibly increased activated stress responses which lead to cell death via induction of apoptosis in vitro. Clearly, abemaciclib affects mTOR signaling resulting in reduction in ATP levels and other essential metabolite which may interfere with IR-induced DNA damage repair. However, the mechanism of mTOR inhibition by abemaciclib is unclear and requires additional studies.

Most interestingly, our study implicates a unique role of CDK4/6 inhibition in IR-induced vasculogenesis in vivo as a contributor to the enhanced IR response. Inhibition of IR-induced vasculogenesis by abemaciclib is in good agreement with the findings of Brown and colleagues, who showed that IR to a tumor can induce tumor vasculogenesis leading to recurrence in a GBM xenograft model (39). He further demonstrated that an inhibitor of vasculogenesis (SDF-1/CXCR4 pathway inhibitor, AMD3100) when administered the second week post-IR resulted in enhanced tumor regrowth delay (39). Phase I/II trials evaluating AMD3100 (plerixafor) after radiation therapy and temozolomide

Figure 5.

Effect of abemaciclib and fractionated radiation on tumor regrowth and radiation induced vasculogenesis. A and B, H460 xenograft showing enhanced tumor regrowth delay by daily administration of 100 mg/kg body weight of abemaciclib through oral gavage for 5 days immediately after 2 Gy × 3 IR dose administered every alternate day, indicated as week 1. The drug dosing was continued for another 5 days, indicated as week 2. A, The significance for tumor regrowth delay was measured when tumor size reached 600 mm³. The statistical significance is derived from 8 animals per group. B, Confocal microscopy showing expression of Ki-67, p-HH3, and p-RB and infiltration of BMDC (bone marrow derived cells) such as CD45, CD11b+ monocytes, and F4/80+ macrophages along with SDF-1 secretion in frozen section of H460 xenograft derived from week 2 treatment of abemaciclib with fractionated radiation. Nucleus is stained with DAPI (shown in blue). All images were taken at a magnification of 100 ×. Scale bar, 10 µm. C, Mean fluorescence intensity (MFI) of immunopositive cells of indicated proteins was measured for approximately eight random fields of the immunostained section using ImageJ. The fold expression was calculated by normalizing the MFI to that of MFI of DMSO-treated tumor section. Error bar represents mean ± SD. *, P < 0.0001; **, P < 0.0000. D, Immunoblot analysis showing expression of HIF1α in H460 xenograft for week 1 and week 2 of abemaciclib administration after fractionated radiation.
in patients with high-grade GBM have shown promising local tumor control [49]. Our data are consistent with these findings in that SDF-1, while not expressed in the tumor samples the 1st week of treatment, was enhanced during the second week post-IR (Fig. 5B and C). Abemaciclib treatment during the second week inhibited SDF-1 induction (Fig. 5B and C), thus inhibiting vasculogenesis. In addition to inhibiting IR-mediated vasculogenesis (Fig. 5) CDK4/6 inhibition also exerted sustained mTOR inhibition and of HIF-1 in vivo. CDK4/6 treatment also prevented tumor cell entry to mitosis as shown by reduced p-HH3 and Ki-67 staining (Fig. 5C). These findings are crucial for radiotherapy because both enhanced tumor hypoxia (induction of HIF-1) and mTOR activation are well-documented mechanisms for altering radiosensitivity in tumors (24, 50). Collectively, our results suggest that there are multiple mechanisms of abemaciclib radiosensitization including inhibition of DNA damage repair, mTOR signaling, and IR-induced vasculogenesis.

In summary, the results of the present study suggest that abemaciclib and IR combination would be a reasonable way to manage NSCLC patients in early and advance disease settings, with implications on minimizing IR-induced tumor recurrence. Our findings clearly warrant consideration of this novel chemoradiation combination in clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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