Abemaciclib, a Selective CDK4/6 Inhibitor, Enhances the Radiosensitivity of Non–Small Cell Lung Cancer \textit{In Vitro} and \textit{In Vivo}

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Abstract

\textbf{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 \textit{in vitro} and \textit{in vivo}.

\textbf{Experimental Design:} IR enhancement by abemaciclib in a variety of NSCLC cell lines was assessed by \textit{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 \textit{in vivo} were studied by xenograft tumor regrowth delay, xenograft lysate immunoblotting, and tissue section immunohistochemistry.

\textbf{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. \textit{In vivo}, 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 signifies inhibiting the CDK4/6 pathway by abemaciclib in combination with IR as a promising therapeutic strategy to treat NSCLC.


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 (DMF) 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 × 106 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.

Metabolic 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 × 106) 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 Facility. All animal experiments were performed at the National Cancer Institute's Animal Production Facility.
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/plateau 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 or KRAS, but dependent on a functional RB and possibly on a functional p53 protein status.

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) compared with untreated control (Fig. 2A) indicating inhibition of DNA damage repair. However, H1299 cells treated with abemaciclib post-IR resulted in similar γH2AX repair kinetics to IR alone treated cells over a 24-hour period, which is 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 or abemaciclib + IR were observed to be reduced in H460 cells when compared with untreated control (Fig. 2B and C). A reduction of phosphorylated ATR (***, P < 0.005) and DNA-PKcs (***, P < 0.005) with abemaciclib and IR combination also suggested a significant DNA-DSB repair inhibition.

Table 1. Summary of abemaciclib’s effect on NSCLC cell line toxicity, cell cycle, and radiation sensitivity

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Histology</th>
<th>EGFR</th>
<th>RB1</th>
<th>KRAS</th>
<th>TP53</th>
<th>p16</th>
<th>PTEN</th>
<th>% G1</th>
<th>% S</th>
<th>% G2</th>
<th>Cell cycle</th>
<th>Radiation effect</th>
<th>Toxicity</th>
</tr>
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<tr>
<td>H460 DN p53</td>
<td>LCC</td>
<td>DEL?</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>78 (66)</td>
<td>4</td>
<td>13</td>
<td>1.71 (0.15)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>H460</td>
<td>LCC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>79 (61)</td>
<td>11</td>
<td>10</td>
<td>1.14 (0.02)</td>
<td>50</td>
<td></td>
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<tr>
<td>A549</td>
<td>NSCLC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>90 (72)</td>
<td>2</td>
<td>8</td>
<td>1.45 (0.02)</td>
<td>60</td>
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</tr>
<tr>
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<td>NSCLC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>77 (55)</td>
<td>13</td>
<td>9</td>
<td>1.01 (0.01)</td>
<td>85</td>
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</tr>
<tr>
<td>H1975</td>
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<td>MUT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>69 (49)</td>
<td>10</td>
<td>21</td>
<td>1.65 (0.02)</td>
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</tr>
<tr>
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<td>NSCLC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>82 (68)</td>
<td>15</td>
<td>3</td>
<td>1.3 (0.51)</td>
<td>66</td>
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<tr>
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<td>NSCLC</td>
<td>MUT</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>DEL</td>
<td>/–/–</td>
<td>64 (51)</td>
<td>16</td>
<td>20</td>
<td>1.0 (0.03)</td>
<td>56</td>
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<tr>
<td>H82</td>
<td>SCLC</td>
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<td>/–/–</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>51 (49)</td>
<td>20</td>
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<td>MCF10A</td>
<td>Normal breast</td>
<td>Amp</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>87 (49)</td>
<td>5</td>
<td>8</td>
<td>1.03 (0.12)</td>
<td>59</td>
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<tr>
<td>H460 plates</td>
<td>LCC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>94 (92)</td>
<td>2</td>
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<tr>
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<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
<td>DEL</td>
<td>WT</td>
<td>89 (79)</td>
<td>7</td>
<td>4</td>
<td>1.04 (0.07)</td>
<td>49</td>
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</table>

NOTE: Values in parentheses are the % G1 of untreated cells. MCF710A are immortalized normal breast epithelial cells. Mutational status of most cell lines determined based on Blanco and colleagues (26) and Molina-Vila and colleagues (48). Toxicity was based on a 10 μmol/L abemaciclib exposure for 24 hours. Abbreviations: Amp, gene amplification; DMF, dose-modifying factor; DN, dominant negative gene; LCC, large cell carcinoma; MUT, Mutated gene; NSCLC, non-small cell lung cancer; PE, plating efficiency; SCLC, small cell lung cancer; SD, standard deviation; WT, wild-type gene; (–/–) represents null status for gene.
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 Ser611/807 (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 H82c 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.

**Figure 2.**
Effect of abemaciclib on radiation induced DNA damage repair and the CDK4/6-RB pathway in NSCLC cells. A, Western blot analysis of phosphorylated γH2AX in isolated histones from H460 and H1299 cell lines as a function of time following treatment with abemaciclib (10 μmol/L) and IR (7.5 Gy). Individual experiment densitometry results were normalized based on the corresponding lane H2A-loading control, and values beneath each row indicate fold change (FC) derived from 3 independent experiments. B and C, Immunoblot showing expression of DNA damage repair pathway proteins in H460 cells as function of time following treatment with abemaciclib + IR (7.5 Gy) combination. Densitometry analysis of three independent experiments in H460 cells (C) and H1299 cells (D) after IR and abemaciclib treatment. The expression of p-ATR, p-ATM, and p-Chk2 was normalized based on their corresponding total protein expression. Error bar, mean ± SEM from three independent experiments. Statistical significance is represented as *P < 0.05; **P < 0.005; and ***P < 0.0005 over DMSO control.

**Figure 3.**
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 3C, 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...
were delivered immediately after the abemaciclib doses (immediately after radiation and 6 hours later) treatment through oral gavage. Initially, two daily fractionated IR (three 2 Gy fractions) with daily abemaciclib treatment. Steady-state levels of 116 metabolites in H460 cells using targeted capillary electrophoresis time of flight (CE-TOF) and tandem triple quadrupole mass spectrometry (QqQMS) was determined. Glycolytic intermediates such as fructose 1,6 bisphosphate were elevated (**, P < 0.005) after 24 hours of abemaciclib treatment, whereas downstream intermediates such as 2-PG (2-phosphoglycerate) and 2-PEP (2-phosphoenolpyruvate) were reduced (**, P < 0.005 and *, P < 0.05 , respectively) suggesting that abemaciclib altered the activity of glyceraldehyde 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 < 0.0001), suggesting a possible amino acid deficiency (nutrient stress) in H460 cells (Fig. A 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 treat-
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 NSCLC have been discouraging (http://www.ascopost.com/News/58135). There are, nonetheless, potential combinatorial therapies where CDK4/6 inhibitors may be beneficial. Preclinical studies have indicated that palbociclib can sensitize KRAS-mutant NSCLC cells to MEK inhibitor and EGFR-TKI (22). Ribociclib was shown to sensitize PIK3CA-mutant breast cancer to PI3K inhibitors (41). Preclinical studies have shown palbociclib to modify its response to breast cancer (42), glioblastoma (43), and medulloblastoma (44). Our preclinical data strongly support abemaciclib, as a potent radiation modifier in NSCLC, whereas our pilot studies indicate that palbociclib and ribociclib did not yield IR enhancement. This could possibly be tumor-type-dependent effect of the drug, drug concentration, and the IR treatment strategy used in our study. Despite CDK inhibitor successes in managing patients with advanced disease (14), there are still challenges in the optimization of CDK inhibitors in clinical practice. A major hurdle is lack of predictive biomarkers to screen appropriate patient populations for better therapeutic outcome. While previous in vitro studies suggested RB, cyclin D, and p16 could predict the response to palbociclib (45), results from phase II/III trials showed no significant correlation between drug response and the expression of p16 (45), Ki-67, cyclin D1 amplification (46), PIK3CA or ESR1 (47) mutational status, leaving no established prognostic or predictive biomarkers.

The preclinical findings of the current study provide the basis for future clinical trials that are biomarker integrated in NSCLC by combining abemaciclib with IR. Clonogenic cell survival data across a panel of cell lines harboring genotypic mutations in key driver proteins involved in the progression of NSCLC such as mutation in KRAS, EGFR-TR domain, p53, RB, and p16 indicated that abemaciclib when administered immediately post-IR primarily radiosensitized cells with functional p53 and RB proteins, but was independent of the mutational status of EGFR, KRAS, and p16 protein (Fig. 1 and Table 1). Interestingly, H1975 cells were radiosensitized by abemaciclib (Fig. 1A) but have a mutated p53 (Table 1; ref. 48). It has been reported for NSCLC that certain p53 mutations can be nondisruptive leading to a Gain-of-function (GOF) and patients with these mutations have poorer prognosis than patients with disruptive p53 mutations (48). H1975 cells used in this study was also found to have the R273H mutation through whole-exon sequencing (data not shown) thus suggesting a possible explanation for our IR sensitization results. Additional research is certainly needed to clarify this observation that in some cases abemaciclib can radiosensitize p53-mutated cells.

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 μM/L for 24 hours leads 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 vivo. 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 vitro 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 trial studies evaluating AMD3100 (plerixafor) after radiation therapy and temozolomide...
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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Enhancement of Radiosensitivity by Abemaciclib in NSCLC

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

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