Antiproliferative Effects of CDK4/6 Inhibition in CDK4-amplified Human Liposarcoma in vitro and in vivo

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Abstract

Well-differentiated/dedifferentiated liposarcomas (WD/DDLPS) are among the most common subtypes of soft tissue sarcomas. Conventional systemic chemotherapy has limited efficacy and novel therapeutic strategies are needed to achieve better outcomes for patients. The cyclin-dependent-kinase 4 (CDK4) gene is highly amplified in over 95% of WD/DDLPS. In this study, we explored the role of CDK4 and the effects of NVP-LEE011 (LEE011), a novel selective inhibitor of CDK4/CDK6, on a panel of human liposarcoma cell lines and primary tumor xenografts. We found that both CDK4 knockdown by siRNA and inhibition by LEE011 diminished RB phosphorylation and dramatically decreased liposarcoma cell growth. Cell cycle analysis demonstrated arrest at G0/G1. siRNA-mediated knockdown of RB rescued the inhibitory effects of LEE011, demonstrating that LEE011 decreased proliferation through RB. Oral administration of LEE011 to mice bearing human liposarcoma xenografts resulted in approximately 50% reduction in tumor 18F-fluorodeoxyglucose uptake with decreased tumor biomarkers including RB phosphorylation and bromodeoxyuridine incorporation in vivo. Continued treatment inhibited tumor growth or induced regression without detrimental effects on mouse weight. After prolonged continuous dosing, reestablishment of RB phosphorylation and cell cycle progression was noted. These findings validate the critical role of CDK4 in maintaining liposarcoma proliferation through its ability to inactivate RB function, and suggest its potential function in the regulation of survival and metabolism of liposarcoma, supporting the rationale for clinical development of LEE011 for the...
treatment of WD/DDLPS.
Introduction

The retinoblastoma (RB) tumor suppressor gene plays a critical role in regulating cellular proliferation, and loss of function of the RB protein is commonly found in human malignancies (1, 2). RB function is regulated by phosphorylation of multiple serine and threonine residues, initiated during the G₁/S cell cycle transition by cyclin-dependent kinases (CDK) 4 or 6 (3-5). These enzymes, in turn, are activated by temporal expression of cyclin D, and inhibited by the CDK inhibitor p16 (6, 7). Multiple mechanisms for dysregulation of this pathway in cancer have been identified, including mutation or loss of expression of RB (2), overexpression of cyclin D (8, 9), loss of p16 (10), and mutation, genomic amplification, or overexpression of CDK4 (11).

Over 95% of human well-differentiated/dedifferentiated liposarcomas (WD/DDLPS) exhibit dysregulation of CDK4 through high level amplification of a region of the long arm of chromosome 12 containing this gene (12, 13). WD/DDLPS represent one of the most common forms of soft tissue sarcomas (14, 15). WDLPS is a typically indolent, non-metastasizing disease that can be cured by surgical excision, although local recurrences are common. DDLPS may arise from WDLPS or de novo, and may also be cured by surgical resection, although its rapid growth rate, predilection for retroperitoneal growth, and ability to be locally destructive or metastasize frequently results in recurrences and patient mortality. Clinical benefit from conventional systemic chemotherapy is limited, transient, and often toxic, indicating that newer therapeutic strategies are needed to better treat this disease (16, 17).
One potential approach to treatment of liposarcoma is through targeting the enzymatic activity of CDK4 with novel small molecule inhibitors. Previous studies have preliminarily demonstrated the in vitro effects of CDK4 inhibition in liposarcoma cells: Helias-Rodzewicz et al first reported that treatment of a liposarcoma cell line with the CDK4 inhibitor NSC625987 decreased cell proliferation and induced adipocytic differentiation (18). Barretina et al showed cell cycle arrest and growth inhibition in two liposarcoma cell lines treated with CDK4 shRNA or the CDK4/6 inhibitor PD0332991 (19). A more recent study also reported that the addition of an IGF1R inhibitor to PD0332991 led to greater inhibition of cell cycle progression and cell metabolic activity (20). However, detailed biochemical analyses and the preclinical in vivo effects of CDK4/6 inhibitors on liposarcoma xenograft models have not yet been described, and predictive biomarkers for CDK4/6 inhibitor activity are still lacking. Recently, several CDK4/6 inhibitors have entered into clinical development, and an exploratory clinical trial suggested activity against liposarcoma (21). In this study, we explore the biochemical, antiproliferative, and antimetabolic effects of CDK4 inhibition in more detail than has previously been reported, using siRNA and the novel small molecule CDK4/6 inhibitor NVP-LEE011 (LEE011) in both cell line and patient-derived tumor xenograft models of WD/DDLPS.

**Materials and Methods**

**Cell Lines and Cell Culture**

Human WD/DDLPS cell lines 449 and 778 (also called 93449 or T449 and
94778 or T778) (22) were kindly provided by Dr. Florence Pedeutour (Université de Nice-Sophia Antipolis, Nice, France) and Dr. David Thomas (Peter MacCallum Cancer Centre, Melbourne, Australia) in 2008; LPS141 (23) was kindly provided by Dr. Jonathan A. Fletcher (Brigham and Women's Hospital, Boston, MA) in 2008; LP3, LP6 (23), and LP8 were generated at Dana-Farber Cancer Institute in 2008. Cell lines were cultured in RPMI 1640 supplemented with 15% fetal bovine serum (Hyclone, Logan, Utah), 1x penicillin-streptomycin-amphotericin B (Invitrogen, Carlsbad, CA) and 1x glutamax (Invitrogen) at 37°C in a humidified incubator with 95% air and 5% CO₂. Human white preadipocyte primary cells were purchased from PromoCell (C-12732, Lot# 0071202.27, Heidelberg, Germany) and cultured in the Preadipocyte Growth Medium (PromoCell). For differentiation of human preadipocytes, confluent preadipocyte cultures were incubated with Preadipocyte Differentiation Medium (PromoCell) for 72 hrs, and then changed to Adipocyte Nutrition Medium (PromoCell) for 14 days. Adipogenic differentiation was confirmed via Oil red O staining as previously described (24). Liposarcoma cell lines were characterized by high-resolution short tandem repeat (STR) profiling with Promega PowerPlex ®1.2 system at the Molecular Diagnostics Laboratory of Dana-Farber Cancer Institute. The cells used for the experiment are passaged for fewer than 6 months after authentication.

Inhibitors and siRNA

The CDK4/6 inhibitor NVP-LEE011 (succinate salt, powder form) (LEE011) was synthesized by the Novartis Institutes for Biomedical Research (Cambridge,
MA)(25). ON-TARGETplus siRNA against *RB1* (#J-003296-10, 13), *CDK4* (#J-003238-12, 13), and scrambled control were purchased from Dharmacon (Layfayette, CO). Cells were transfected with siRNA at a final concentration of 3.12-6.25 nM with RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

**Copy Number Analysis**

Gene copy number was determined by probe-based quantitative real-time PCR using StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). Human male normal genomic DNA was purchased from PE Biosystems (Foster City, CA). Genomic DNA from cell cultures was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). Primers and probes were obtained from the TaqMan® copy number assays catalog of Applied Biosystems (CDK4, Hs06345580_cn; RNase P, 4403326). The *Rnase P* gene was used as an internal normalization reference. Each reaction was performed in a total volume of 20 μl, containing 1× Taqman® Genotyping Master Mix, 1× CDK4 Primer-Probe Mix, 1× RNase P Primer-Probe Mix and 20 ng genomic DNA. All reactions were performed in quadruplicate and repeated at least three times. PCR thermocycling conditions consisted of an initial step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The threshold cycle (Ct) level for tested genes was automatically determined by the StepOnePlus™ Software. *CDK4* copy number was determined by CopyCaller software (version 1.0, Applied Biosystems) using human normal genomic DNA as a calibrator sample.
**Immunoblotting**

Cells were lysed on ice in lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 10% Glycerol, 1% Triton X-100, 100 mM NaF, 10 mM Na₄P₂O₇⋅10 H₂O, 1 mM Na₃VO₄, and 1x Complete® Protease Inhibitor Cocktail (Roche Diagnostics, Germany). Antibodies for immunoblotting were purchased from Santa Cruz, Santa Cruz, CA (CDK4 #sc-260); Sigma, St. Louis, MO (α-tubulin #T9026); and Cell Signaling, Danvers, MA (p-RB (Ser780) #9307, p-RB (Ser807/811) #9308, RB #9309, CDK6 #3136, Cyclin D1 #2978, Cyclin D2 #3741, Cyclin D3 #2936, p15 #4822, p16 #4824, p18 #2896). Chemiluminescent signal was captured with X-ray film.

**Cell Proliferation Assay**

Cells were exposed to various treatments (inhibitor, siRNA or vehicle control) for times as indicated. Cell numbers were determined using a Neubauer haemocytometer.

To determine the half maximal growth inhibitory concentration (GI₅₀) values of LEE011 in liposarcoma cells, cell numbers were counted before treatment (T₀) and after 3 days of vehicle control (C) or LEE011 treatment (T). Drug response was calculated following the formula: Percentage Growth=100 × (T - T₀)/(C - T₀). GI₅₀ values were determined using sigmoidal dose-response (variable slope) curve fitting with Prism 5 software (GraphPad Software).

**Cell Cycle Analysis by Flow Cytometry**

Cells were exposed to inhibitors or 0.1% DMSO for 24 hr and harvested.
washing with ice-cold PBS, cells were fixed in 70% ethanol at 4 ºC for at least 2 hr. Fixed cells were stained in PBS containing 10 μg/mL RNase A and 20 μg/mL propidium iodide (Sigma, St. Louis, MO) in the dark. DNA content analysis was performed by flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA) with CellQuest and ModFIT LT software (BD Biosciences).


The LPS3 primary human liposarcoma xenograft model was established by implanting fresh human liposarcoma tissue fragments into the subcutaneous tissue of female nude mice (Nu/Nu, Charles River Laboratories, Wilmington, MA) after signed informed consent was previously obtained from a patient undergoing surgery, according to an Institutional Review Board-approved research protocol. LPS3 tumor xenografts were serially passaged as subcutaneous implants of tumor fragments approximately 2 mm in diameter derived from tumors less than 1000 mm³.

The LP6 cell line was derived from the same patient’s liposarcoma tissue, and for cell line xenograft experiments approximately 1 x 10⁶ cells were suspended in PBS, mixed 1:1 with Matrigel (BD Biosciences), and subcutaneously injected into female nude mice (Nu/Nu, Charles River Laboratories) in a final volume of 100 μl.

The HSAX2655 tumor was obtained under collaboration with the National Cancer Institute (Bethesda, MD), and originated from a retroperitoneal liposarcoma. Tumor xenografts were serially passaged in female nude mice.
Harlan Laboratories, Indianapolis, IN) as subcutaneous implants of tumor fragments approximately 3 mm in diameter.

**In vivo Bromodeoxyuridine (BrdU) Incorporation Assay.**

Mice bearing LP6 tumors (>200 mm³) were treated with 250 mg/kg LEE011 formulated as a 25 mg/ml suspension in 0.5% methylcellulose (#S80080, Fisher Scientific, Fair Lawn, NJ) or vehicle control by oral gavage for three daily doses. BrdU solution (10 mg/ml, 0.2 ml/mouse) was injected intraperitoneally 16 hours after the last dose of study drug or control. After 2 additional hours, mice were sacrificed and tumors were fixed in 10% formalin for immunohistochemical analysis or snap frozen for immunoblot analysis.

**18F-FDG-PET Functional Imaging Study**

Matched cohorts of mice with LP6 tumors (300 mm³ average) were randomly assigned to treatment with LEE011 (250 mg/kg) or vehicle control by oral gavage for three daily doses. Before randomization (T₀) and after 3 days of therapy (T₃), mice were evaluated by ¹⁸F-fluorodeoxyglucose (FDG)-PET imaging (26). Each mouse was fasted overnight before imaging and then was administered ~400 uCi ¹⁸F-FDG (~0.2 ml) through intraperitoneal injection. Mice were warmed and awake during a 60 min tracer uptake period, and then anesthetized before undergoing 10 min PET scan. The maximum standardized uptake value (SUVmax) in tumor was recorded, and the change in SUVmax after therapy was calculated as % Change SUVmax= 100x (SUVmax (T₃)- SUVmax (T₀))/SUVmax (T₃).
In vivo Efficacy Studies

Mice bearing LPS3 tumor xenografts (>2 mm in diameter and with volumes <100 mm³) were randomized into statistically identical cohorts (6 mice/group), and treated with 250 mg/kg LEE011 or with vehicle alone by oral gavage following a 5 days on/2 days off schedule for three weeks. Mice with established LP6 cell line xenograft tumors (average tumor volume >250 mm³) were randomized into statistically identical cohorts (≥8 mice/group), and treated with 250 mg/kg LEE011 or with vehicle alone by oral gavage daily for 21 days. Tumor size was measured by caliper every 3-6 days, and volume was calculated using the formula: Volume = 0.5xLxW². Mouse body weight was recorded every 3-7 days. Mice were sacrificed when the tumor diameter reached 2 cm.

Mice bearing HSAX2655 tumor xenografts were treated with 250 mg/kg LEE011 or vehicle control daily by oral gavage beginning 35 days post-implantation (4 mice in control group, 12 mice in LEE011 group), when the tumor volume reached an average size of 258 mm³, and continued for 80 days at which point drug treatment was suspended. Mice with tumors that regrew were retreated with 250 mg/kg LEE011 daily when tumors reached a volume of >500 mm³. Body weight and tumor volume were recorded twice weekly.

All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Dana-Farber Cancer Institute or Novartis Biomedical Research Institutes.
Immunohistochemistry

Immunohistochemistry was performed on four-micron sections of formalin-fixed paraffin-embedded (FFPE) samples. Tissue sections were deparaffinized, and rehydrated, and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) in a 750 W microwave oven at 199 °C for 30 minutes for BrdU staining, and in a pressure cooker at 120°C for 5 minutes followed by 90°C for 10 seconds for phospho-RB staining. Phospho-RB (Ser780) (Abcam, Cambridge, MA, Ab47763), or BrdU primary antibody (BD Biosciences, #347580) was added at a dilution of 1:100 and incubated for 1 hour at room temperature. Sections were further processed with horseradish peroxidase-conjugated secondary antibody. The reaction was detected by 3,3-diaminobenzidine and hematoxylin staining. Images were obtained with an Olympus CX41 microscope and QCapture software (QImaging, Surrey, Canada).

Statistical Analysis

Comparisons between groups were made using the two-tailed unpaired t-test. Differences in means ± SEM with $P < 0.05$ were considered statistically significant.

Results

RB is Highly Expressed and Strongly Phosphorylated in Human Liposarcoma Cells

We determined $CDK4$ copy number in the 6 liposarcoma cell lines and the
LPS3 and HSAX2655 primary human liposarcoma xenografts and found high-level CDK4 gene amplification (Fig. 1A and data not shown) in each, recapitulating the setting of liposarcoma tumors. Correspondingly, CDK4 protein was expressed at a higher level in liposarcoma cells in comparison to normal preadipocytes and adipocytes (Fig. 1B).

We then examined the expression and phosphorylation level of RB as well as the status of other G1-S transition regulatory proteins in liposarcoma cells. As shown in Fig. 1B, RB was highly expressed and strongly phosphorylated at the CDK4/6-specific sites Ser780 and Ser807/811 (5) in liposarcoma cells in comparison to normal preadipocytes and adipocytes. Cyclin D1, p15, and p16 were expressed in all of the liposarcoma cells lines. CDK6 and Cyclin D2 were only detectable in a subset. These data demonstrate increased CDK4 and RB expression/activity in human liposarcoma cells.

**CDK4 Knockdown Inhibits RB phosphorylation and Cell Growth in Liposarcoma Cells**

To explore the role of CDK4 in regulation of RB phosphorylation and cell growth, we applied CDK4 siRNAs to LP6 liposarcoma cells and investigated their biological effects. As shown in Fig. 2A, the expression of CDK4 was dramatically decreased by CDK4 siRNAs. CDK4 knockdown resulted in a decrease in RB phosphorylation at the CDK4-specific sites Ser807/811 compared to controls (Fig. 2A). Moreover, growth in CDK4 siRNA transfected cells was completely blocked at 24 hr after transfection (Fig. 2B). These results demonstrate that CDK4 is a
major regulator of RB phosphorylation and cell growth in liposarcoma cells.

**LEE011, a Selective CDK4/6 Inhibitor, Inhibits RB Phosphorylation, and Blocks Cell Proliferation in Liposarcoma Cells**

LEE011 is a novel, selective CDK4/6 inhibitor in clinical development (Supplementary Fig. S1) (25, 27). In order to demonstrate its impact on tumor cells in vitro, we examined the effects of LEE011 in liposarcoma cells. LEE011 reduced RB phosphorylation at Ser780 and Ser807/811 in both a concentration- and time-dependent manner with complete inhibition at 3.33 μM (Fig. 3A and B, Supplementary Fig. S2). Correspondingly, 0.04-3.33 μM LEE011 decreased LP6 cell growth in a concentration-dependent manner, with sustained growth arrest following 24 hrs of treatment of 1.11 and 3.33 μM LEE011 (Fig. 3C and D). Cell cycle analysis demonstrated cell cycle arrest at G0/G1 and a decreased proportion of cells in S phase following 24 hr of exposure to LEE011 (Fig. 3E).

The growth inhibitory potential of LEE011 was examined in five additional liposarcoma cell lines, and similar effects were observed in each (Fig. 3D and 3F). LEE011 inhibited cell growth in a concentration-dependent manner with GI50 value of 0.13-0.24 μM (Fig. 3D) and complete inhibition at 3.33 μM, and dramatically decreased the proportion of cells in S phase (Fig. 3F). These data demonstrate the ability for LEE011 to induce cell cycle arrest and inhibit cell growth in a variety of liposarcoma cell lines.
LEE011 Inhibited Liposarcoma Cell Cycle Progression in a RB-Dependent Manner

To determine the specificity of the inhibitory effects of LEE011 on cell cycle progression, we transfected cells with two independent siRNA constructs to RB and then examined the effects of LEE011 on the RB-depleted liposarcoma cells. As shown in Fig. 4A and 4B, the observed induction of cell cycle arrest by LEE011 were dramatically abrogated in LP6 cells transfected with RB siRNA, in comparison to cells transfected with control siRNA or without siRNA. Therefore, RB is a key mediator of LEE011-induced cell cycle arrest and diminished cell growth in liposarcoma cells.

LEE011 Reduces RB Phosphorylation, BrdU Incorporation and Tumor FDG Uptake in vivo

In order to demonstrate the impact of LEE011 on tumor behavior in vivo, we employed a xenograft model of liposarcoma cell line LP6 and further investigated the effects of LEE011 treatment on RB phosphorylation and cell proliferation. As shown by immunoblot and immunohistochemistry staining, following treatment with 3 daily doses of LEE011 (250 mg/kg/d), RB phosphorylation at Ser780 was dramatically reduced and RB protein shifted to its hypophosphorylated form (Fig. 5A and B). In addition, in vivo BrdU incorporation into tumors was significantly decreased (Fig. 5B), indicative of reduced cell proliferation. We attempted to determine the in vivo effects on cellular proliferation using 3'-deoxy-3'[^18]F]-fluorothymidine (^18F-FLT)-PET imaging, but there was no significant baseline
FLT-PET signal in the liposarcoma xenograft models examined (data not shown).

In order to assess the impact of LEE011 treatment on tumor metabolism, we evaluated the in vivo $^{18}$F-FDG-PET response to LEE011 in mice bearing the LP6 liposarcoma tumor xenograft model. Baseline $^{18}$F-FDG-PET scans consistently identified aberrantly high tumor glucose utilization in the LP6 models (SUVmax: ~2.0 or higher). After 3 days of treatment, the SUVmax in the LP6 tumors of those mice treated with LEE011 dropped significantly by approximately 50%. In contrast, the tumor SUVmax in vehicle-treated mice was either unchanged or slightly increased (Fig. 5C and D). These results suggest that LEE011 substantially alters tumor metabolism in the liposarcoma xenograft model.

**LEE011 Inhibits Tumor Growth in vivo**

To determine whether the observed biological effects of LEE011 treatment could be translated into clinically relevant antitumor activity, liposarcoma growth was studied in established LP6 cell line xenografts as well as in LPS3 and HSAX2655 primary human liposarcoma tumor xenografts following LEE011 treatment. As shown in Fig. 5E and 5F, LEE011 significantly decreased growth of both LP6 (250 mg/kg/d, 21 days, p.o.) and LPS3 xenografts (250 mg/kg, 5 days on/2 days off for 3 weeks, p.o.) during the treatment ($p<0.001$ for LP6 xenografts, $p<0.05$ for LPS3 xenografts). No significant weight loss of the mice was observed in the mice treated with LEE011 (Supplementary Fig. S3A and S3B). The aggressively growing LP6 cell line xenograft eventually grew despite continued treatment with LEE011, albeit at a significantly slower rate than in mice treated
with vehicle control (Fig. 5E). In contrast, the LPS3 primary liposarcoma xenograft continued to respond over the treatment period. Daily oral treatment with LEE011 (250 mg/kg) resulted in dramatic and durable tumor regression of the HSAX2655 primary liposarcoma xenografts, reaching 90% after 80 days of continuous treatment (Fig. 5G). No significant weight loss of the mice was observed in the HSAX2655-carrying mice treated with LEE011 (Supplementary Fig. S3C). We continued to monitor animals for tumor regrowth after suspension of drug treatment. Two out of 12 mice did not have palpable tumors at study termination on day 206 (91 days post-treatment cessation) and were considered complete regression. Tumors regrew in the remaining animals with varying sensitivity to retreatment with LEE011 (data not shown).

**Cell Cycle Re-Entry Following Continuous Chronic CDK4/6 Inhibition in vitro**

To further determine the possible causes of LP6 cell line xenograft tumor growth in the setting of prolonged LEE011 administration in contrast to the effects seen with short-term dosing, we examined the phosphorylation status of RB and the cell cycle dynamics. Chronic continuous exposure to LEE011 led to gradual recovery of RB hyperphosphorylation at the CDK4/6-specific sites S780 and S807/811 and release from cell cycle arrest after 4, 7, or 17 days (Fig. 6A). Concomitant with these changes, increases in cyclins D1, D2, and D3 expression were observed (Fig. 6B), which may reflect a compensatory feedback mechanism driving cell cycle progression. The appearance of RB
hyperphosphorylation and escape from cell cycle arrest were completely reversible: following a 3 day washout period from LEE011, repeat exposure to drug again resulted in suppression of RB phosphorylation and induction of G₀/G₁ arrest (Fig. 6C). Cyclin D2 expression similarly reverted to normal levels following drug washout but was re-induced in the presence of LEE011 (Fig. 6D). These data suggest a dynamic mechanism that may be induced with chronic exposure and which permits escape from cell cycle arrest independent from presumed genetic selection of treatment-resistant subclones.

Discussion

In this report, we define the critical role of CDK4 overexpression and activity in regulating liposarcoma growth in vitro and in vivo. By using siRNA or a small molecule inhibitor of CDK4/6, we observed RB hypophosphorylation, cell cycle arrest, decreased DNA synthesis, decreased tumor glucose metabolism, and model-dependent tumor growth arrest or regression. The CDK4 gene is significantly amplified in the vast majority of WD/DDLPS. Our findings validate the critical role of CDK4 in maintaining liposarcoma proliferation (18), metabolism, and survival, supporting the rationale for clinical development of CDK4 inhibitors for the treatment of WD/DDLPS. The inhibitory effects of LEE011 on cell cycle progression required RB; therefore, the status of RB may be useful as a selective or predictive biomarker for clinical studies of CDK4/6 inhibitors.

Distinct from the cell cycle inhibitory effects seen in vitro, LEE011 induced tumor regression in one primary patient tumor xenograft model. This may reflect
a greater dependency on CDK4/6 signaling in the context of the tumor/stromal environment, distinct from cell lines selected for their growth on plastic. Alternatively, this finding may indicate that in some tumor models the endogenous apoptosis rate may become revealed by way of tumor regression once cell proliferation is abrogated and the cell growth/death balance is altered. A similar observation has been reported in studies with PD0332991 in other cancer types (28), wherein no cell death was observed in vitro, while in vivo treatment resulted in complete regressions in some tumor models. In addition, a CDK4/6 inhibitor caused apoptosis in Notch1-driven T cell acute lymphoblastic leukemia (T-ALL) (29), suggesting that synthetic lethal interactions between CDK4/6 inhibition and other pathways may drive cell death.

Based both on the proposed mechanism of action as well as our observations reported herein, the clinical consequences of CDK4 inhibition by LEE011 treatment may range from cell cycle arrest to tumor regression. This should be factored into translationally-focused clinical studies to determine the activity of CDK4 inhibitors in liposarcoma: the clinical trials should be designed primarily to detect changes in tumor growth rates rather than to solely measure radiographic shrinkage of tumor (conventional rates of anatomic tumor “response”). Indeed, a recent clinical study of the CDK4/6 inhibitor PD0332991 in patients with liposarcoma demonstrated a median progression free survival of 18 weeks, with a radiographic response rate of only 3.4% (21). The significance of disease control rates in uncontrolled, single arm Phase 2 studies is difficult to assess, however, and appropriate comparisons to the pre-study tumor growth rate or to
control treatment arms should be included (30).

Additionally, our findings suggest that chronic treatment with a CDK4/6 inhibitor can lead to gradual recovery of RB hyperphosphorylation at the CDK4/6-specific sites S780 and S807/811 and re-entry into the cell cycle in one liposarcoma model. The breakthrough of cell cycle arrest does not appear to be due to selection of subclones of LP6 cells that are resistant to LEE011 on the basis of genetic alterations. Instead, removal of LEE011 led to rapid re-establishment of sensitivity to drug, suggesting a dynamic feedback mechanism that may support some level of cell cycle re-entry in this model, although the durable tumor control seen in the LPS3 and HSAX2655 xenografts indicates that this may not be a universal effect. The Phase II study of PD0332991 in liposarcoma used a 14 day on/7 day off dosing schedule, selected on the basis of determination of the maximum tolerated dose in a Phase I study (31). Our observations suggest that this intermittent schedule may have been a fortuitous study design that enhanced anti-tumor activity by avoiding this potential feedback mechanism, although further exploration of dosing schedules with correlative pharmacodynamic studies will be essential to maximize RB hypophosphorylation, cell cycle arrest, and anti-tumor activity.

Compensatory upregulation of cyclin D1, D2, and D3 expression was also observed following LEE011 treatment. The precise mechanism of cyclin D induction and its biochemical consequences remain to be determined, although this was not the trivial result of accumulation of cells at the G1/S boundary, since flow cytometry analysis showed a decreased proportion of cells at this point in
the cell cycle as they again re-established a normal cell cycle profile.

Deregulated expression of cyclin D has been shown to promote mitogen-independent proliferation as well as other cellular processes (9, 32). Whether the elevated level of cyclin D following LEE011 treatment contributed to the recovery of RB hyperphosphorylation and re-entry into the cell cycle requires further investigation.

WD/DDLPS characteristically also have amplification of the MDM2 gene (12), the product of which inhibits the activity of the p53 tumor suppressor and targets it for proteasomal degradation (33-35). By reactivating p53, small molecule inhibitors of the MDM2-p53 interaction induce cell cycle arrest and apoptosis (36). Inhibitors of this sort are currently in clinical development, and a recent study showed limited activity in patients with liposarcoma (37). Whether combined inhibition of CDK4 and MDM2 would lead to greater anti-tumor activity remains an area of active exploration.

In summary, our study reveals the important CDK4-RB signaling axis that directly regulates cell cycle and cell proliferation for the growth of liposarcoma in vitro and in vivo. Treatment with a CDK4/6 inhibitor is effective, but the waning response with continuous exposure suggests a reversible feedback mechanism that should be further explored and taken into account in the design of rationally-designed translational clinical trials employing small molecule CDK4/6 inhibitors.

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Figure Legends

Figure 1. CDK4 Copy Number and Expression of Cell Cycle Regulatory Proteins in Liposarcoma Cells

A. CDK4 copy number in liposarcoma cells and a patient-derived xenograft (PDX) was determined by quantitative real-time PCR with the Rnase P gene as an internal normalization reference and human normal genomic DNA as a calibrator sample. Values represent mean ± SD (n ≥3)

B. Protein expression was analyzed by immunoblots. PA, preadipocytes; A, adipocytes.

Figure 2. Effects of siRNA-mediated Knockdown of CDK4 on RB Phosphorylation and Cell Growth in Liposarcoma Cells

A. Effects of CDK4 knockdown on RB phosphorylation were examined by immunoblot at 40 hr in LP6 liposarcoma cells.

B. Effects of CDK4 knockdown on growth of LP6 cells were monitored daily by cell counting.

Figure 3. Effects of CDK4/6 Inhibitor LEE011 on RB Phosphorylation and Cell Growth in Liposarcoma Cells

A-B. Effects of LEE011 on RB phosphorylation in LP6 (A) and other liposarcoma cells (B) were evaluated by immunoblot at 24 hr.

C. Growth curves of LP6 cells treated with vehicle or LEE011 at indicated concentrations. Values represent mean ± SEM (n=2)
D. Response of liposarcoma cells to 3 days treatment of LEE011. Values represent mean ± SD (n=2).

E-F. Effects of LEE011 on cell cycle distribution of liposarcoma cells at 24 hr. In Figure E, LP6 cells were used; in Figure F, Values represent mean ± SD (n=2).

**Figure 4. siRNA-mediated Knockdown of RB Rescues the Inhibitory Effects of LEE011 in Liposarcoma Cells.**

A. siRNA-mediated knockdown of RB expression at 48 hr.

B. LP6 cells were transfected with RB siRNA, control siRNA, or buffer only. LEE011 was added 48 hrs after transfection and its effects on cell cycle distribution were examined by flow cytometry analysis at 24 hr. Data are representative of three independent experiments.

**Figure 5. Effects of LEE011 Treatment on RB Phosphorylation, BrdU Incorporation, Tumor FDG Uptake, and Tumor growth in vivo.**

A-B, After 3 doses of LEE011 (250 mg/kg/d) or vehicle control, tumor samples were analyzed for in vivo BrdU incorporation. A. Protein expression and phosphorylation in frozen LP6 tumor specimens were evaluated by immunoblots. B. Representative examples of immuno-histochemistry staining for BrdU and phospho-RB (Ser780) in LP6 tumor xenografts. Original magnification: 200×.

C-D, FDG-PET response after 3 doses of LEE011 (250 mg/kg/d) or vehicle
control. **C.** Change in $^{18}$F-FDG SUVmax of LP6 tumors. Each bar represents a tumor lesion. **D.** Representative PET imaging of LP6 xenografts. Small arrows indicate the anatomical location of tumor xenograft. Other areas of $^{18}$F-FDG signal represent the brain, heart, and bladder.

**E.** Established LP6 tumors were treated with 250 mg/kg LEE011 or with vehicle alone daily for 21 days by oral gavage. Tumor size was measured by caliper every 3-6 days. Mice were sacrificed when the tumor diameter reached 2 cm. Values represent mean volume ± SEM (n≥8). *, p<0.05; **, p<0.01; ***, p<0.001; compared with respective control group treated with vehicle.

**F.** Primary human liposarcoma xenograft LPS3 was treated with 250 mg/kg LEE011 or with vehicle alone by oral gavage following a 5 days on/2 days off schedule for 3 weeks. Tumor size was measured by caliper every 3-4 days. Values represent mean volume ± SEM (n=6). *, p<0.05; compared with respective control group treated with vehicle.

**G.** Primary human liposarcoma xenograft HSAX2655 was treated with 250 mg/kg LEE011 or with vehicle alone daily by oral gavage beginning 35 days after implantation and continued for 80 days. Tumor size was measured by caliper twice weekly. Values represent mean volume ± SEM (n=4 in control group, n=12 in LEE011 group).

**Figure 6. Continuous Exposure to LEE011 Leads to Enhanced RB Phosphorylation and Reversible Cell Cycle Re-Entry.**

**A-B.** LP6 cells were continuously exposed to LEE011 for the indicated time and
concentrations. RB phosphorylation (A, upper panel) and the expression of cyclin D (B) were examined by immunoblots 24 hours after the last dose, and cell cycle distribution was determined by flow cytometry analysis (A, lower panel).

C-D. LP6 cells with or without pretreatment were exposed to LEE011 for 24 hrs. RB phosphorylation (C, upper panel) and the expression of cyclin D2 and CDK4 (D) were examined by immunoblots. Cell cycle distribution was determined by flow cytometry analysis (C, lower panel).
Figure 1

A. CDK4 Copy number

B. Normal Cells Liposarcoma Cells

<table>
<thead>
<tr>
<th>Normal Cells</th>
<th>Liposarcoma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>A</td>
</tr>
</tbody>
</table>

CDK4
CDK6
p-RB (S780)
p-RB (S807/811)
RB
α-Tubulin

Normal Cells Liposarcoma Cells

<table>
<thead>
<tr>
<th>Normal Cells</th>
<th>Liposarcoma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>A</td>
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</tbody>
</table>

Cyclin D1
Cyclin D2
p15
p16
α-Tubulin
Figure 2

A

<table>
<thead>
<tr>
<th>Ctrl siRNA</th>
<th>CDK4 siRNA</th>
<th>A</th>
<th>B</th>
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</thead>
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<tr>
<td>CDK4</td>
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<td></td>
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<tr>
<td>p-RB (S807/811)</td>
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<td></td>
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</tr>
<tr>
<td>RB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

B

Cell Number (x10^5)

Days of Treatment

- Ctrl siRNA
- CDK4, siRNA
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>A</th>
<th>B</th>
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</thead>
<tbody>
<tr>
<td>RB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing % S phase vs LEE011 (µM)]

Key:
- No siRNA
- Control siRNA
- RB siRNA

Legend:
- No siRNA
- Control siRNA
- RB siRNA
**Figure 5**

**A**

<table>
<thead>
<tr>
<th>LEE011</th>
<th>-</th>
<th>+</th>
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<tbody>
<tr>
<td>p-RB (S780)</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>RB</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Mouse #</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>LEE011</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td><img src="image13" alt="Image" /></td>
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<tr>
<td>p-RB (S780)</td>
<td><img src="image15" alt="Image" /></td>
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<td>Mouse #</td>
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</tbody>
</table>

**C**

FDG-PET response after 3 doses

%Change SUV

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<tr>
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<th>Vehicle</th>
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<tbody>
<tr>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
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**D**

<table>
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<tr>
<td>Mouse ID #1</td>
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**E**

LP6 Cell Line Xenografts

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<th>Tumor Volume (mm³)</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td><img src="image23" alt="Image" /></td>
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<tr>
<td>Days of treatment</td>
</tr>
</tbody>
</table>

**F**

LPS3 Primary Tumor Xenografts

<table>
<thead>
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<th>Tumor Volume (mm³)</th>
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<tbody>
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<td>0</td>
</tr>
<tr>
<td><img src="image29" alt="Image" /></td>
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<tr>
<td>Days of treatment</td>
</tr>
</tbody>
</table>

**G**

HSAX2655 Primary Tumor Xenografts

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
<td><img src="image35" alt="Image" /></td>
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<tr>
<td>Days post implantation</td>
</tr>
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</table>
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>1.1 μM</th>
<th>3.3 μM</th>
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<tbody>
<tr>
<td>Day</td>
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<td>1</td>
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<tr>
<td>p-RB (S780)</td>
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<td></td>
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<tr>
<td>p-RB (S807/811)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>1.1 μM</th>
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<tbody>
<tr>
<td>Day</td>
<td>0</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
</tr>
<tr>
<td>Cyclin D2</td>
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<tr>
<td>Cyclin D3</td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
</tr>
</tbody>
</table>

C

Pretreatment: None 20 d
Treatment: 0 1.1 3.3 17 d at 1.1 μM 3.3 μM then 3 d washout

D

Pretreatment: None 20 d
Treatment: 0 1.1 3.3 1.1 3.3 0 1.1 0 3.3 μM

% of Cells

G0/G1

S

G2/M

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Antiproliferative Effects of CDK4/6 Inhibition in CDK4-amplified Human Liposarcoma in vitro and in vivo

Yi-Xiang Zhang, Ewa Sicinska, Jeffrey T. Czaplinski, et al.

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