Pharmacologic inhibition of cyclin-dependent kinase 4/6 activity arrests proliferation in myoblasts and rhabdomyosarcoma-derived cells


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Abstract
Myoblast cell cycle exit and differentiation are mediated in part by down-regulation of cyclin D1 and associated cyclin-dependent kinase (Cdk) activity. Because rhabdomyosarcoma may represent a malignant tumor composed of myoblast-like cells failing to exit the cell cycle and differentiate, we considered whether excess Cdk activity might contribute to this biology. Cyclin D-dependent Cdk4 and Cdk6 were expressed in most of a panel of six human rhabdomyosarcoma-derived cell lines. Cdk4 was expressed in 73% of alveolar and embryonal rhabdomyosarcoma tumors evaluated using a human tissue microarray. When challenged to differentiate by mitogen deprivation in vitro, mouse C2C12 myoblasts arrested in G1 phase of the cell cycle, whereas four in the panel of rhabdomyosarcoma cell lines failed to do so. C2C12 myoblasts maintained in mitogen-rich media and exposed to a Cdk4/Cdk6 inhibitor PD 0332991 accumulated in G1 cell cycle phase. Similar treatment of rhabdomyosarcoma cell lines caused G1 arrest and prevented cell accumulation in vitro, and it delayed growth of rhabdomyosarcoma xenografts in vivo. Consistent with a role for Cdk4/Cdk6 activity as a regulator of myogenic differentiation, we observed that PD 0332991 exposure promoted morphologic changes and enhanced the expression of muscle-specific proteins in cultured myoblasts and in the Rh30 cell line. Our findings support the concept that pharmacologic inhibition of Cdk4/Cdk6 may represent a useful therapeutic strategy to control cell proliferation and possibly promote myogenic differentiation in rhabdomyosarcoma.

Introduction
In mammalian cells, biochemical events in the nucleus promote the transition from G1 phase into S phase of the cell cycle past the “restriction point,” beyond which cell cycle progression can continue in the absence of extracellular stimuli (reviewed in ref. 1). The sequential activation of cyclins and their catalytic partners cyclin-dependent kinases (Cdk) is central to this transition. Activated cyclin/Cdk complexes phosphorylate the retinoblastoma gene product (Rb), thereby blocking its ability to control the G1-S phase transition. In the prevailing model, biochemically similar D-type cyclins (D1, D2, and D3) with their specific partners Cdk4 and Cdk6 function early in G1 phase followed by activation of cyclin E/Cdk2 as cells enter the S phase (reviewed in refs. 1, 2). It is likely that functional redundancy among these cyclins allows some mammalian cells to proliferate, although they lack a full complement of the genes (3–5). Nonetheless, their general importance in cell proliferation suggests that Cdk inhibition may represent a useful therapeutic strategy for cancer.

In addition to promoting cell proliferation, cyclin/Cdk activity couples extracellular cues to the onset of terminal differentiation in certain types of cells (reviewed in refs. 6, 7). This regulatory pathway is particularly well established for skeletal myoblasts, in which differentiation can be induced in vitro by cultivation in mitogen-poor media. Myogenic lineage commitment and differentiation are controlled by the expression and activity of four members of the MyoD family of basic helix-loop-helix transcription factors, MyoD, Myogenin, Myf5, and Mrf4 (8–10), which work with members of the Mef2 family of transcription factors (9, 11) to induce their own expression and that of other skeletal muscle proteins in carefully orchestrated patterns (reviewed in refs. 9, 12, 13). A number of observations indicate that D-type cyclins and Cdk4/Cdk6 control the activity of myogenic transcription factors in proliferating myoblasts. Cyclin D1 decreases (14–16) and several Cdk inhibitors increase during muscle differentiation (17–21). Ectopically expressed cyclin D1, and to a lesser extent cyclin D2, inhibits the expression of skeletal muscle genes (14, 15), whereas forced expression of p21Waf1/Cip1 or the Cdk4/Cdk6-specific inhibitor p16INK4a reverses mitogen-dependent repression of muscle-specific
promoters (15). Although Rb is required for differentiating myocytes to exit the cell cycle and to efficiently induce the expression of a number of skeletal muscle genes (22, 23), cyclin D–mediated inhibition of muscle gene expression is not strictly dependent on Rb protein hyperphosphorylation (24). Because of the importance of cyclin D–associated Cdk4/Cdk6 activity as a regulator of myogenic differentiation, we considered whether its pharmacologic inhibition might be useful in cancer derived from this lineage.

Rhabdomyosarcoma, a childhood cancer thought to be derived from skeletal myoblasts or their progenitors, is the most common soft tissue sarcoma in children (25). Most rhabdomyosarcoma fall into one of two biologically distinct subgroups represented by alveolar and embryonal histology (26). Regardless of this distinction, rhabdomyosarcoma-derived cells seem to represent myoblast-like cells arrested in a partially differentiated state. Supporting molecular evidence includes the facts that (a) most rhabdomyosarcoma tumor samples and rhabdomyosarcomaderived cell lines express MyoD and Myogenin mRNA and protein (27–29), but (b) although endogenous and ectopically expressed MyoD can bind consensus DNA elements in rhabdomyosarcoma-derived cell lines, it fails to efficiently activate muscle-specific promoters when challenged to differentiate in vitro (30). Interestingly, this “molecular phenotype” for MyoD is similar to what occurs when cyclin D1 is ectopically expressed in myoblasts (24). Despite a multidisciplinary approach, including surgical resection, irradiation, and intensive chemotherapy, most children with metastatic rhabdomyosarcoma die of their disease (31). New therapeutic strategies based on better understanding of rhabdomyosarcoma biology are needed.

We have taken advantage of archived pathology material and established mouse myoblast and human rhabdomyosarcoma-derived cell lines to begin to address whether Cdk4/Cdk6 inhibition is effective in preclinical rhabdomyosarcoma models. We specifically focused on determining (a) whether Cdk4 or Cdk6 expression could be detected in human rhabdomyosarcoma tumor samples and cell lines; (b) if the inability to exit the cell cycle and differentiate is correlated with expression of Cdk4 or Cdk6 in rhabdomyosarcoma-derived cell lines; (c) whether the exposure of myoblasts or rhabdomyosarcoma cells to a selective Cdk4/Cdk6 inhibitor PD 0332991 (32, 33) forces their exit into G1 phase of the cell cycle; and (d) if this compound induces morphologic or molecular evidence of enhanced muscle differentiation in myoblasts or rhabdomyosarcoma-derived cells.

Materials and Methods

Cell Lines and Growth Conditions

C2C12 mouse skeletal myoblasts, obtained from the American Type Culture Collection (Manassas, VA), were maintained as previously described (15). Human alveolar rhabdomyosarcoma cell lines Rh18 (derived from a tumor of mixed alveolar/embryonal histology), Rh28, Rh30, and Rh41 and embryonal rhabdomyosarcoma cell lines Rh36, RD, and JR-1 were previously described (34–39). They were cultivated at 37°C and 5% CO2 in 10% fetal bovine serum (FBS) in RPMI 1640 (GM; Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Cells were induced to differentiate by culture for 24 to 96 hours in DM (2% HS in DMEM or 1% FBS in RPMI 1640 for myoblasts and rhabdomyosarcoma cells, respectively). For C2C12 myoblasts, serum deprivation using 1% FBS in RPMI was done in experiments primarily evaluating cell cycle effects, whereas 2% HS was used for promoting differentiation. PD 0332991 (32), provided by Pfizer Global R&D, Michigan Labs (Ann Arbor, MI), was resuspended in DMSO and used at a concentration of 1,000 nmol/L in cell culture medium.

Flow Cytometry for Cell Cycle Analysis

For cell cycle analysis, rhabdomyosarcoma cells and myoblasts were cultured with 10% or 1% FBS, with PD 0332991 or vehicle, for 48 hours. Trypsinized cells and cells floating in the medium were stained with propidium iodide (0.05 mg/mL; Sigma-Aldrich, St. Louis, MO) in 0.1% sodium citrate, 0.1% Triton X-100. For bromodeoxyuridine (BrdUrd) staining, cells were cultured in medium containing 10 μmol/L BrdUrd (BD Biosciences, San Jose, CA) for 2 hours before harvest. Cells were trypsinized and labeled with anti-BrdUrd primary and species-specific secondary antibodies using a commercially available kit (BD Biosciences, San Jose, CA). Stained cells were counted using either a FACSscan or FACScalibur (BD Biosciences) with histogram analysis by ModFitLT software (v.3.0; Verity Software House, Topsham, ME). Data shown are representative of two or three independent experiments with duplicate or triplicate samples.

Cell Accumulation Assay

RD, Rh30, and Rh36 cells were seeded onto six-well plates at 5 × 103 per well and cultured at 37°C and 5% CO2 in 10% FBS. Six sets of triplicate wells were plated for each cell line. The following day (day 0), half of the wells of each cell line were refed with GM containing PD 0332991 (1,000 nmol/L) in DMSO or an equivalent amount of vehicle. On days 3, 6, and 9, triplicate wells were harvested, and the total number of cells were counted via hemocytometer; remaining wells were refed with either PD 0332991 or DMSO as above.

Xenograft Studies

The ability of PD 0332991 to control rhabdomyosarcoma growth in vivo was evaluated in Rh18, Rh28, and Rh30 cells growing as xenografts in CB17/Scid female scid−/− mice (Charles Rivers, Wilmington, MA). To accomplish this, single tumor fragments were injected s.c. into the mice, as described previously (40, 41). Tumor-bearing mice were randomized into groups of 5 to 10 before therapy and maintained under barrier conditions. All experiments were conducted using protocols and conditions approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee. When the tumors were ~0.2 to 1 cm diameter, mice bearing tumors were treated with 150 mg/kg of PD 0332991 given by oral gavage twice a day for 1 week, then every other day. Tumor diameters were
measured every 7 days using Vernier calipers interfaced with a Macintosh computer. Tumor volumes were calculated, assuming tumors to be spherical, using the formula: \((\pi/6) \times d^3\), where \(d\) is the mean diameter. Tumor volumes were determined for 12 weeks after starting treatment or until individual tumors reached quadruple their volume from the initiation of therapy.

**Western Blot Analysis**

Cells were harvested in cold lysis buffer [20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% NP40 (Igepal), 10% glycerol, 0.1% SDS, supplemented with 10 \(\mu\)g/mL leupeptin, 1 \(\mu\)g/mL pepstatin, 10 \(\mu\)g/mL aprotinin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethanol sulfonate fluoride, 1 mmol/L DTT, and 20 mmol/L \(\beta\)-glycerophosphate], sonicated, and clarified by centrifugation. For immunoblotting of D-type cyclins, cell lysates were prepared by suspending cells in universal lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 25 mmol/L \(\beta\)-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 0.1 mmol/L phenylmethanol sulfonate fluoride, 5 \(\mu\)g/mL leupeptin, 0.2% Triton X-100, 0.5% NP40] on ice for 30 minutes followed by centrifugation and protein quantitation, determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein (20–100 \(\mu\)g) were fractionated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Blotted proteins were detected using mouse anti-Myogenin (F5D; BD Biosciences), mouse anti-Cdk4 (DCS-35; Santa Cruz Biotechnology), mouse anti-cyclins D1, D2, and D3 (BD PharMingen: DCS-6, G132-43, G107-205), and rabbit polyclonal anti-Cdk4 antibody (C-22, Santa Cruz Biotechnology). In some experiments, 100 nmol/L PD 0332991 or an equal amount of volume of vehicle was added to the kinase reaction mix. Products of the kinase reaction were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Blotted proteins were detected using mouse anti-Myogenin (F5D; BD Biosciences), mouse anti-MyoD (5.8A; ref. 28); mouse anti-Myosin heavy chain (MF-20; American Type Culture Collection); mouse anti-Cdk4 (DCS-35; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-cyclins D1, D2, and D3 (BD PharMingen: DCS-6, G132-43, G107-205, respectively); and goat anti-HSC 70 (Santa Cruz Biotechnology), used as a loading control. Primary antibodies were detected with the species-specific horseradish peroxidase–coupled secondary antibodies (Kirkegaard and Parry Laboratories, Gaithersburg, MD) and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

**Antibody-Based Staining**

Rh30 cells (4 \(\times\) 10^5) were plated on 10-cm tissue culture dishes. One day later, culture medium was replaced with GM or DM with or without 1,000 nmol/L PD 0332991. Twenty-four to 48 hours later, cells were fixed with 2% paraformaldehyde, permeabilized with 0.25% Triton X-100 in PBS, blocked using 4% nonfat dry milk in PBS, and probed with anti-Myogenin antibody followed by rhodamine red-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Dual immunofluorescence staining for Myogenin and BrdUrd was done essentially as described (42). Stained cells, covered with aqueous mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), were visualized by immunofluorescence microscopy using a BX60 Olympus microscope (Olympus America, Inc., Melville, NY) equipped with a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI). Quantitative analyses were done by counting stained cells in 5 to 10 fields at \(\times 400\) magnification; statistical significance was assessed by unpaired t test.

**Immunohistochemical Staining for human Cdk4**

Immunohistochemical staining for human Cdk4 was done using two human rhabdomyosarcoma tissue microarrays obtained from the Cooperative Human Tissue Network (Columbus, OH). Experiments using human rhabdomyosarcoma tissue microarrays were approved by the St. Jude Children’s Research Hospital Institutional Review Board.

Monoclonal antibodies reactive with human Cdk4 were obtained from Santa Cruz Biotechnology (C-22). Heat-induced epitope retrieval was done, and 3,3'-diaminobenzidine/H_2O_2 (Biogenex, San Ramon, CA) was used as a chromogen with hematoxylin as the counterstain. Immunostaining was done in duplicate on sections from tissue microarray blocks comprising one to several representative tissue cores from embryonal or alveolar rhabdomyosarcoma tumors. Immunostaining was considered positive if neoplastic cells exhibited unequivocal nuclear, cytoplasmic, or both nuclear and cytoplasmic immunostaining. The extent of immunostaining was determined by counting at least 200 neoplastic cells in each of the tissue cores and calculating the average percentage for each case. Cases were considered positive if, on average, \(\geq 20\%\) of neoplastic cells were positive for Cdk4. Statistical significance was assessed by Fisher’s exact test.

**Immunoprecipitation and In vitro Cdk4 Assay**

Immunoprecipitation and kinase assays were done essentially as described (43) using subconfluent C2C12 cells and rabbit polyclonal anti-Cdk4 antibody (C-22, Santa Cruz Biotechnology). In some experiments, 100 nmol/L PD 0332991 or an equal amount of volume of vehicle was added to the kinase reaction mix. Products of the kinase reaction were resolved by 10% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue, destained, and dried. Proteins were visualized by autoradiography of the dried gel. Immunoprecipitation of Rb was done by harvesting cells into radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, with 10 \(\mu\)g/mL leupeptin, 1 \(\mu\)g/mL pepstatin, 10 \(\mu\)g/mL aprotinin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethanol sulfonate fluoride, 1 mmol/L DTT, and 20 mmol/L \(\beta\)-glycerophosphate] followed by sonication, centrifugation, and protein quantitation. Cell lysates, normalized to protein content, were immunoprecipitated with anti-Rb antibody (BD PharMingen, G3-245) and protein A/G-Sepharose beads. Immunoprecipitated proteins were separated using 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Rb protein was detected using the same antibody; Rb phosphorylation status was assessed with anti-phospho-Ser780 antibody (Cell Signaling Technology, Beverly, MA).
Results

Expression of Cyclin D–Dependent Cdns in Alveolar and Embryonal Rhabdomyosarcoma Cells and Tumors

Deregulation of the cyclin/Cdk pathway was previously implicated as an important part of rhabdomyosarcoma biology (44). However, little work has directly addressed that in human rhabdomyosarcoma tissue. We determined whether cyclin D–dependent Cdk4 or Cdk6 might contribute to rhabdomyosarcoma biology by evaluating their expression in rhabdomyosarcoma-derived cell lines and human tumor samples. Relatively high expression of Cdk4 was detectable in Rh18, Rh30, and Rh36; all six rhabdomyosarcoma-derived cell lines expressed high levels of Cdk6 (Fig. 1A, lanes 2–7). Little to no Cdk4 or Cdk6 was detectable in Rh1 cells, which are derived from a primitive neuroectodermal tumor (ref. 45; Fig. 1A, lane 1). D-type cyclins were detectable in seven rhabdomyosarcoma-derived cell lines, with each line expressing at least two of the three (Fig. 1B). Immunohistochemical staining for Cdk4 was done on two human rhabdomyosarcoma tissue microarrays containing 39 and 35 unique cases of alveolar and embryonal rhabdomyosarcoma, respectively. Cdk4 was detected in 32 of 39 (82%) cases of alveolar rhabdomyosarcoma and 22 of 35 (63%) cases of embryonal rhabdomyosarcoma (Fig. 1C). The difference in Cdk4 expression between embryonal and alveolar tumors was not statistically significant ($P = 0.07$). The majority of Cdk4-positive cases exhibited strong nuclear and cytoplasmic immunostaining in the tumor cells.

Defective Cell Cycle Exit and Differentiation in Most Rhabdomyosarcoma-Derived Cells

If a component of rhabdomyosarcoma biology was driven by deregulated Cdk4/Cdk6 expression, we reasoned that rhabdomyosarcoma-derived cell lines would fail to arrest when cultured in mitogen-poor media. We evaluated this using seven rhabdomyosarcoma-derived cell lines. When exposed to mitogen-poor media for 48 hours, four of seven tested cell lines failed to accumulate in G1 phase of the cell cycle (Fig. 2A). One was derived from an embryonal rhabdomyosarcoma; three were derived from alveolar tumors. The three rhabdomyosarcoma cell lines showing G1 accumulation included two derived from embryonal rhabdomyosarcoma and one derived from a tumor of mixed alveolar/embryonal histology. The G1 accumulation in rhabdomyosarcoma cells ($\sim 2–15\%$ change) was smaller than that in serum-deprived mouse myoblasts ($\sim 26\%$ change; Fig. 2A). Pulse labeling with BrdUrd verified that serum deprivation did not decrease the relative amount of DNA synthesis in Rh30 cell line (Fig. 2B), which was the focus of additional work (see below). The expression of Cdk4 or Cdk6 did not correlate with the ability of these cells to arrest with serum deprivation; all six cell lines tested had equivalent levels of Cdk6. One of the three lines with relatively low Cdk4 (RD) and two of the three lines with relatively high levels (Rh18 and Rh36) showed some G1 arrest.

Myogenic differentiation is closely coupled with G1 phase cell cycle arrest. When cultivated in serum-deficient medium, normal skeletal myoblasts arrest and induce the expression of Myogenin within 24 hours (Fig. 2C, lanes 7, 8, 13, and 14). Rh18 and Rh36, both of which exhibited an increase in the fraction of G1-arrested cells, also displayed small increases in Myogenin (Fig. 2A and C). Myogenin protein was not induced in three other rhabdomyosarcoma cell lines, including RD cells that also showed small G1 arrest with serum deprivation. As with cell cycle arrest, the
relative expression of Cdk4 or Cdk6 did not correlate with
the capacity to induce Myogenin. Because enhanced
Myogenin represents an early event in skeletal muscle
differentiation, this finding suggests that certain rhado-
myosarcoma cell lines retain early aspects of the differen-
tiation program. The differentiation is abortive, however, as
we did not observe enhanced Myosin heavy chain
expression, a terminal differentiation marker, in any of
the rhabdomyosarcoma cells (negative data not shown).

**Exposure of Myoblasts and Rhabdomyosarcoma-
Derived Cells to a Novel Cdk4 Inhibitor Causes G₁ Cell
Cycle Arrest**

Although neither D cyclins nor Cdk4/Cdk6 activity is
strictly required for cell proliferation during mouse
embryogenesis (5, 46), their abrupt disruption by ectopic
expression of p16 Ink4a arrests cell proliferation in an Rb-
dependent manner (47). Because Cdk4/Cdk6 expression
was frequently detectable in rhabdomyosarcoma-derived
cell lines and tumors, we considered whether its pharma-
cologic inhibition might alter their biology. PD 0332991 is a
pyridopyrimidine that reversibly blocks cyclin D–associ-
ated Cdk4 and Cdk6 (33). In vitro studies indicate that PD
0332991 is remarkably specific with IC₅₀s of <0.015 μmol/L
for Cdk4 or Cdk6 and IC₅₀s >10 μmol/L for many other
kinases, including Cdk1, Cdk2, and Cdk5 (33). We,
therefore, determined whether exposure of myoblasts and
rhabdomyosarcoma-derived cells to PD 0332991 would
arrest their proliferation and block their accumulation
in vitro and in vivo.

Cdk4 protein could be immunoprecipitated from C2C12
mouse myoblasts cultured in serum-replete growth medi-
um (Fig. 3A, left). Immunoprecipitated Cdk4 retained the
ability to phosphorylate bacterial-expressed glutathione
S-transferase-Rb protein in vitro, and this activity increased
in myoblasts cultured in GM versus DM (Fig. 3A, right). The
addition of 100 nmol/L PD 0332991 to the kinase reaction
blocked Cdk4-mediated glutathione S-transferase-Rb phos-
phorylation (Fig. 3A, right). When PD 0332991 was added
directly to culture medium, we were unable to detect a
decrease in Cdk4 activity because the reversible inhibition
of Cdk4 by PD 0332991 is likely disrupted with multiple
washes of the immunoprecipitated proteins. Nonetheless,
consistent with its capacity to block Cdk4 activity in vitro,
exposure of C2C12 myoblasts to 1,000 nmol/L PD 0332991
for 48 hours caused cells to accumulate in G₁ phase at the
expense of cells in S phase (Fig. 3B), and it decreased the
fraction of cells incorporating BrdUrd (Fig. 3C). PD 0332991
had little effect on the cell cycle arrest achieved by culture in
mitogen-poor medium (Fig. 3B), which suggests that serum
depression and PD 0332991 disrupt the same pathway.

PD 0332991 displayed a similar capacity to arrest
rhabdomyosarcoma-derived cells. In all seven cell lines, it
caused accumulation in G₁ phase at the expense of cells in S
or G₂-M phase (or both) despite the presence of serum-rich
medium (Fig. 4A). As in myoblasts, little apoptosis was
observed (data not shown). The greatest effect was
observed in Rh18 cells, with relatively high Cdk4 expres-
sion (Fig. 1A), in which the fraction in G₁ phase increased
by 40%. Even in Rh30 cells where the G₁ arrest was modest,
PD 0332991 decreased phosphorylation of Rb at residue
S²⁷⁸⁰ (Fig. 4B), which is a Cdk4-specific phosphorylation
site (48). The effect seemed to be durable, as evidenced by
persistent cell cycle arrest 6 days after exposure to PD
0332991 in Rh18, Rh30, RD, and JR-1 cells (data not shown).
Moreover, the arrest seemed to be significant because
continuous exposure of cells to PD 0332991 over a 9-day
period blocked their accumulation in vitro (Fig. 4C).
Blocked cell accumulation, which was more dramatic than
expected given the relatively small changes in cell cycle
profile (Fig. 4A), could have been due to greater cumulative
inhibition of Cdk4/Cdk6 activity or more subtle proapop-
totic effects not observed with transient drug exposure.

Whether PD 0332991 could inhibit tumor growth in vivo
was tested using Rh18, Rh28, and Rh30 rhabdomyosarco-
ma-derived cell lines implanted as xenografts into

![Figure 2](image-url)
immunodeficient mice. Administration of PD 0332991 to xenograft-bearing mice hindered tumor growth (Fig. 4D). Similar to what was observed in vitro, it slowed Rh18 growth to a greater extent than Rh28 and Rh30 cells. That the tumors did not regress and that growth accelerated upon drug withdrawal implied that the drug has cytostatic effects in vivo. PD 0332991 Enhances the Expression of Muscle-Specific Genes Associated with Differentiation in Myoblasts and Rh30 Cells

As mentioned above, changes in cyclin D–associated Cdk activity can influence myogenic differentiation. We considered whether PD 0332991 could augment the expression of early or later markers of myogenic differentiation. Both MyoD and Myogenin increased between 24 and 48 hours in myoblasts maintained in mitogen-rich GM (Fig. 5A, lanes 1 and 4). Myogenin increased in myoblasts exposed to mitogen-poor DM (Fig. 5A, lane 2 versus lane 1 and lane 5 versus lane 4). These changes are consistent with an autoregulatory loop enhancing the expression of these transcription factors as an early response to cell confluence and mitogen deprivation (12). PD 0332991 did not enhance the expression of either myogenic regulatory factor in cells cultured in DM (Fig. 5A, lanes 3 and 6 versus lanes 2 and 5; Fig. 5B, lanes 4 and 8 versus lanes 3 and 7), but it did promote earlier expression of Myosin heavy chain, a marker of late muscle differentiation, under these conditions (Fig. 5A, lane 6 versus lane 5 or lane 11 versus lane 12). At 96 hours, though, PD 0332991 did not augment the robust differentiation induced by prolonged mitogen deprivation (Fig. 5A, lane 9 versus lane 8 or lane 14 versus lane 15). Earlier evidence of differentiation might be due to earlier or more complete cell cycle arrest observed in myoblasts cultured in DM and PD 0332991 (Fig. 5C). However, this did not measurably alter the expression of myogenic regulatory factors.

Figure 4. Established rhabdomyosarcoma cell lines and C2C12 myoblasts accumulate in G1 when exposed to the Cdk4 inhibitor PD 0332991. A, chart showing data compiled from fluorescence-activated cell sorting analysis of the indicated propidium iodide–stained cells cultured in GM (PD 0332991 for 48 h). Columns, means of duplicates from a representative experiment for each cycle phase; bars, SD. B, immunoprecipitation-Western blot for Rb and Rb phosphorylation at Ser780, from Rh30 cells cultured for 48 h with or without PD 0332991. C, representative charts showing numbers of the indicated rhabdomyosarcoma cells when plated (day 0) and after cultivation for 3, 6, and 9 d in the presence of PD 0332991 (PD) or the drug vehicle DMSO (Vh). D, chart showing rhabdomyosarcoma xenograft growth in untreated mice (a, c, and e) or mice treated (b, d, and f) with 2 or 6 wks of PD 0332991 (black columns). Tumors from individual mice are represented by single lines.
lanes 4 after culture in GM or DM with or without PD 0332991 for the indicated experiments. C, not due to sample spillover. Representative Western blots of two separate differentiations was observed.5 The Rh30 cell line was cultured with PD 0332991, little morphologic evidence of Mhc transcript was notable exception: elongated cells emerged when exposed to 1% FBS with PD 0332991 (Fig. 6A). The ability of the compound to induce a G1 cell cycle arrest in Rh30 cells was produced a more robust G1 arrest (Fig. 5C).

We also evaluated whether PD 0332991 enhanced Myogenin expression when myoblasts were maintained in mitogen-rich GM. In this case, we were concerned that potential positive effects of PD 0332991 on Myogenin expression due to its Cdk4/Cdk6 inhibition might be masked by negative effects due to consequent lower cell confluence. To control for this, the effect of PD 0332991 was evaluated in subconfluent myoblasts plated so that at the time of analysis, equivalent cell number and confluence were present with or without PD 0332991. Under these conditions, 48 hour of exposure to PD 0332991 in GM-fed myoblasts increased Myogenin over those cultured in GM alone (Fig. 5B, lane 6 versus lane 5 or lane 10 versus lane 11). Of note, the modest differentiation-promoting effects of PD 0332991 under these conditions may be due to the fact that cell cycle arrest induced by the drug is not entirely equivalent to that induced by cultivation in DM, which produced a more robust G1 arrest (Fig. 5C).

When rhabdomyosarcoma-derived cell lines were cultured with PD 0332991, little morphologic evidence of differentiation was observed.5 The Rh30 cell line was a Cdk4/Cdk6 inhibitor p16 Ink4a in increasing Myogenin expression when myoblasts were maintained in 10% or 1% FBS (data not shown). Myogenin increased in Rh30 cells maintained with PD 0332991 in either GM or DM (Fig. 6B). This compound did not induce Myosin heavy chain, though, under these conditions (negative data not shown). Increased Myogenin expression was also evident as increased number of Myogenin-expressing cells detected by immunofluorescence staining (Fig. 6C). In addition to increased numbers of Myogenin-expressing cells, PD 0332991 increased the fraction of Myogenin-positive cells that were BrdUrd negative from 52.9% to 92.3% (P = 0.047) for cells cultured in GM without or with PD 0332991, respectively (Fig. 6D).

Discussion

Based on our findings, we propose that Cdk4/Cdk6 inhibition has potential as a therapeutic strategy for rhabdomyosarcoma. Most alveolar and embryonal rhabdomyosarcoma-derived cell lines and tumor samples expressed cyclin D–dependent Cdk4 and Cdk6. Consistent with deregulation of this pathway, rhabdomyosarcoma-derived cell lines did not efficiently exit the cell cycle or undergo complete myogenic differentiation when challenged to do so in vitro. Cdk4/Cdk6 inhibition with PD 0332991, however, forced their arrest in G1 phase of the cell cycle and slowed their accumulation in vitro and in vivo. Moreover, this facilitated skeletal muscle differentiation in mitogen-stimulated myoblasts and in one rhabdomyosarcoma cell line. This was manifested by enhanced morphologic changes, increased expression of Myogenin, and increased numbers of Myogenin-expressing cells, most of which had exited the cell cycle. The cell cycle arrest induced by Cdk4/Cdk6 inhibitors may offer therapeutic benefit to patients with rhabdomyosarcoma; whether subtle effects on cellular differentiation are also relevant is not clear.

It is important to recognize that cell cycle arrest by genetic or pharmacologic strategies to block Cdk4/Cdk6 depends on functional Rb protein (33, 47). The status of the Rb gene has not been systematically evaluated in rhabdomyosarcoma, but the fact that PD 0332991 arrested all seven of the rhabdomyosarcoma cell lines in our studies implies that the gene is intact. This is consistent with previous findings that the Rb protein is detectable by immunohistochemical staining in ~83% of embryonal rhabdomyosarcoma and 65% of alveolar rhabdomyosarcoma (49). Existing dogma holds that when the Rb gene is not mutated, the Rb “pathway” is disrupted by other mechanisms like increased cyclin/Cdk activity or loss of function of cyclin/Cdk inhibitors (2). Our observation that most rhabdomyosarcoma tumors express Cdk4 and all tested cell lines expressed Cdk6 supports this idea. In at least some cases, enhanced Cdk4 expression in rhabdomyosarcoma may be due to amplification of its chromosomal locus (12p13; refs. 26, 50). The activation of both Cdk4 and Cdk6 is likely driven by cyclins D1 and D2, either or both of which we found to be expressed in each of the rhabdomyosarcoma-derived cell lines in our study, as was shown by other investigators as well (51). Loss of expression of the Cdk4/Cdk6 inhibitor p16 Ink4a in ~33% (49) and the

5J.L. Bills and S.X. Skapek, unpublished observation.
deletion of the gene encoding it (CDKN2A) in 25% (52) of human rhabdomyosarcoma provide further ways to deregulate Cdk4/Cdk6 activity. That rhabdomyosarcoma tumors display numerous mechanisms to disrupt the Rb pathway suggests that frequent Rb gene mutations may not limit the applicability of Cdk4/Cdk6 inhibition in rhabdomyosarcoma.

There is no reason to predict that expression of Cdk4 or Cdk6 in childhood cancers should be limited to rhabdomyosarcoma. Cdk4 is detectable in 74% of osteosarcoma (53); Cdk4, Cdk6, or both are overexpressed, relative to normal kidney, in 63% of Wilms’ tumors (54). Both Rb and Cdk4 are detectable in 13 Ewing’s sarcoma cell lines examined (55). Cdk4 mRNA is detectable in 19 neuroblastoma cell lines and in all six primary tumors tested (56). Targeted therapeutic strategies depend on the expression of the relevant protein, but the expression of the protein itself may not necessarily correlate with effects of its inhibition. For example, in our studies, the relative expression Cdk4 or Cdk6 did not strictly correlate with how well PD 0332991 arrested cell proliferation. Cdk activity is positively and negatively regulated by a number of different proteins. Hence, its expression level may not always correlate with its activity. Better characterization of the functional status of Cdk4/Cdk6 in other childhood cancers seems to be warranted as one considers the broader use of this therapeutic approach.

Targeting cyclin D–dependent Cdk4 or Cdk6 in rhabdomyosarcoma is particularly intriguing because of its potential to also influence the myogenic differentiation program, a component of which includes irreversible cell cycle arrest when Rb is intact (22, 57). Although inhibition of Cdk4 and/or Cdk6 would be expected to induce a hypermitogenic G1 arrest rather than a hypomitogenic G0 arrest, a hypermitogenic arrest can still be associated with cell differentiation or senescence (58). We observed some evidence for a promyogenic effect of PD 0332991 in Rh30 cells exhibiting enhanced Myogenin expression and proliferation arrest in Myogenin-expressing cells following drug exposure. Admittedly, these changes do not represent terminal differentiation, but even subtle changes in differentiation status may still be biologically relevant. Emerging findings from gene expression profiling indicate that patients with rhabdomyosarcoma characterized by molecular evidence of a more differentiated state have a better clinical outcome, independent of histology and other covariates. Whether and how prodifferentiation effects of Cdk4/Cdk6 inhibition might alter rhabdomyosarcoma biology to improve prognosis should be further evaluated using available preclinical models.

That inhibition of Cdk4/Cdk6 did not promote complete myogenic differentiation in rhabdomyosarcoma was not unexpected. First, as mentioned above, hypomitogenic and hypermitogenic cell cycle arrest achieved by culture in mitogen-poor media and exposure to PD 0332991 may

6 T.J. Triche, Children’s Hospital Los Angeles, personal communication.
not be exactly equivalent. Early myoblast studies found that different phases of G1 are more or less permissive for myogenic differentiation (59). Hence, the inability of PD 0332991 to robustly force myogenic differentiation in myoblasts and to only promote differentiation in one rhabdomyosarcoma cell line may be due to subtle, cell line–specific differences in the nature of the arrest. Our fluorescence-activated cell sorting analyses cannot distinguish “early” and “late” G1 arrest that would be more or less permissive for myogenic differentiation. Second, although PD 0332991 decreased Rb phosphorylation in Rh30 cells, residual Ser780 phosphorylation (Fig. 4B) implied that Cdk4/Cdk6 inhibition was not complete. Third, cyclin D/Cdk4/Cdk6 represents only one of a number of regulatory mechanisms for myogenic transcription factors (reviewed in reference ref. 13). Abnormalities in others may also influence rhabdomyosarcoma cell biology and impede myogenic differentiation. For example, expression of the Twist gene, which inhibits MyoD and Mef2-dependent gene expression (60), is detectable in rhabdomyosarcoma tissue samples (61) and some rhabdomyosarcoma-derived cell lines.7 The Mdm2 gene is amplified in some rhabdomyosarcoma tissue samples and cell lines (39, 62), and its gene product can block myogenic differentiation (13), but this fails to occur in some rhabdomyosarcoma-derived cell lines (64). Finally, the capacity of MyoD to promote myogenic differentiation may be impeded in rhabdomyosarcoma by associated histone deacetylase activity, present in undifferentiated myoblasts (65, 66). Conceivably, more complete differentiation might be achieved in rhabdomyosarcoma by combining Cdk4/Cdk6 inhibition with strategies to manipulate other regulatory pathways.

Ultimately, it will be important to define the optimal use of Cdk4/Cdk6 inhibitors in appropriate preclinical rhabdomyosarcoma models. In our experiments, PD 0332991 as a single agent showed relatively modest, likely cytostatic effects on grossly evident xenografts. It will be critical to assess whether this dosing schedule was able to effectively distinguish “early” and “late” G1 arrest that would be more or less permissive for myogenic differentiation. It will be critical to consider the role of Cdk4/Cdk6 inhibition in combination with other cytotoxic drugs or in the setting of maintenance therapy for minimal residual disease. Additional issues are relevant as one considers developing this as a method to induce terminal differentiation. Extensively passed rhabdomyosarcoma–derived cell lines may not represent the best model for this because cumulative genetic or epigenetic changes acquired during cell culture may prevent their myogenic differentiation. The use of early passage or primary cultures of rhabdomyosarcoma–derived cells or transgenic mice with spontaneous rhabdomyosarcoma (67–69) might better reflect what is likely to happen in a clinical scenario.

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7 S.X. Skapek, unpublished observation.
Pharmacologic inhibition of cyclin-dependent kinase 4/6 activity arrests proliferation in myoblasts and rhabdomyosarcoma-derived cells


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