Discovery and Evaluation of Dual CDK1 and CDK2 Inhibitors

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Abstract

In eukaryotic cells, cyclin-dependent kinase (CDK) complexes regulate the temporal progression of cells through the cell cycle. Dereegulation in the cell cycle is an essential component in the evolution of cancer. Here, we validate CDK1 and CDK2 as potential therapeutic targets using novel selective small-molecule inhibitors of cyclin B1/CDK1 and cyclin E2/CDK2 enzyme complexes (CDKi). Flow cytometry–based methods were developed to assess intracellular retinoblastoma (Rb) phosphorylation to show inhibition of the CDK pathway. Tumor cells treated with CDK inhibitors showed an overall decrease in cell proliferation, accumulation of cells in G1 and G2, and apoptosis in a cell line–specific manner. Although CDK inhibitors activate p53, the inhibitors were equipotent in arresting the cell cycle in isogenic breast and colon tumor cells lacking p53, suggesting the response is independent of p53. In vivo, the CDK inhibitors prevented the growth of colon and prostate tumors, blocked proliferation of tumor cells, and inhibited Rb phosphorylation. The discovery and evaluation of novel potent and selective CDK1 and CDK2 inhibitors will help delineate the role that CDK complexes play in regulating tumorigenesis. (Cancer Res 2006; 66(8): 4299-308)

Introduction

Deregulation of the cell cycle is one of the hallmarks of tumor formation and progression. Cyclins E1 and E2 bind and activate the catalytic activity of CDK2, and overexpression of cyclin E transforms immortalized cells in culture, induces chromosome instability, and stimulates tumor formation in transgenic mice (1–4). Recent reports have shown that elevated expression of cyclin E1 in primary tumors correlates with poor survival rates for breast cancer patients (5, 6). In addition, cyclin E2 is associated with a group of 70 genes whose expression in node-negative primary breast carcinomas correlates with poor prognosis. The expression of this gene set has outperformed all other clinical variables in predicting the likelihood of distant metastases within 5 years (7, 8). The expression and catalytic activity of cyclin E/CDK2 complexes is elevated in multiple tumor types, including primary breast tumors (9–12). Cyclin B1/CDK1 complexes have been implicated in a number of cell cycle processes, including the monitoring of both the DNA structure checkpoints during late G2 and the spindle assembly checkpoint during mitosis (13, 14). Ablation of CDK1 and cyclin B1 has been described for a number of primary tumors and, in some cases, seems to correlate with patient survival rates (15–17).

Recent reports have shown that ablation of CDK2 expression by RNA interference (RNAi), antisense, and genetic knockout does not alter cell cycle progression, and, surprisingly, the CDK2 homozygous null mice are viable with normal life spans (18–21). Although CDK2 knockout data show that CDK2 may not be required for cell proliferation, a recent report suggests that melanocytes may be uniquely dependent on CDK2 for proliferation (19). Retroviral-mediated RNAi suppression of CDK2 in melanoma tumor-derived cell lines inhibits cell cycle progression, proliferation, and colony formation, strongly suggesting that CDK2 may be an essential cell cycle regulator in melanomas (18, 19). Although CDK1 activity is clearly required for G2 and mitosis in lower organisms and tumor-derived cell lines, the effect of deleting CDK1 in transgenic mice has not yet been reported. CDK1 is the only mammalian CDK complex present and active during late G2 and mitosis; therefore, the loss of CDK1 would likely result in embryonic lethality.

CDK complexes regulate cell cycle progression by phosphorylating downstream substrates required for G2-S and G2-M transit. One of the substrates for G1 cyclin/CDK complexes is the retinoblastoma protein (Rb), a tumor suppressor implicated in negatively regulating E2F-mediated transcriptional responses (22–24). Although it is not clear if individual CDK complexes recognize specific phosphorylation sites on Rb, it is clear that CDK-mediated Rb phosphorylation is a critical step in regulating E2F transcriptional responses. Cyclin B1/CDK1 kinase complexes regulate late G2 and mitosis by phosphorylating substrates and triggering dramatic structural reorganization of the nuclear envelope, spindle apparatus, and actin cytoskeleton and induction of chromosomal DNA condensation (25). One of the substrates for CDK1 complexes is PP1-α phosphatase, which is phosphorylated at Thr286 by cyclin B1/CDK1 during metaphase. Phosphorylation on Thr286 is believed to inhibit PP1-α phosphatase activity and thereby contribute to the increased phosphorylation of proteins critical in orchestrating mitotic progression (26).

To date, the majority of the published data suggest that inhibition of cyclin/CDK complexes may prevent or delay tumor progression in cancer patients. There are currently a number of ongoing clinical trials using CDK inhibitors, including Flavopiridol, UCN-01, CYC202 (racemic Roscovitine), and BMS-387032 (27). The compounds have a range of potencies against recombinant CDK enzyme complexes and tumor cells in vitro and in vivo assays...
(28, 29). However, many of these small-molecule CDK inhibitors either lack potency, selectivity, or suitable physical properties. For example, a small-molecule CDK inhibitor (BMS-387032) currently in clinical trials has IC_{50} of 480, 48, and 925 nmol/L against recombinant cyclin B1/CDK1, cyclin E/CDK2, and cyclin D/CDK4 kinase complexes, respectively (30). In addition, the maximum tolerated dose (MTD) of BMS-387032 is 48 mg/kg, and efficacy is observed in an A2780 xenograft model at 36 mg/kg dosed once a day for 8 days, showing a narrow therapeutic index (31). Another small-molecule CDK inhibitor currently in clinical trials, racemic Roscovitine, has an IC_{50} of 0.65 μmol/L against CDK1, an average IC_{50} of 0.7 μmol/L against CDK2 complexes, and an average IC_{50} of 16 μmol/L in growth inhibition assays against a panel of 60 tumor-derived cell lines from the National Cancer Institute (31). The R-isomer of Roscovitine, CYC202, has slightly greater potency against CDK2 complexes with an average IC_{50} of 0.4 μmol/L, is less potent against CDK1 complexes with an IC_{50} of 2.7 μmol/L, and has an average IC_{50} of 15.2 μmol/L in tumor cell growth inhibition assays (32, 33).

In vivo growth inhibition assays (32, 33). In vivo tumor experiments showed CYC202 dosed thrice a day i.p. at 100 mg/kg for 5 days resulted in a 45% reduction in tumor volume at day 27 in a human colorectal LoVo tumor model (32). Therefore, there is still a need for potent and selective CDK inhibitors to test the idea that CDK complexes are required for the proliferation and survival of primary tumors. In this report, we describe the discovery and characterization of novel potent small-molecule inhibitors of cyclin E/CDK2 and cyclin B1/CDK1 complexes and discuss the possible therapeutic implications for CDK inhibition.

Materials and Methods

Biochemical assays. A primary biochemical screen was completed with recombinant cyclin E2/CDK2 (in house, baculovirus) using homogenous time-resolved fluorescence technology platform. The kinase reaction [10 μmol/L Tris-HCl (pH 7.5), 1 μmol/L MgCl2, 2 μmol/L glycero phosphate, 1 μmol/L DTT, 5 μmol/L ATP, 1 μmol/L biotinylated histone H1, 1 μmol/L EGTA, and 0.2 mg/mL bovine serum albumin (BSA)] followed by the addition of 2 μmol/L cyclin E2/CDK2 was incubated for 60 minutes at room temperature. Detection of substrate phosphorylation was determined using streptavidin allopolyoxynan (Prozyme, Saneado, CA) and europium anti-phosphothreonine probe antibody (Perkin-Elmer, Boston, MA). Compounds potent in the primary screen were tested using recombinant cyclin D3/CDK6 (Upstate Cell Signaling, Waltham, MA), cyclin B1/CDK1 (Biomol, Plymouth Meeting, PA), p25/CDK5 (in house, baculovirus), and GSK-3β (in house, baculovirus). Kinase activities were determined using substrates histone H1 and/or COOH-terminal Rb protein fragment (Upstate Cell Signaling). The kinase reactions [20 μmol/L Tris-HCl (pH 7.5), 12.5 μmol/L MgCl2, 100 μmol/L NaCl, 1 μmol/L DTT, 125 μg/mL BSA, 2-3 μM K ATP, 2-3 μM ATP, 5-10 μM substrate] were incubated for 60 minutes at room temperature. The compounds were serially diluted over the appropriate concentration range and added to the kinase buffer before the addition of recombinant enzyme. The data were expressed as percent inhibition = 100 − [(signal with inhibitor − background) / (signal with DMSO vehicle − background)] × 100.

Human cells and cell culture. The tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Human primary bone marrow mononuclear cells were obtained from Cambrex BioScience (Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5.0% CO2.

CDK1 and CDK2 RNAi and kinase assay. RNAi triggers were synthesized for CDK1, CDK2, and single mismatch controls for both CDK1 and CDK2. RNAi triggers were transfected into cells using the liposome LipofectAMINE 2000 (Invitrogen). After 16 hours of incubation, the transfection medium was removed and replaced with fresh complete medium. The transduced U2OS cells were harvested on the third day after a 1-hour pulse with bromodeoxyuridine (BrDU/rd; Invitrogen). Immunoprecipitations, kinase reactions, and Westerns were done as previously described (12). The control for the kinase assay was U2OS cells treated with 1 μmol/L CDKi 277 for 24 hours.

Human p53 knockout cell lines. Plasmid vectors harboring small interfering RNA (siRNA) triggers for either human p53 or scrambled sequence control were transfected into MCF-7 cells and HCT116 using the liposome Fugene 6 (Roche, Indianapolis, IN). The cells were clonally selected in complete media supplemented with 400 μg/mL of G418 (Sigma, St. Louis, MO). Individual clones were then assessed for p53 protein levels.

Mitotic-arrested HeLa cell extract preparation. HeLa cells were treated with 0.1 μg/mL Nocodazole (Sigma) for 12 hours; the semi-adherent, mitotic cells were collected and removed by pipetting. Cells were then treated with inhibitor compounds at indicated concentrations for 1 hour and harvested for Western analysis as previously described (12).

Antibodies and Western blotting. Mouse monoclonal antibodies anti-total RB, anti-underphospho-RB, and anti-poly(ADP-ribose)polymerase (PARP, Asp214) were obtained from BD Biosciences (San Jose, CA). Rabbit polyclonal antibodies anti-phospho-Rb (Ser286/288), anti-total PPI-α, anti-phospho-PI-π (Thr286), anti-total P53, and anti-phospho-p53 (Ser15) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies anti-CDK2, anti-CDK1, and mouse monoclonal anti-p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody anti-actin (Sigma) was used as a protein loading control.

In vitro cell line extracts were prepared for Western analysis using a modified radioimmunoprecipitation assay buffer (12).

Flow cytometry. The cell lines were treated with CDKi 277 [0.004-2.5 μmol/L (hypophosphorylated Rb, hypo-Rb), 0.0019-5 μmol/L (BrDU/rd), and 0.5 μmol/L (time course)] or Roscovitine (Calbiochem EMD Biosciences, Inc., San Diego, CA; 0.41-100 μmol/L/BrDU/rd) for 24 hours of continuous exposure or harvested at 4, 8, 12, and 24 hours. The cells were pulsed with BrDU/rd for 1 hour before cell harvest when appropriate. The cells were fixed, permeabilized, acid treated (only BrDU/rd), and neutralized in preparation for intracellular staining. The cells were stained with antibodies for 2 hours using anti-BrDU/rd-FITC (BD Biosciences) or anti-BrDU/rd- alexa 647 (Invitrogen), anti-cyclin B1-FITC (BD Biosciences), active caspase 3/FTTC (BD Biosciences), and anti-histone H3 p-Ser28-Alexa 488 (US Biological, Swampscott, MA) followed by DNA counterstaining with propidium iodide. The 96-well hypo-Rb assay was developed using the Colo205 cells. The cells were treated with inhibitor compounds (12-pt. dose range, 0.0025-50 μmol/L) for 5 hours. The cells were fixed and permeabilized in preparation for intracellular staining. The fixed cells were stained with anti-hypo-Rb-alexa 488 antibody for 2 hours at room temperature and counterstained with 7-AAD (BD Biosciences). Data was acquired using a 96-well plate AMS auto-sipper (Cytek, San Jose, CA) with FACSscan flow cytometer (BD Biosciences).

Tumor xenografts. All in vivo experiments were conducted in accordance with institutional animal care and use committee. Eight- to 10-week-old female CD1 nude mice (Charles Rivers Laboratories, Wilmington, MA) were used in all studies. Mice were injected s.c. with 2 × 10^6 tumor cells. Tumor volume was calculated as length × (width)^2 and expressed in mm^3. Mice were euthanized with CO2 asphyxiation. Results are described as mean ± SE. The data were statistically analyzed with factorial ANOVA followed by Scheffe's post hoc analysis for repeated measurements (Statview v.5.0.1, SAS Institute, Cary, NC). The mice for the short-term tumor xenografts were treated i.p. with CDKi 277 at 50 mg/kg once a day (QD) and vehicle QD for 4 days, or Taxotere at 20 mg/kg QD for 2 days. Tumors were harvested on day 5, 2 hours before the harvest mice were injected i.v. through the tail vein with 0.2 mL of BrDU/rd at 2 mg/mL (Invitrogen). Tumors were excised, minced, and processed in preparation for flow cytometry and Western analysis (12).
Results

Inhibition of CDK Expression Reduces Tumor Cell Proliferation

To determine the cellular effect of selectively inhibiting CDK complexes, RNAi-based suppression methods were used to specifically target CDK1 or CDK2. As shown in Fig. 1, RNAi-mediated inhibition of CDK2 expression and kinase activity modestly increased the percentage of cells in G2-M and had little or no effect on BrdUrd incorporation or the overall cell cycle profile of asynchronously dividing U2OS osteosarcoma cells. These data are consistent with previous results targeting CDK2 with antisense oligonucleotides in U2OS cells (18). In contrast, inhibition of CDK1 expression decreased the percentage of U2OS cells in the S phase by 26% and increased the percentage of cells in G2-M by almost 3-fold when compared with mismatch-treated controls. Inhibition of both CDK2 and CDK1 expression produced results similar to U2OS cells treated with CDK1 RNAi alone (Fig. 1C).

Antisense-based suppression of CDK2 and CDK1 in breast tumor-derived MDA-MB-453 cells showed that inhibition of CDK2 expression led to a 29% decrease in BrdUrd-positive, S-phase cells relative to the mismatch antisense control (data not shown). Suppression of CDK1 expression in MDA-MB-453 cells resulted in a 48% decrease in BrdUrd-positive, S-phase cells, a 47% decrease in G2-M, and a 164% increase in the sub-G1 DNA content relative to the mismatch antisense control (data not shown). The decrease in S and G2-M phases with CDK1 antisense was likely due to an increase in cell death. Overall, these results show that selective inhibition of CDK2 expression had a very modest effect on cell proliferation, whereas suppression of CDK1 expression decreased the percentage of cells in the S phase for both U2OS and MDA-MB-453 cells. Inhibition of CDK1 expression also led to either cell death in MDA-MB-453 cells or an increase in the percentage of U2OS cells in the G2-M phase.

Discovery of Small-molecule CDK Inhibitors (CDKi)

Suppression of CDK1 using RNAi suggests an essential role in cell cycle progression; CDK2 suppression alone could not inhibit cell cycle progression, suggesting the possibility of CDK pathway redundancy. However, previous reports clearly showed that both dominant-negative mutant CDK2 and CDK2 inhibitory peptides blocked tumor cell proliferation (34, 35). Therefore, we designed a high-throughput screen for dual CDK2 and CDK1 inhibitors. To discover novel small-molecule inhibitors for CDK2 a high-throughput screen was completed using active recombinant cyclin E2/CDK2 complexes. As shown in Table 1A and B, a representative thiazole urea [(N2(4-pyridinyl)1,3-thiazol-4-yl)N'-(6-(((2R,2-pyrrolidinylmethyl)oxy)-2-pyridinyl)urea)] small-molecule compound, CDKi 277, potently inhibited CDK2, CDK1, and CDK5 kinase activity with IC50s below 10 nmol/L. CDKi 277 is 10-fold more selective for CDK2 than CDK6 or GSK-3β.

Inhibition of GSK-3β enzyme with CDKi 277 is consistent with the fact the GSK-3 kinases, and CDKs have structural similarities in the
ATP binding domains. To determine the selectivity of CDKi 277 against additional kinases, we screened a panel of 41 serine/threonine and tyrosine kinases and showed that CDKi 277 is selective against these additional kinases (Table 1B). The thiazole urea structural series of compounds are competitive with respect to ATP, noncompetitive with respect to substrate and are reversible (data not shown).

**Table 1.**

A. Structure of CDKi 277, a representative thiazole urea small-molecule CDK inhibitor

![Structure of CDKi 277](image)

B. Biochemical enzyme IC\textsubscript{50}s and kinase selectivity

<table>
<thead>
<tr>
<th>Biochemical assay</th>
<th>IC\textsubscript{50} (\textmu mol/L)</th>
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<tr>
<td>CDK2-E2</td>
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</tr>
<tr>
<td>CDK1-B1</td>
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<tr>
<td>CDK5-p25</td>
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<tr>
<td>CDK6-D3</td>
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<tr>
<td>GSK-3β</td>
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<td>9 Serine/threonine kinases</td>
<td>&gt;5.0</td>
<td>&gt;500</td>
</tr>
<tr>
<td>5 Tyrosine kinases</td>
<td>&gt;5.0</td>
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<tr>
<td>10 Serine/threonine kinases</td>
<td>&gt;50 POC at 10 \textmu mol/L</td>
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<tr>
<td>17 Tyrosine kinases</td>
<td>&gt;50 POC at 10 \textmu mol/L</td>
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C. Inhibition of Rb, PP1\textalpha phosphorylation, and cell proliferation

<table>
<thead>
<tr>
<th>In cell phosphorylation assays</th>
<th>In vitro cell proliferation assays</th>
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<tr>
<td>Rb phosphorylation cell assay IC\textsubscript{50} = 0.163 \textmu mol/L</td>
<td>Mean BrdUrd IC\textsubscript{50} = 0.234 \textmu mol/L (cell lines tested, n = 21)</td>
</tr>
<tr>
<td>PP1\textalpha phosphorylation cell assay IC\textsubscript{50} = 0.305 \textmu mol/L</td>
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Inhibitors Block Phosphorylation of CDK Substrates in Tumor Cells

**pRb phosphorylation.** CDK inhibitors having IC\textsubscript{50} <10 nmol/L in the CDK1 and CDK2 kinase assays were tested for their ability to inhibit CDK activity in cells using a novel 96-well plate flow cytometry–based Rb phosphorylation assay. Rb is a proximal substrate for G\textsubscript{1} CDK complexes and the phosphorylation state of Rb correlates with its ability to function as a tumor suppressor. A monoclonal antibody recognizing the hypophosphorylated or underphosphorylated form of Rb was chosen to develop the flow cytometry assay. CDK inhibitors shifted Rb to a hypophosphorylated (hypo-Rb) state where it is recognized by the hypo-Rb–specific monoclonal antibody. The whole cell flow cytometry approach using threshold gating provided superior sensitivity and reproducibility, compared with cell extract–based methods. A panel of cell lines were evaluated for expression of total Rb protein; the colon tumor-derived cell line Colo205 proved to be the best candidate based on Rb protein levels (Fig. 2A).

Colo205 cells treated with CDKi 277 show a clear dose-dependent increase in hypo-Rb (red, y axis) using the phospho-Rb flow cytometry assay (Fig. 2B). The cellular phospho-Rb IC\textsubscript{50} for CDKi 277 was 0.163 \textmu mol/L (Fig. 2B). Time course experiments have shown that Rb shifted to a hypophosphorylated state within 30 minutes of compound treatment (data not shown). To reduce the likelihood that inhibition of Rb phosphorylation was due to a cell cycle arrest, cells were treated with compound for 5 hours, which is before any substantial CDK inhibitor, mediated changes in cell proliferation (Fig. 3B). Western blot analysis with phospho-Rb (Ser\textsuperscript{807} and Ser\textsuperscript{811}) and hypo-Rb antibodies confirmed the flow cytometry–based IC\textsubscript{50} for CDKi 277 (Fig. 2B).

**PP-1\textalpha phosphatase phosphorylation.** PP-1\textalpha phosphatase is a direct substrate for cyclin B1/CDK1 complexes; the CDK inhibitor olomoucine has been shown to block the phosphorylation of PP-1\textalpha on Thr\textsuperscript{320} in mitotic cell extracts (26). To determine if CDKi 277 inhibited intracellular CDK1 activity, HeLa cells were synchronized in mitosis with nocodazole, and the semiadherent mitotic cells were harvested and treated with CDKi 277 for 1 hour. As shown in Fig. 2C, proliferating HeLa cells show...
very little phosphorylation of PP-1α, whereas the nocodazole-treated cells show a dramatic increase in PP-1α phosphorylation. Figure 2C shows that CDKi 277 clearly triggered a dramatic dose-dependent decrease in phosphorylation of PP-1α in cells released from a mitotic nocodazole block. At doses of ≥0.625 μmol/L, the phosphorylation on Thr320 was completely inhibited with no change in the total amount of PP-1α. Densitometry measurements of signal intensity for the bands depicting phosphorylation of PP-1α on Thr320 indicate an IC50 of 0.3 μmol/L for CDKi 277 (Fig. 2C).

Our results establish the use of screening CDK inhibitors using endogenous cellular substrates in their native forms. The phospho-Rb flow cytometry assay identified small molecules capable of inhibiting CDK-mediated phosphorylation of intracellular Rb. In addition, phosphorylation of PP-1α on Thr 320 has proven to be an excellent secondary assay to show inhibition of CDK1 activity in a tumor-derived cell line.

**CDK Inhibitors Block Cells in G1 and G2 and Induce Apoptosis**

To determine the effect of CDK inhibition on cell proliferation, we treated a panel of tumor cell lines and normal nontransformed cells with CDKi 277 and Roscovitine and examined their cell cycle profiles after 24 hours of treatment. CDKi 277 and Roscovitine inhibited BrdU/rd uptake in all of the cell lines. For CDKi 277 the IC50s ranged from 0.09 to 0.7 μmol/L with a mean IC50 of 0.243 μmol/L, whereas Roscovitine IC50s ranged from 12 to 35 μmol/L with a mean IC50 of 19.7 μmol/L. On average, the CDKi 277 inhibitor was ~77-fold more potent compared with Roscovitine (Table 2; Fig. 3A). The 24-hour cellular phenotypes were classified as either cytostatic (G1 and G2-M arrest) or cytotoxic (>20% cell death measured by % sub-G1 DNA content). Although the CDK inhibitors differ dramatically in terms of potency, both compounds triggered similar cell cycle responses in both tumor cell lines.
cell lines and in normal bone marrow–derived leukocytes, the one exception was the uterine tumor-derived cell line MES-SA (Table 2). Interestingly, osteosarcoma cell lines SaSO2 (Rb+/C0) and U2OS (Rb+) treated with CDKi 277 had comparable IC50 (0.3 μmol/L) and cellular phenotypes (cytotoxic), indicating that CDKi 277 inhibits cell cycle progression irrespective of Rb status. Additionally, MCF-7 and HCT116 p53+/− isogenic tumor cell lines treated with CDKi 277 exhibited nearly identical cell cycle responses, suggesting that p53 may not play an essential role in regulating the cell arrest phenotype in G1 or G2 (Table 2; Fig. 3).

Figure 3. CDK inhibitors block cells in G1 and G2 and induce apoptosis. A, 24-hour full dose-response using CDKi 277 was done on tumor cell lines Colo205, MCF-7 p53 (+), MCF-7 p53 (−), and normal nontransformed foreskin fibroblasts. Flow cytometry BrdUrd-coupled DNA staining was used to simultaneously measure cell proliferation and cell death (sub-G1 DNA content). B, time course of Colo205 cells treated with CDKi 277 at 0.5 μmol/L. Cell cycle stage and sub-G1 were determined (boldface number represents % cells/phase or sub-G1). For the 12-hour sample, the arrow (→) indicates complete inhibition of G1 cells traversing into the S phase (BrdU−). C, time course of Colo205 cells treated with either 0.5 μmol/L CDKi 277 or DMSO. Flow cytometry analysis of the same Colo205-treated cells using DNA-coupled staining with cyclin B1, phospho-histone H3 (Ser10) and active caspase-3 antibodies. D, Western blot analysis was done on protein extracts using the following cleaved-PARP, histone H2AX (Ser139), and actin. Actin antibody was used as a protein loading control.
To determine the kinetics of cell cycle inhibition, Colo205 cells were treated with CDKi 277 over a 24-hour time period, harvested at various time points, and evaluated using the following end points; cell cycle profiles, BrdUrd analysis of S phase, apoptosis markers, mitotic markers, and p53 pathway activation. Figure 3B shows that Colo205 cells treated with CDKi 277 show a time-dependent decrease in % S phase (BrdUrd) and an increase in % G2-M and % sub-G1. G1 cells decreased only modestly; however, by 12 hours, G1 cells were completely blocked from entering into the S phase. To determine the cause of the precipitous drop in proliferation upon treatment with CDKi 277, Colo205 cells were assessed for the following apoptosis end points: cleaved caspase-3, cleaved PARP, and phosphorylation of histone H2AX on Ser139 (Fig. 3D). The phosphorylation of histone H2AX on Ser139 by ATM occurs in response to double-strand DNA breaks and apoptosis-mediated genome fragmentation (36, 37). Colo205 cells treated with CDKi 277 show a time-dependent increase in all three apoptotic indicators (Fig. 3B and D).

To gain a clearer understanding of whether cells were arrested in G2 or mitosis, tumor cells were exposed to CDKi 277 and analyzed by flow cytometry using antibodies against cyclin B1, phosphorylated histone H3, and MPM-2. Figure 3C shows a dramatic decrease in cyclin B1 and phospho-histone H3 staining, indicating that the cell cycle arrest occurred in G2 before entering into mitosis. The decrease in phospho-histone H3 staining is likely caused by inhibition of downstream CDK1 effectors. As an additional measure, cells treated with compound were stained for tubulin and DNA and analyzed by fluorescent microscopy. We did not observe condensed chromosomes or metaphase plate formation in compound-treated cells, which is consistent with cells arrested in G2 and not mitosis (data not shown).

### Cell Cycle Arrest Mediated by CDK Inhibitors Is p53 Independent

To determine if the antiproliferative effects of CDKi 277 were dependent upon activation of p53, isogenic MCF-7 breast tumor cells lacking p53 were generated using a stable transducer vector harboring a siRNA targeting p53 gene expression. As a control, scrambled siRNA was stably transfected into the parental MCF-7 cells. MCF-7 p53-positive cells treated with CDKi 277 showed a time-dependent accumulation of both total p53 protein and phosphorylated p53 protein on Ser15 (Fig. 4A). Ser15 is a major site of p53 phosphorylation in response to DNA damage, and ATM/ATR kinases have been shown to phosphorylate p53 on Ser15 in response to CDK2 inhibition (38, 39). As expected, treatment of MCF-7 p53-negative cells with CDKi 277 did not increase the expression of p53 or p21 protein (Fig. 4A). In the MCF-7 p53-positive cells, p21 induction was visible only after 24 hours of compound treatment, clearly showing the decrease in BrdUrd incorporation resulting from CDK inhibition preceded the increase in p21 protein (Fig. 4A and B). Interestingly, the flow cytometry analysis showed similar G1 and G2 cell cycle arrest profiles for both MCF-7 p53-positive and p53-negative cells (Fig. 4B), indicating that although CDKi 277 activates the p53 pathway, the activation of p53 is not required for CDKi 277 to inhibit the cell cycle.

### Inhibition of CDK Activity Suppresses the Growth of Human Tumors In vivo

**Short-term human tumor cell in vivo proliferation assay.** CDK inhibitors exhibiting potent inhibitory activity in the cell-based proliferation and substrate phosphorylation assays and having acceptable pharmacokinetic and physical properties were tested in a short-term in vivo tumor cell proliferation assay. Colo205

<table>
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<th>Roscovitine</th>
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<tbody>
<tr>
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<td>IC50 (μmol/L)</td>
<td>Cellular phenotype</td>
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<td>0.137</td>
<td>Cytostatic</td>
</tr>
<tr>
<td>MES-SA (uterine)</td>
<td>0.31</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>MES-SA/Dx5 (uterine, MDR1)</td>
<td>0.7</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>Normal foreskin fibroblast (fibroblast)</td>
<td>0.24</td>
<td>Cytostatic</td>
</tr>
<tr>
<td>Mean BrdUrd IC50 (μmol/L)</td>
<td>0.243</td>
<td>19.73</td>
</tr>
</tbody>
</table>
tumor-bearing CD1 nude mice were treated with CDKi 277 for 4 days. Tumor samples were harvested and processed for flow cytometry–based BrdUrd analysis. The mice treated with either CDKi 277 at 50 mg/kg QD or taxotere at 20 mg/kg QD showed a significant decrease in Colo205 tumor cell proliferation when compared with the vehicle control-treated mice (see Supplementary Fig. S1).

**Figure 5** shows that CDKi 277 reduced the rate of Colo205 tumor growth when dosed twice a day (BID) at 12.5 and 25 mg/kg for three cycles of 4 days on and 1 day off. Tumor volumes for CDKi 277–treated mice (n = 10 per dose group) showed stasis at 12.5 mg/kg and regression at 25 mg/kg when compared with the vehicle control group. The mice seemed to tolerate the 12.5 mg/kg dose with 100% survival and no significant weight loss; but at 25 mg/kg, survival rates dropped dramatically after the start of the third cycle of treatment. A follow-up study using the Colo205 tumor model was completed using a less frequent dosing schedule; CDKi 277 was dosed BID at 17.5 and 12.5 mg/kg for 3 days per week for 3 weeks. CDKi 277 induced complete tumor stasis over the course of treatment with no adverse effects on body weights or survival (data not shown). In addition, Fig. 5B shows in an established PC-3 prostate tumor xenograft model that treatment with CDKi 277 dosed at 12.5 mg/kg BID for 2 days on and 4 days off for five cycles inhibited tumor growth. In a separate arm of the study, we observed tumor regressions in mice treated with Taxotere. Both cell cycle inhibitors were well tolerated over the course of the study. Studies we conducted with CDK inhibitor Roscovitine showed 35% tumor growth inhibition in an established PC-3 tumor model (data not shown). Roscovitine was given as a continuous s.c. infusion using osmotic minipumps at 10 mg/mL (2 mg/wk) for 3 weeks. Osmotic minipumps were chosen because of the demanding dose and schedule requirements for i.p. administration and formulation considerations (32).

To determine if inhibition of CDK signaling correlated with suppression of tumor growth, we examined Rb phosphorylation in tumor extracts from the Colo205 tumor xenograft study. Figure 5C shows that CDKi 277 dosed at 25 and 12.5 mg/kg inhibited or reduced, respectively, Rb phosphorylation in the Colo205 tumors when compared with the vehicle control. Overall, these results show that blocking CDK activity in vivo results in inhibition of tumor cell proliferation, inhibition of Rb phosphorylation, and suppression of tumor growth. In addition, by altering the dose and schedule of CDKi 277, we have shown that in vivo efficacy can be achieved with an acceptable therapeutic index.

**Discussion**

The increased expression of cyclins and activation of CDK complexes regulate the normal cell cycle, and their deregulation...
in tumors contributes to tumor progression and poor patient survival rates. Although advancements have been made in developing CDK inhibitors with the appropriate properties for clinical development, many of the CDK inhibitors currently in clinical studies still have poor CDK selectivity, potency, and physical properties. There is a clear need for more potent and selective CDK inhibitors with the appropriate physical properties to determine if inhibition of CDK activity will prevent tumor progression in cancer patients.

Suppression of gene expression is one way to determine the functional role that specific protein plays in regulating the cell cycle. The CDK2 antisense and RNAi results are consistent with a recent report showing that inhibition of CDK2 expression had little effect on proliferation in several tumor-derived cell lines in cell culture (18); however, our results did indeed detect reproducible cell cycle effects (G2-M increase) by coupling BrdUrd and DNA staining, whereas the recently reported data showed no cell cycle phenotype effect using CDK2 suppression methodologies. Our results show that CDK1 expression was required for osteosarcoma and breast tumor cell proliferation and that suppression of CDK1 decreased S phase while markedly increasing G2-M. Techniques, such as RNAi, antisense, and overexpression of kinase-dead mutants, are commonly accepted methods for validation of drug targets. However, the cell phenotypes generally seen with small-molecule kinase inhibitors are consistently more dramatic than RNAi or kinase-dead mutants over a variety of kinase targets with distinct phenotypes. Perhaps a small-molecule inhibitor with a high affinity for the ATP-binding pocket of CDK2 may act more like a dominant-negative inhibitor and titrate out cyclins as well as proteins required for the catalytic activity of additional CDK complexes. In this respect, a CDK2 small-molecule inhibitor may have a different cellular phenotype compared with cells that have lost expression of CDK2. In support of this concept, reports have shown that in U2OS osteosarcoma cells suppression of CDK2 expression by antisense had no effect on cell cycle progression, whereas a dominant-negative CDK2 mutant increased the percentage of U2OS cells in G1 and decreased the S-phase fraction (18, 34).

Our results show that CDKi 277 potently inhibited CDK2 and CDK1 enzyme activity with IC50s of 4 and 8 nmol/L, respectively. In addition, when tested in a 24-hour proliferation assay, CDKi 277 had an average cellular IC50 of 0.243 nmol/L against a broad panel of tumor cell lines; furthermore, CDKi 277 was significantly more potent than other CDK inhibitors against CDK2 and CDK1.

Figure 5. CDKi 277 treatment results in tumor growth inhibition for both Colo205 and PC-3 xenograft models. CD1 nude tumor-bearing mice (n = 10 per dose group) were dosed using intermittent dosing regimens. A, Colo205 tumor-bearing mice were treated BID i.p. with vehicle and CDKi 277 at 25 or 12.5 mg/kg (mpk) for 4 days on and 1 day off, for three consecutive cycles. Tumor volumes for CDKi 277 at 25 and 12.5 mg/kg showed either complete regression (25 mg/kg) or stasis (12.5 mg/kg) compared with the vehicle control. CDKi 277 (25 mg/kg) animal mortality (M) during the third cycle of treatment. B, PC-3 tumor-bearing mice treated BID i.p. with vehicle or CDKi 277 at 12.5 mg/kg for 2 days on and 4 days off for 5 cycles. Taxotere was treated at 20 mg/kg QW (twice weekly) for five cycles. Tumor volumes for CDKi 277–treated group at 12.5 mg/kg showed tumor stasis, and Taxotere showed tumor regression compared with vehicle control. C, Western blot analysis of CDKi 277–treated Colo205 xenograft tumors using anti-phospho-Rb (Ser807 and Ser811) and anti-total Rb antibodies. Densitometry measurements of anti-phospho-Rb signal intensities (RU) show statistically significant inhibition of in vivo Rb phosphorylation at CDKi 277 at 25 mg/kg (P = 0.0003) and 12.5 mg/kg (P = 0.023).
more potent than Roscovitine, a CDK inhibitor currently in clinical trials. The proliferation assay correlated well with the IC50 generated in the short-term Rb and PP-1a cellular phosphorylation assays. The ability of CDKi 277 to arrest cells in the G1 and G2 phase of the cell cycle and to induce tumor cell apoptosis is consistent with the reported role that CDK2 and CDK1 complexes play in regulating the cell cycle. The CDKi 277 apoptotic response was marked by an increase in the active forms of PARP and caspase-3, which also coincided with the phosphorylation of histone H2AX. CDKi 277 also inhibited Rb phosphorylation and E2F-mediated transcriptional activation, which is again consistent with the role that CDK complexes play in regulating Rb and its interaction with E2F complexes (data not shown). Our results show that the inhibition of Rb phosphorylation correlates with the ability of compounds to block tumor cell proliferation. However, CDKi 277 was equipotent in Rb-positive and Rb-negative tumor cells, suggesting that modulation of additional proximal CDK substrates was required for the cell cycle arrest. Additionally, CDKi 277 was equipotent on both pS3-positive and pS3-negative isogenic tumor cells and exhibited similar cell cycle arrest phenotypes. It is important to highlight that whereas our results with CDKi 277 were consistent with the biochemical and cellular phenotype expected for inhibition of CDK complexes (40), the possibility does exist that our CDK inhibitors may affect other signaling pathways.

CDKi 277 exhibited potent antitumor activity in vivo. By adjusting the dose and schedule for CDKi 277, we minimized toxicities and achieved tumor growth inhibition in both Colo205 and PC-3 tumor xenografts. In addition, the compound-treated mice showed a reduction in both Rb phosphorylation and BrdUrd incorporation in the tumor. Although CDKi 277 showed significant tumor inhibition as a monotherapy, in the clinic, CDK inhibitors will most likely be used in combination with either standard of care chemotherapeutics or with biologic-based therapies.

The precise mechanisms determining tumor cell fate after treatment with CDK inhibitors still remains elusive, gaining a better understanding of the survival and checkpoint adaptations will help elucidate the underlying factors regulating the phenotype response. Ultimately, CDK inhibitors with increased potency, better selectivity, and favorable drug-like properties have the greatest potential for clinical advancement.

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Discovery and Evaluation of Dual CDK1 and CDK2 Inhibitors
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