In vitro antitumor properties of a novel cyclin-dependent kinase inhibitor, P276-00

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Abstract
Cyclin-dependent kinases (Cdk) and their associated pathways represent some of the most attractive targets for the development of anticancer therapeutics. Based on antitumor activity in animal models, a variety of Cdk inhibitors are undergoing clinical evaluation either as a single agent or in combination with other approved drugs. In our anticancer drug discovery program, a novel series of flavones have been synthesized for evaluation against the activity of Cdk4-D1. This enzyme catalyzes the phosphorylation of retinoblastoma protein, thus inhibiting its function. We have identified a series of potent Cdk4-D1 inhibitors with IC50 below 250 nmol/L. In this report, we have described the properties of one of the best compounds, P276-00, of the flavone’s series. P276-00 shows 40-fold selectivity toward Cdk4-D1, compared with Cdk2-E. The specificity toward 14 other related and unrelated kinases was also determined. P276-00 was found to be more selective with IC50s <100 nmol/L for Cdk4-D1, Cdk1-B, and Cdk9-T1, as compared with other Cdk5, and less selective for non-Cdk kinases. It showed potent antiproliferative effects against various human cancer cell lines, with an IC50 ranging from 300 to 800 nmol/L and was further compared for its antiproliferative activity against cancer and normal fibroblast cell lines. P276-00 was found to be highly selective for cancer cells as compared with normal fibroblast cells. To delineate its mechanism of action, the effect of P276-00 on cell cycle proteins was studied in human breast cancer cell line (MCF-7) and human non-small cell lung carcinoma (H460). A significant down-regulation of cyclin D1 and Cdk4 and a decrease in Cdk4-specific pRb Ser780 phosphorylation was observed. P276-00 produced potent inhibition of Cdk4-D1 activity that was found to be competitive with ATP and not with retinoblastoma protein. The compound also induced apoptosis in human promyelocytic leukemia (HL-60) cells, as evidenced by the induction of caspase-3 and DNA ladder studies. These data suggest that P276-00 has the potential to be developed as an anti-Cdk chemotherapeutic agent. [Mol Cancer Ther 2007;6(3):918–25]

Introduction
The ability of eukaryotic cells to proliferate in response to a growth signal is tightly controlled by a complex network of ordered biochemical events collectively known as the cell cycle (1–3). The cell cycle can be divided into four phases: G1 (Gap1) during which cellular events are preparing the cell for DNA synthesis; S (Synthesis) phase in which DNA replication occurs; G2 (Gap2), preparing the cell for division, and M (Mitosis), which is the effective division of the mother cell into two daughter cells (3). Cyclin-dependent kinases (Cdk) are critical regulators of cell cycle progression and are constitutively expressed throughout the cell cycle (3). Expression and interaction of Cdk5, cyclins, and their activators and inhibitors, account to a great extent for the timing of events in cell cycle. Specific Cdk5 operate in distinct phases of the cell cycle. The major regulatory checkpoint in this process controls the transition from G1 to S phase and is characterized by inactivation and phosphorylation of the retinoblastoma (Rb) gene product (4). Inactivation of Rb leads to the release of the transcriptional factor E2F1 and the activation of E2F-responsive genes necessary for progression to S phase (5–8). Complexed with their respective D-type cyclins, Cdk4 and Cdk6 are responsible for the progression of the cells through G1 (9–11). A complex of Cdk2 and cyclin E is required for the progression of the cells from G1 to S phase.

A single, most defining feature of cancer is unchecked growth. In malignant cells, altered expression of Cdk5 and their modulators, including overexpression of cyclins and loss of expression of natural Cdk5 inhibitors, results in deregulated Cdk5 activity, providing a selective growth advantage (12–14). Because of their critical role in cell cycle progression, as well as the association of their activities with the processes of differentiation and apoptosis, the Cdk5 comprise an attractive set of targets for novel antineoplastic.

Several drug discovery programs have produced potent small-molecule Cdk5 inhibitors (15–20). A variety of chemical classes, which include purine analogues (21–25), pyrimidine analogues (26–28), indenopyrazoles (29, 30), pyridopyrimidines (31–33), pyrazolopyridines (34, 35), indolocarbazoles (36), pyrrolocarbazoles (37, 38), oxindoles
(39, 40), and aminothiozoles (41) have been developed as Cdk inhibitors. Several compounds that inhibit Cdk activity are currently in clinical trials, including flavopiridol, R-rosocvitine (CYC-202), UCN-01 (7-hydroxyxastrosporine), and BMS-387032 either as single agents or in combination. Most of these compounds, however, inhibit multiple Cdks, with Cdk2 being a particularly common target. The present report describes a potent inhibitor of Cdk4-D1, Cdk1-B, and Cdk9-T1 with ~40-fold selectivity over Cdk2-E in in vitro enzyme assays. Furthermore, we describe the antiproliferative activity of this compound in a broad spectrum of human cancer cell lines. We also provide evidence of down-regulation of cyclin D1 and Cdk4 protein levels with decreased phosphorylation of pRb at Ser^780, an event that was preceded by the loss of Cdk4-D1 activity. The ability of the compound to induce apoptosis and the effect on Cdk4-D1 enzyme kinetics were also studied.

Materials and Methods

Compound Used for the Studies

Compounds P276-00 and flavopiridol were synthesized at Nicholas Piramal Research Centre, Nicholas Piramal India Limited, Mumbai, India.

Cell Lines and Cell Culture Conditions

All human cancer cell lines were obtained either from the American Type Culture Collection (Manassas, VA) or from National Centre for Cell Sciences, Pune, India. Cells were maintained in the appropriate media such as RPMI 1640, DMEM, McCoy’s or F12 Hams modified medium supplemented with fetal bovine serum, 2 mmol/L L-glutamine, and penicillin (100 units/mL) and streptomycin (10 units/mL) in a humidified 37°C incubator with 5% CO2.

Cdk4-Cyclin D1 and Cdk2-Cyclin E Enzyme Assays

Human cyclin-dependent kinase-4/2 was coexpressed with cyclin D1/E in a baculovirus expression system. To accomplish this, 1 × 10^7 Sf9 cells were coinfectected with baculoviruses containing human glutathione S-transferase (GST)–tagged Cdk-4/2 and cyclin D1/E genes. The cells were lysed after 72 h in 500 μL of the lysis buffer [50 mmol/L HEPES (pH, 7.5), 10 mmol/L MgCl2, 1 mmol/L DTT, 5 μg/mL of aprotinin, 5 μg/mL of leupeptin, 0.1 mmol/L NaF, 0.2 mmol/L phenylmethylsulfon fluoride, and sodium orthovanadate]. The cell lysate was centrifuged, and the supernatant was purified on a GST-sepharose column. Purity of the proteins was checked by SDS-PAGE followed by Western blots using antibodies specific to Cdk4 or Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA).

GST-retinoblastoma (amino acids 776–928) fusion protein was expressed in Escherichia coli and purified by reduced glutathione (GSH)-Sepharose affinity chromatography. GST-Rb bound to these beads served as the substrate in the assay.

The Cdk4-D1/Cdk2-E enzyme assay was run in 96-well format using Millipore Multiscreen filtration plates (Uni-filter plates, Packard, Meriden, CT). All assay steps are done in a single filter plate. The filtration wells were pretreated with 100 μL of kinase buffer [50 mmol/L HEPES (pH, 7.5), 2.5 mmol/L EGTA, 1 mmol/L DTT, 0.4 mmol/L NaF, 0.4 mmol/L sodium orthovanadate] diluted in kinase buffer was added to each well. The test compound (4× final concentration in kinase buffer) or kinase buffer alone (control) was then added in an additional 25 μL volume. To each well, 50 μL (100 ng) of human Cdk4-D1/ Cdk2-E enzyme in kinase buffer was added to initiate the reaction, which was allowed to continue for 30 min at 30°C. When the reaction was completed, vacuum was applied again, and the plate was washed with the TNEN buffer [20 mmol/L Tris (pH, 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% nonidet-P40] three times; the filter plate was air-dried and was placed in a Multiscreen adapter plate. Packard Microsecent-O cocktail (30 μL) was added, and the plate was covered with a Top-Count A film. Quantitation of 32P-GST-Rb in 96-well filter plates was carried out by Top Count scintillation counter (Packard). All compounds were tested initially at 1 μm/L concentration. Compounds showing more than or equal to 50% inhibition were further profiled for IC^50 determination. Flavopiridol, a pan-Cdk inhibitor, was used as a standard in all experiments.

Other In vitro Kinase Assays for Specificity Analysis

In vitro kinase assays in the presence of increasing concentrations of P276-00 and appropriate substrates were carried out using a Kinase Profiler (Protocol Guide) at Upstate (Dundee, United Kingdom).

In vitro Cell Proliferation Assay

Exponentially growing cultures of human cancer cell lines were used to assess the effect of compounds on proliferation and survival. All the compounds showing IC^50 ≤ 1 μmol/L in Cdk4-D1 enzyme assay were evaluated in the in vitro cell proliferation ³H-thymidine uptake assay. The cells were seeded at a density of 3,000–5,000 cells per well, depending on cell type in 180 μL of culture medium in 96-well plate and incubated overnight to allow the cells to adhere. Varying concentrations of compounds were added to the wells and incubated for 48 h at 37°C. ³H-thymidine (0.25 μCi) was added to each well, and incorporation of the radioactivity was allowed to proceed for 5 to 7 h. Following this incubation, cells were harvested onto GF/B unifilter plates (Packard) using a Packard Filtermate Universal harvester, and the plates were counted in a Packard Top Count 96-well liquid scintillation counter.

Immunoprecipitation

H-460 and MCF-7 cells were treated with 1.5 μmol/L P276-00 for 3, 6, 9, 12, and 24 h. Following this incubation, the cells were lysed in chilled cell lysis buffer [50 mmol/L HEPES (pH, 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 1 mmol/L DTT, 0.1% Tween 20, 10% glycerol, 0.1 mmol/L phenylmethylsulfonyl
fluoride, 100 μg/mL leupeptin, 20 μg/mL aprotonin, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, and 0.1 mmol/L sodium vanadate. The cell lysate was cleared by centrifugation at 10,000 × g for 15 min at 4°C. Cdk4 was immunoprecipitated using specific Cdk4 antibody (Santa Cruz Biotechnology) with protein A-sepharose beads at 4°C from 400 μg protein from cell lysates. The immune complexes were washed thrice with NET-N [20 mmol/L Tris-HCl (pH, 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% NP40] and twice with kinase buffer. Kinase assay was then done as described in the method above using this immune complex and the Rb protein as a substrate.

Western Blot Analysis

H-460 and MCF-7 cells were treated with P276-00 at 1.5 μmol/L for 3, 6, 9, 12, and 24 h. HL-60 cells were treated with 0.5, 1.0, 2.5, and 5.0 μmol/L concentrations of P276-00 for 6 and 24 h. Cells were lysed, and protein content of the lysate was estimated using the Bradford reagent. An equal amount of protein was loaded on SDS-PAGE, and Western blot analysis was carried out as described previously (42) using specific antibody to cyclin D1, Cdk4, pRb, and phospho-pRb Ser780 antibodies (Santa Cruz Biotechnology) for H-460 and MCF-7 cell lysate. Similarly, specific antibody to cleaved caspase-3 (Santa Cruz Biotechnology) was used for HL-60 cell lysate.

Caspase-3 Cellular Activity

HL-60 (human promyelocytic leukemia) cells were treated with different concentrations of P276-00 for 24 h and lysed, and the lysate was assayed using the caspase-3 cellular activity kit (Calbiochem, La Jolla, CA).

DNA Ladder Formation

HL-60 cells were treated with different concentrations of P276-00 for 24 and 48 h. Cells were lysed, and DNA was isolated and subjected to 1.5% agarose gel electrophoresis as described previously (43).

Quantitation of DNA Fragmentation

The amount of DNA fragmentation was quantified using the method of Xia et al. (44). This method separates fragmented and genomic DNA and stains it with DNA intercalating fluorescent dye, 4′-6-diamidino-2-phenylindole. Excitation at 365 nm and emission at 454 nm were read using a Flurometer (POLARstar Optima from BMG, Labtach GmbH, Offenburg, Germany).

Enzyme Kinetic Studies

Cdk4-D1 kinase activity was measured by varying the concentrations of Rb 200 μg/mL (1, 2, 4, 6, 8, and 10 μL) or ATP (10, 30, 50, 70, 100, and 150 μmol/L) as substrates in the presence of different concentrations of P276-00 (30, 60, and 90 nmol/L). The Cdk4-D1 assay was carried out as described above. The enzyme inhibitory activity of P276-00 was determined by measuring the phosphorylation of Rb in comparison with the absence of inhibitors.

Results

Inhibition of Cdk4-D1 and Cdk2-E Enzyme Activity

In an effort to generate novel anti-Cdk4 inhibitors, several flavone derivatives (Fig. 1) were evaluated for their in vitro kinase activity as part of the anticancer-screening program. Several compounds exhibiting below 200 nmol/L in vitro anti-Cdk4 activity were selected. Optimization of the leads with structure-activity relationship studies yielded the most potent compound P276-00 with an IC50 of 63 ± 18.5 nmol/L in Cdk4-D1 enzyme assay. This compound was then screened for inhibition of Cdk2 activity. The results of these experiments confirmed that P276-00 exhibited the highest potency and selectivity for Cdk4-D1 in comparison with Cdk2-E (Table 1). For Cdk4-D1 and Cdk2-E enzyme assays, flavopiridol is used as standard and showed IC50 of 40 ± 15 and 150 ± 50 nmol/L, respectively.

Selectivity of P276-00 against Different Kinases

To ascertain the selectivity of P276-00 for cyclin-dependent and non-Cdks, it was tested against a panel of Cdks and kinases other than Cdks by performing in vitro kinase assays in the presence of increasing concentrations of P276-00 and the appropriate substrates as per the Kinase Profiler at Upstate (Table 1). P276-00 exhibited greater selectivity (IC50s below 100 nmol/L) to Cdk4-D1, Cdk1-B, and Cdk9-T1, as compared with other Cdks tested. P276-00 showed the highest potency for Cdk9-T1 with an IC50 of 20 nmol/L. When compared with other Cdks that are directly involved in cell cycle progression, it was found to be more selective for Cdk4-D1 and Cdk1-B inhibition than Cdk2-E and Cdk7-H (as 40 times specific for Cdk4-D1). In addition, it was not active against many non-Cdk enzymes such as mitogen-activated protein kinase 1 (MAPK1), MAPK2, PKCa, PKA, PKC, LCK, and GSK3β and cSRC (Table 1).

Potency against Human Tumor Lines

Based on the potency in in vitro Cdk4-D1 enzyme assay, P276-00 was selected as the best compound for further studies. Its antiproliferative effect was also determined in a panel of 12 tumor cell lines in comparison with two normal fibroblast cell lines using the 3H-thymidine uptake assay. The range of IC50 values for the cell line panel spanned from 300 to 800 nmol/L in the 3H-thymidine uptake assay. To ascertain whether the compound had any selectivity for normal versus cancer cells, human normal lung fibroblast WI-38 and MRC-5 were treated with P276-00. The IC50 value for normal fibroblast cell lines was significantly higher than those obtained for human tumor cell lines (Table 2).

Loss of Cdk4 Activity Induced by P276-00 Preccedes Depletion of Cyclin D1, Cdk4, and Rb Hypophosphorylation

Cdk4, its activators, and natural inhibitors are responsible for the regulation of the cell cycle. Cell homeostasis is
maintained as long as these factors are in the right proportions. Deregulation of these proteins may lead to cancer. P276-00 was studied for its effect on the regulation of levels of cell cycle proteins in human non–small cell lung cancer (H-460) and human breast cancer (MCF-7) cell lines. To address whether P276-00 decreases the abundance of cyclin D1 indirectly because of effects on G1 Cdk function, catalytic Cdk4 enzyme activity was measured from intact H-460 and MCF-7 cell lines exposed to P276-00. Cdk4 kinase activity immunoprecipitated from growing MCF-7 and H-460 cells following exposure to 1.5 μmol/L P276-00 for various times is shown in Fig. 2. Shortly after addition of P276-00 to H-460 cells (3, 6, 9, 12, and 24 h), significant reduction in enzyme activity was seen at 6 h onward (Fig. 2). This decline in Cdk4-D1 activity in H-460 was followed by a reduction in cyclin D1, Cdk4, and Rb levels as seen by Western blot analysis (Fig. 3A). However, decrease in Rb phosphorylation at Ser780 was seen at 3 h itself (Fig. 3A). Similar treatment of MCF-7 cells shows a loss in Cdk4-D1 enzyme activity at 3 h onward (Fig. 2), whereas protein levels of cyclin D1 and Cdk4 levels starts decreasing at 6 and 9 h, respectively (Fig. 3B). This was also accompanied by a decrease in phosphorylation of Rb at Ser780 from 6 h onward, followed by reduced Rb levels at 24 h (Fig. 3B). Therefore, from these results, it seems that the loss of Cdk4-D1 activity at the early time point is not due to the specific decline in cyclin D1 and Cdk4 levels. Instead, loss of Cdk4 activity precedes cyclin D1 and Cdk4 depletion.

Table 1. Activity against a panel of cyclin-dependent and non-Cdks in the presence of compound P276-00

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Kinase</th>
<th>P276-00 IC50 (μmol/L)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Cdk4-D1</td>
<td>0.063 ± 0.018</td>
</tr>
<tr>
<td>2</td>
<td>Cdk2-E</td>
<td>2.540 ± 0.409</td>
</tr>
<tr>
<td>3</td>
<td>Cdk1-B</td>
<td>0.079</td>
</tr>
<tr>
<td>4</td>
<td>Cdk2-A</td>
<td>0.224</td>
</tr>
<tr>
<td>5</td>
<td>Cdk6-D3</td>
<td>0.396</td>
</tr>
<tr>
<td>6</td>
<td>Cdk7-H</td>
<td>2.870</td>
</tr>
<tr>
<td>7</td>
<td>Cdk9-T1</td>
<td>0.020</td>
</tr>
<tr>
<td>8</td>
<td>C-Src tyrosine kinase</td>
<td>&lt;10</td>
</tr>
<tr>
<td>9</td>
<td>Glycogen synthase kinase-3β</td>
<td>2.771</td>
</tr>
<tr>
<td>10</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>Mitogen-activated protein kinase 1</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>Mitogen-activated protein kinase 2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>13</td>
<td>Protein kinase Ca</td>
<td>10</td>
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<tr>
<td>14</td>
<td>Protein kinase A</td>
<td>575</td>
</tr>
<tr>
<td>15</td>
<td>Protein kinase C (total)</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2. Inhibition of proliferation of human cancer cells by P276-00 as determined by thymidine uptake assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>3H-thymidine (IC50) (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>Colon carcinoma</td>
<td>310</td>
</tr>
<tr>
<td>U2OS</td>
<td>Osteosarcoma</td>
<td>400</td>
</tr>
<tr>
<td>H-460</td>
<td>Lung carcinoma</td>
<td>800</td>
</tr>
<tr>
<td>HL-60</td>
<td>Promyelocytic</td>
<td>750</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon carcinoma</td>
<td>600</td>
</tr>
<tr>
<td>SiHa</td>
<td>Cervical carcinoma</td>
<td>420</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast carcinoma</td>
<td>520</td>
</tr>
<tr>
<td>Colo-205</td>
<td>Colon carcinoma</td>
<td>650</td>
</tr>
<tr>
<td>SW-480</td>
<td>Colon carcinoma</td>
<td>760</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate carcinoma</td>
<td>560</td>
</tr>
<tr>
<td>Caco2</td>
<td>Colon carcinoma</td>
<td>650</td>
</tr>
<tr>
<td>T-24</td>
<td>Bladder carcinoma</td>
<td>390</td>
</tr>
<tr>
<td>WI-38</td>
<td>Normal lung fibroblast</td>
<td>16,500</td>
</tr>
<tr>
<td>MRC-5</td>
<td>Normal lung fibroblast</td>
<td>11,500</td>
</tr>
</tbody>
</table>

Figure 2. Effect of P276-00 on the Cdk4 enzyme activity in human breast carcinoma (MCF-7) and human non–small cell lung carcinoma (H-460) cells by immunoprecipitation. Lane 1, control; lane 2, 1.5 μmol/L P276-00.

Figure 3. Effect of P276-00 on cell cycle protein levels. A, cyclin D1, Cdk4, pRb, and the phosphorylation status of pRb at Ser780 in human non–small cell lung carcinoma cell line (H-460). Lane 1, control; lane 2, 1.5 μmol/L P276-00. B, cyclin D1, Cdk4, pRb, and the phosphorylation status of pRb at Ser780 in human breast carcinoma cell line (MCF-7). Lane 1, control; lane 2, 1.5 μmol/L P276-00.
Caspase-3 Cellular Activity and Western Blot Analysis

Caspase-3, an executioner caspase, is activated during apoptotic signaling events by upstream proteases, including caspase-6, caspase-8, and cytotoxic T cell–derived granzyme B. Caspase-3 is one of the principal caspases found in apoptotic cells. Hence, the ability of P276-00 to induce caspase-3 activity in HL-60 cells was examined. HL-60 cells were treated with 0.5, 0.75, and 1 μmol/L of P276-00 for 24 h. Actinomycin, a known inducer of apoptosis, was used as a positive control. As compared with untreated control, a dose-dependent increase was seen in cellular caspase-3 activity after 24 h of treatment with P276-00 (Fig. 4). The specific activity of caspase-3 at 0.5, 0.75, and 1 μmol/L of P276-00 were 17.2, 116.5, and 138.2 pmol/min/μg, respectively. The caspase activity increases from 3.6- to 29-fold over the control activity of 4.763 pmol/min/μg. Western blot analysis of caspase-3 protein using cleaved caspase-3–specific antibody indicates an increase in the levels of cleaved caspase-3 after 24 h of treatment with P276-00 (Fig. 5).

DNA Ladder Formation

During apoptosis, cells undergo widespread morphologic changes (chromatin condensation and cytoplasmic blebbing), which is associated with the incidence of nucleosome excision from chromatin. This endonuclease-mediated nucleosome excision is observed as a DNA ladder (multimers of ~180-200 bp) in agarose gels. This characteristic form of DNA degradation is a major biochemical hallmark of apoptosis. HL-60 cells treated with 1 μmol/L of P276-00 for 24 h and 2.5 μmol/L for 24 and 48 h induced apoptosis as evident by the DNA ladder formation (Fig. 6).

Quantitation of DNA Fragmentation

HL-60 (promyelocytic leukemia) cells treated with 0.25, 0.5, and 0.75 μmol/L of P276-00 for 48 h showed increase in apoptosis from 30% to 80% at 48 h with increasing concentration of P276-00 (Fig. 7). The induction of DNA fragmentation was also compared with the normal lung fibroblast cell line WI-38 versus the cancerous non–small cell lung carcinoma cell line H-460. H-460 cells showed 85% and 73% apoptosis at 2.5 and 5.0 μmol/L, respectively, as

Figure 4. Induction of caspase-3 in human promyelocytic leukemia (HL-60) cells by treatment with 0.5, 0.75, and 1.0 μmol/L of P276-00 for 24 h. Actinomycin served as a positive control.

Figure 5. Western blot analysis of caspase-3 following 6 and 24 h treatment of human promyelocytic leukemia (HL-60) cells with 0.5 μmol/L (lane 1), 1.0 μmol/L (lane 2), 2.5 μmol/L (lane 3), 5 μmol/L (lane 4) of P276-00, and C-control (lane 5).

Figure 6. DNA fragmentation as seen by ladder formation in human promyelocytic leukemia (HL-60) cells after treatment with 1 and 2.5 μmol/L P276-00. Lane 1, untreated control; lane 2, 1 μmol/L P276-00, 24 h; lane 3, 2.5 μmol/L P276-00, 24 h; lane 4, 2.5 μmol/L P276-00, 48 h.

Figure 7. Quantitation of DNA fragmentation in HL-60 following treatment with 0.25, 0.5, and 0.75 μmol/L of P276-00. Actinomycin was used as a positive control.
compared with 5% apoptosis in the normal cell line at 5.0 μmol/L of P276-00 (Table 3). These results further confirm our previous observations that P276-00 is not active in the normal fibroblast cell line.

**Kinetics of Inhibition of Cdk4-D1 by P276-00**

In an attempt to study the role of P276-00 with respect to Cdk4 inhibition, enzyme kinetics was studied. The enzyme kinetics (competitive, uncompetitive, or mixed type) was determined from inhibition measured against varying concentrations of one substrate (Rb or ATP) and fixed concentration of the other substrate. As seen from the Lineweaver-Burk plot (45), \( V_{\text{max}} \) remains constant with increasing concentration of ATP, indicating that P276-00 is a competitive inhibitor with respect to ATP (Fig. 8A). In the case of Rb at varying concentrations of the inhibitor, all have the same slope, indicating a proportional decrease in the value of \( K_m \) and \( V_{\text{max}} \). This indicates that it is an uncompetitive inhibition relative to Rb (Fig. 8B).

**Discussion**

It is well established that eukaryotic cell proliferation is a tightly regulated system controlled by a network of cyclin-Cdk complexes (46–48). In epithelial cells, the major regulatory checkpoint in this process is the transition from G\(_1\) to the S phase. This transition is characterized by the phosphorylation of pRb, and the Cdk4-D1 enzyme complex catalyzes the reaction. Because most carcinos are of epithelial origin, they exhibit a general dependence on Cdk4-D1 activity for their proliferation. Inhibition of Cdk4-D1 results in the inhibition of cell proliferation as evidenced by the experiments using antisense or antibodies to cyclin D1 (49–53). These observations suggest that an agent that can block the activity of this enzyme complex may be of therapeutic value against most carcinomas.

In this study, we report the results of our efforts to find inhibitors of Cdk4s and their possible therapeutic utility. We identified potent compounds from a series of synthetic flavones screened in an *in vitro* Cdk4-D1 enzyme assay. The best compound P276-00 was profiled. To establish the selectivity of P276-00, it was tested against a representative panel of serine, threonine, and tyrosine kinases. It potently inhibited Cdk9-T1 enzyme activity, which is a known transcription activator. Other than poten activity IC\(_{50}\) below 100 nmol/L in Cdk4-D1, Cdk1-B, and Cdk9-T1, the compound had little or no activity against other Cdk5 and non-Cdks. These *in vitro* enzyme assay results also suggested that the compound P276-00 would be the most potent inhibitor of cell proliferation. It was further tested in cell proliferation assay, i.e., \(^3\)H-thymidine uptake assay in a panel of 12 cancerous cell lines and two normal fibroblast cell lines. We were able to show a selective antitumor effect, with an average potency of 568 nmol/L against a panel of human tumor cell lines, that compared with potency of almost 5-fold or higher against nonproliferating cells, suggesting that a therapeutic window could be achieved because they might have a selective cytotoxic effect on rapidly proliferating cells over quiescent or slowly proliferating normal cells, a property that would make them suitable for use in anticancer therapies.

With P276-00 being a potent Cdk4-D1 inhibitor, the cellular levels of cyclin D1, Cdk4, Rb, and phospho-Rb Ser\(^{\text{780}}\) were investigated. The phosphorylation mediated by Cdk4 on Rb protein is required for the cells to progress from G\(_1\) to S phase in those cells possessing a functional phospho-Rb. As an indication of P276-00 inhibiting the Cdk4 within the cells, we have determined the phosphorylation status of Rb gene product following exposure to P276-00 in exponentially growing H-460 and MCF-7 cells. Over a 24-h exposure to P276-00 in both the cell lines, we found that Cdk4 enzyme activity is inhibited as seen by immunoprecipitation (Fig. 2). This is also confirmed by Western blot, which shows that the Rb protein changed from mostly hyperphosphorylated pRb at 3 h in H-460 and at 6 h in MCF-7 cells to a completely dephosphorylated form at 24 h (Fig. 3). In this study, we show that P276-00, a potent Cdk inhibitor, induces a decline in cyclin D1 and Cdk4 levels. The depletion of cyclin D1 and Cdk4 is preceded by the loss of Cdk4-D1 activity and Rb phosphorylation, thus indicating that the P276-00 may exert its antiproliferative effect at an early stage through inhibition of Cdk4-D1 in a competitive manner with ATP. This evidence is also provided by enzyme kinetic studies, which show that P276-00 competes with ATP for binding at the catalytic site of the Cdk4 enzyme and, thus, inhibiting its kinase activity. Alternatively, inhibition of Cdk9-T1 globally affects cellular transcription with the most profound effects on synthesis of mRNA with short lives, including those encoding growth and apoptosis regulators. Effect of Cdk9-T1 may underlie the transcriptional repression of cyclin D1 by P276-00 as seen in flavopiridol (54, 55), which needs to be proven. To define the mechanism by which P276-00 induces cell death, caspase-3 cellular activity, DNA ladder formation and quantitation of DNA fragmentation were studied. The results showed that caspase-3 activity increased with increasing concentrations of P276-00. This was further confirmed by demonstrating DNA ladder formation, a biochemical hallmark of apoptosis. Western blot analysis also indicated the increase in caspase-3 protein levels as an executioner protein for apoptosis. Apoptotic DNA was quantitated using the fluorescent dye 4′,6-diamidino-2-phenylindole. An increase in apoptotic DNA was seen on treatment of the cells with P276-00. These results showed that the anti-proliferative effect of P276-00 is through the induction of apoptosis in tumor cell lines. The enzyme kinetic studies

**Table 3. Percentage DNA fragmentation in H-460 versus WI-38 after 48 h of treatment with P276-00**

<table>
<thead>
<tr>
<th>Samples</th>
<th>H-460 (%)</th>
<th>WI-38 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>P276-00 (2.5 μmol/L)</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>P276-00 (5.0 μmol/L)</td>
<td>73</td>
<td>5</td>
</tr>
</tbody>
</table>
indicatethat P276-00 is a competitive inhibitor of Cdk4-D1 enzyme and competes with ATP for the ATP binding site on the enzyme.

P276-00, like flavopiridol, belongs to the flavone class of compounds. However, unlike flavopiridol, it shows better selectivity toward Cdk1-B, Cdk4-D1, and Cdk9-T1 as compared with Cdk7-H and Cdk2-E. It inhibits Cdk4-D1, Cdk9-T1, and Cdk1-B with almost the same potency as flavopiridol. However, the inhibition of tumor cell growth in culture is twice to thrice higher than flavopiridol, which may be attributed to its not being a potent pan-Cdk inhibitor and, hence, is also less toxic than flavopiridol (56), although in comparison to other Cdk inhibitors like kenpaullone, olomoucin, and roscovitine, P276-00 is substantially more potent in inhibiting tumor cell growth in culture.

The results presented in this paper show that Cdk5s are a viable target for anticancer therapy. By focusing on specific mechanisms of tumorigenesis, we have developed a primary screening assay for discovering compounds that may be developed for cancer chemotherapy. We have also illustrated that it is necessary in this process to incorporate both in vitro enzyme and cell-based proliferation assays to evaluate compounds for their therapeutic potential. Predictive evaluations of potency and efficacy in cell systems and/or in animal models are essential adjuncts to primary screening in the drug discovery process. Compound P276-00, a novel Cdk inhibitor, is currently in phase I/II clinical trials. Outcome of clinical trial results will decide the beneficial effects of Cdk inhibitors.

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In vitro antitumor properties of a novel cyclin-dependent kinase inhibitor, P276-00

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