

In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1

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Abstract

Polo-like kinase 1 (PLK1) plays key roles in the regulation of mitotic progression, including mitotic entry, spindle formation, chromosome segregation, and cytokinesis. PLK1 expression and activity are strongly linked to proliferating cells. Many studies have shown that PLK1 expression is elevated in a variety of tumors, and high expression often correlates with poor prognosis. Using a variety of methods, including small-molecule inhibition of PLK1 function and/or activity, apoptosis in cancer cell lines, cell cycle arrest in normal cell lines, and antitumor activity *in vivo* have been observed. In the present study, we have examined the *in vitro* biological activity of a novel and selective thiophene benzimidazole ATP-competitive inhibitor of PLK1 and PLK3 (5-(5,6-dimethoxy-1*H*-benzimidazol-1-yl)-3-{[2-(trifluoromethyl)-benzyl]oxy}-thiophene-2-carboxamide, called compound 1). Compound 1 has low nanomolar activity against the PLK1 and PLK3 enzymes and potently inhibits the proliferation of a wide variety of tumor cell lines. In the lung adenocarcinoma cell line NCI-H460, compound 1 induces a transient G₂-M arrest, mitotic spindle defects, and a multinucleate phenotype resulting in apoptosis, whereas normal human

diploid fibroblasts arrest in G₂-M and show little apoptosis. We also describe a cellular mechanistic assay that was developed to identify potent intracellular inhibitors of PLK1. In addition to its potential as a therapeutic agent for treating cancer, compound 1 is also a useful tool molecule for further investigation of the biological functions of PLK1 and PLK3. [Mol Cancer Ther 2007;6(2):450–9]

Introduction

The development of anticancer agents directed against mitotic cells has given rise to clinically important therapies (1). Microtubule disrupters like the taxanes and *Vinca* alkaloids disrupt the mitotic spindle, preventing mitotic progression and inducing apoptosis, and have been used successfully in the clinic for the treatment of a variety of cancers. However, there are significant dose-limiting toxicities associated with the use of these drugs (e.g., peripheral neuropathy; ref. 2). Thus, it would be useful to identify specific mitotic molecular targets whose disruption would result in tumor cell apoptosis without the side effects of antimicrotubule drugs. Polo-like kinase 1 (PLK1) plays key roles in the regulation of mitotic progression, including mitotic entry, spindle formation, chromosome segregation, and cytokinesis (3), and has been postulated as a potential antimitotic cancer target for a number of reasons (4). For example, PLK1 is often overexpressed in many different tumor types, and overexpression often correlates with poor prognosis (5). In addition, interference with PLK1 activity and/or function by a variety of methods (antisense oligonucleotides, small interfering RNA, and various dominant negatives) results in tumor cell apoptosis in culture (6–13) and *in vivo* (14–18); normal cells survive severe PLK1 depletion whereas tumor cells do not (19). Finally, because PLK1 expression and activity are tightly coupled to mitosis, nondividing cells should not be affected by PLK1 inhibition, perhaps mitigating the side effects seen with antimicrotubule drugs. There are increasing efforts to identify non-ATP-competitive and ATP-competitive small-molecule inhibitors for PLKs (20). Recently, ON01910 was reported to be a non-ATP-competitive inhibitor of PLK1, induce apoptosis in cancer cells, and have *in vivo* activity in xenograft tumor models (21). It was also recently reported that an ATP-competitive inhibitor of PLK1, BI 2536, has been advanced to phase I clinical trials (22, 23).

We were interested in developing small-molecule inhibitors of PLK1 and report here the characterization of a novel and selective thiophene benzimidazole ATP-competitive inhibitor of PLK1 (5-(5,6-dimethoxy-1*H*-benzimidazol-1-yl)-3-{[2-(trifluoromethyl)-benzyl]oxy}thiophene-2-carboxamide, hereafter called compound 1; ref. 24). Compound 1 is at least 100-fold selective for PLK1 against other non-PLK kinases tested. We show that compound 1 potently inhibits the proliferation of a variety of human tumor cell lines and

Received 8/31/06; revised 12/1/06; accepted 12/13/06.

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doi:10.1158/1535-7163.MCT-06-0543

has other characteristics predicted for a PLK1 inhibitor. To show inhibition of PLK1 inside the cell, we developed a mechanistic assay using a tetracycline (*tet*)-inducible HeLa (human cervical carcinoma) cell line that expressed a chimeric protein consisting of the complete p53 protein fused to the kinase domain of PLK1. In addition to compound 1, this assay also allowed us to identify many potent PLK1 inhibitors. Because compound 1 is also a potent inhibitor of PLK3, it can also be a useful tool molecule for further investigation of the biological functions of PLK1 and PLK3.

Materials and Methods

Preparation of Compound 1

Compound 1 was prepared as described in ref. 24.

Cells and Cell Culture

All cell culture media were purchased from Invitrogen (Carlsbad, CA) and fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Human diploid fibroblasts (HDF) were isolated by digesting neonatal human foreskins in 2.5% trypsin/1 mmol/L EDTA and were grown in low-glucose DMEM containing 10% FBS. NCI-H460 lung adenocarcinoma (H460) cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 containing 10% FBS. HeLa Tet-On (cervical carcinoma) cells (Clontech, Mountain View, CA) expressing *tet*-inducible p53-PLK1 protein were grown in RPMI 1640 containing 10% Tet System Approved FBS (Clontech) supplemented with 100 µg/mL geneticin (G418, Invitrogen) and 100 µg/mL hygromycin B (Calbiochem, San Diego, CA) to maintain the expression plasmid. MES-SA and MES-SA/DX5 uterine carcinoma cells were obtained from American Type Culture Collection and grown in McCoy's 5A medium containing 10% FBS.

In vitro Kinase Assays

PLK1 and PLK3 proteins were prepared from baculovirus-infected *Trichoplusia ni* cells. Enzyme activity for PLK1 and PLK3 was determined as follows. All measurements were obtained under conditions where signal production increased linearly with time and enzyme. Test compounds were added to white 384-well assay plates (0.1 µL for 10 µL and some 20 µL assays, 1 µL for some 20 µL assays) at variable known concentrations in 100% DMSO. DMSO (1–5% final, as appropriate) and EDTA (65 mmol/L) were used as controls. Reaction Mix contained the following components at 22°C: 25 mmol/L HEPES (pH 7.2); 15 mmol/L MgCl₂; 1 µmol/L ATP; 0.05 µCi/well [γ -³³P]ATP (10 Ci/mmol); 1 µmol/L substrate peptide (Biotin-Ahx-SFNDTLDFD); 0.15 mg/mL bovine serum albumin; 1 mmol/L DTT; and 2 nmol/L PLK1 kinase domain or 5 nmol/L full-length PLK3. Reaction Mix (10 or 20 µL) was quickly added to each well immediately following addition of enzyme via automated liquid handlers and incubated for 1 to 1.5 h at 22°C. The 20-µL enzymatic reactions were stopped with 50 µL of stop mix [50 mmol/L EDTA, 4.0 mg/mL streptavidin SPA beads in Dulbecco's PBS (without Mg²⁺ and Ca²⁺), 50 µmol/L ATP]

per well. The 10-µL reactions were stopped with 10 µL of stop mix [50 mmol/L EDTA, 3.0 mg/mL streptavidin-coupled SPA Imaging Beads ("LeadSeeker") in Dulbecco's PBS (without Mg²⁺ and Ca²⁺), 50 µmol/L ATP] per well. Plates were sealed, spun at 500 × g for 1 min or settled overnight, and counted in Packard TopCount for 30 s/well (regular SPA) or imaged with a Viewlux imager (Lead-Seeker SPA). Signal above background (EDTA controls) was converted to percent inhibition relative to that obtained in control (DMSO-only) wells.

Methods for additional kinase assays can be found in Supplementary methods.⁷

In vitro Growth Inhibition Assays

Assays were carried out and data analyzed as described by Rusnak et al. (25). In these assays, H460 cells were plated at a density of 2,000 per well, HDF cells were plated at 5,000 per well, and the drug-resistant cell line MES-SA/DX5 and its sensitive parent line MES-SA were plated at 7,000 and 6,000 per well, respectively, in a 96-well plate. These densities allowed vehicle controls to grow logarithmically during the course of the 3-day assay. All cells were exposed to 3-fold dilutions of the compound (30–0.00152 µmol/L) in low-glucose DMEM containing 5% FBS, 50 µg/mL gentamicin, and 0.3% (v/v) DMSO (HDF cells); RPMI 1640 containing 5% FBS, 50 µg/mL gentamicin, and 0.3% (v/v) DMSO (H460); or McCoy's 5A containing 5% FBS, 50 µg/mL gentamicin, and 0.3% (v/v) DMSO (MES-SA and MES-SA/DX5).

Tet-Inducible Construct and PLK1 Mechanistic Assay

A *tet*-inducible plasmid was constructed to allow regulatable expression of a p53-PLK1 chimeric protein. A mammalian expression plasmid for p53-PLK1 was constructed and consists of the entire p53 sequence, a "flexible" linker of four tandem repeats of the amino acid sequence GGGS, and the kinase domain of PLK1 (amino acids 3–345; Fig. 1A). The p53-PLK1 sequence was subsequently subcloned into a BacMam vector (26). Using PCR, a *Hind*III (site added with the PCR amplimer)-*Not*I fragment (from the BacMam vector) containing the p53-PLK1 sequence was isolated and subcloned in-frame into the prokaryotic biotinylation sequence vector Pinpoint Xa-1 (Promega, Madison, WI). The PBS-p53-PLK1 sequence was excised by cutting with *Eco*RI and *Not*I and subcloned into the *tet*-inducible vector pTRE-Tight (BD Clontech) to create pTRE-Tight Biotin-p53-PLK1. The prokaryotic biotinylation sequence was replaced with the eukaryotic biotinylation sequence from pcDNA6/BioEase-DEST (Invitrogen) as follows. pTRE-Tight Biotin-p53-PLK1 was cut with *Eco*RI and *Nru*I and the ends blunted using T4 DNA polymerase. The eukaryotic biotinylation sequence was excised from pcDNA6/BioEase-DEST by cutting with *Bam*HI and *Hind*III, the ends blunted with T4 DNA polymerase, and ligated in frame into the blunt-ended vector. The activated mutant T210D (ACC→GAC) in the *PLK1* gene was then made using the Transformer Site-Directed Mutagenesis Kit

⁷ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

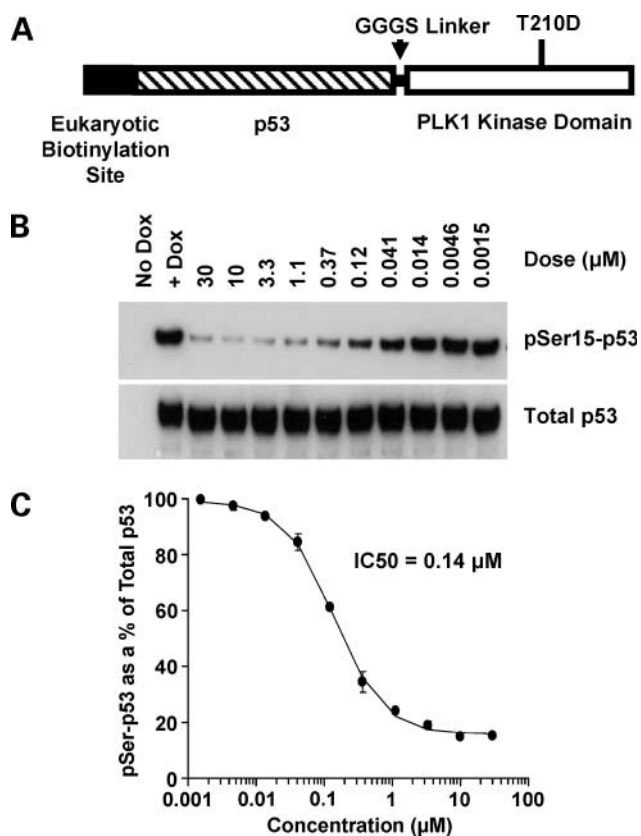


Figure 1. Mechanistic cellular assay for PLK1. **A**, schematic diagram of the *tet*-inducible chimeric p53-PLK1 protein. The chimeric protein has a eukaryotic biotinylation site at the NH₂ terminus, which allows binding to streptavidin, and a flexible GGGG linker to facilitate intramolecular phosphorylation. The p53 comprises the entire protein, and the PLK1 sequence encompasses amino acids 3 to 345, which include the kinase domain but not the polo boxes. **B**, Western immunoblots of HeLa cells treated with compound 1 following induction of the p53-PLK1 fusion. *Top*, phospho-p53 (Ser¹⁵); *bottom*, total p53. **C**, compound 1 inhibition curve from an ELISA developed using the *tet*-inducible p53-PLK1 fusion protein expressed in HeLa cells (IC₅₀, 0.14 μmol/L).

(BD Clontech) according to the manufacturer's instructions. This construct was transfected into the HeLa Tet-On cell line, which expresses the reverse *tet* transactivator, and stable cell lines were selected using hygromycin B (100 μg/mL). An ELISA using these cells was carried out as follows. Cells were seeded onto wells of a 96-well plate at 5,000 per 100 μL of high-glucose DMEM containing 10% Tet System Approved FBS and 125 ng/mL doxycycline (Sigma, St. Louis, MO; the last column of the plate contains cells with media but no doxycycline) and grown overnight. High-binding, half-well 96-well assay plates (Corning, Corning, NY) were coated overnight at 4°C with 50 μL/well of 0.5 mg/mL anti-p53 antibody (BD Transduction Laboratories, San Jose, CA) in PBS (containing Ca²⁺ and Mg²⁺). The next day, cells were treated with ten 3-fold dilutions of compound (final concentrations, 30–0.00152 μmol/L, the last two columns receiving only DMSO) in high-glucose DMEM containing 5% Tet System Approved

FBS, 0.3% DMSO, and 125 ng/mL doxycycline for 1 h. Assay plates were washed four times with PBS containing 0.05% Tween 20 (PBST) and then blocked with PBST containing 5% milk at room temperature. The medium was aspirated from the dosed cells, the wells were washed once with PBS, and the cells were lysed in 100 μL/well of lysis buffer [137 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 20 mmol/L Tris-HCl (pH 8)] containing Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) and a 1:100 dilution of phosphatase inhibitor cocktail (Calbiochem). The lysed cells were kept on ice for ~1 h. Duplicate samples of 50-μL lysate were distributed to assay plates, one each for total p53 and phospho-p53 (Ser¹⁵), and incubated overnight at 4°C. Assay plates were washed five times with PBST and incubated for 1 h at room temperature with either a 1:500 dilution of phosphospecific Ser¹⁵-p53 antibody (Cell Signaling Technology, Beverly, MA) or anti-p53 antibody (Cell Signaling Technology) in PBST containing 5% milk. The plates were washed as before and incubated for 1 h at room temperature with a 1:10,000 dilution of antirabbit immunoglobulin G horseradish peroxidase antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBST containing 5% milk. The plates were washed as before and incubated for ~10 min in 50-μL TMB horseradish peroxidase substrate solution (BioFX Labs, Owing Mills, MD) and the reaction was stopped with 50 μL of 2 mol/L H₂SO₄. Absorbance was measured at 450 nm and data were analyzed using a macro written for Microsoft Excel. IC₅₀ values were interpolated using the equation $Y = V_{\max} X \{1 - [x / (k + x)]\} + Y_2$, where K equals the IC₅₀ and Y_2 is the minimum value on the Y axis.

Western Immunoblotting

HeLa cells expressing the *tet*-inducible p53-PLK1 were incubated overnight with 125 ng/mL doxycycline to induce expression of the p53-PLK1 protein. The cells were treated for 6 h with 3-fold dilutions of compound 1 (30–0.00152 μmol/L) and lysates were prepared. The proteins were resolved on 4% to 12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose, and then blotted either with the phospho-specific Ser¹⁵-p53 or total p53 antibodies previously noted.

Fluorescence-Activated Cell Sorting Analysis

Cells were seeded onto six-well plates to allow logarithmic growth over the course of the assay [H460 cells: 100,000 per well (24 h), 50,000 per well (48 h), and 25,000 per well (72 h); HDF cells: 120,000 per well (24 h), 60,000 per well (48 h), and 30,000 per well (72 h)] and treated with 1 and 3 μmol/L of compound 1 for 24, 48, and 72 h. Cells were collected by trypsinization and centrifugation along with any cells floating in the media. The cells were fixed in ethanol, permeabilized with Triton X-100, and stained for DNA content with propidium iodide (Calbiochem). Samples were analyzed using a Becton Dickinson FACSsort flow cytometer.

Outgrowth Assays

HDF and H460 cells were seeded onto 96-well plates at densities of 1,300 and 600 per well, respectively. After 24 h, the cells were exposed to vehicle (0.3% DMSO) or compound 1 at the concentrations indicated in the figure

legend for Fig. 3. After 72 h, the medium was removed, the wells were washed once with PBS, and fresh medium containing 50 $\mu\text{g}/\text{mL}$ gentamicin was added. Plates were stained with methylene blue, as previously described, at the time points indicated in Fig. 3 (a total of 13 days), and the data were analyzed as previously described.

Apoptosis Assays

H460 cells were plated at 320,000 per well on six-well plates and treated with 5 $\mu\text{mol}/\text{L}$ compound 1 for 7, 22, 26, 30, 46, 51, 55, and 72 h. Lysates were prepared in caspase assay buffer [10 mmol/L Tris-HCl, 10 mmol/L $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$ (pH 7.5), 130 mmol/L NaCl, 1% Triton X-100, 10 mmol/L sodium pyrophosphate] and the lysates were assayed for cleavage of the caspase-3 fluorogenic substrate peptide Ac-DEVD-AMD using the Caspase 3 Assay Kit (BD PharMingen, San Diego, CA). Cleavage of the fluorescent AMD from the DEVD peptide was monitored using a plate reader with an excitation wavelength of 380 nm and an emission wavelength range of 420 to 460 nm. To examine poly(ADP-ribose) polymerase (PARP) cleavage, H460 cells were plated as before and treated with 5 $\mu\text{mol}/\text{L}$ compound 1 for 22, 30, 46, 55, and 72 h. The cells were lysed in caspase assay buffer and proteins were resolved on 4% to 12% Bis-Tris gels, transferred to nitrocellulose, and blotted with an anti-PARP antibody (BD PharMingen) that recognizes both the 116-kDa complete protein and the 85-kDa cleavage fragment.

Immunofluorescence Microscopy

H460 cells were grown in chamber slides and treated with 0.5 or 1 $\mu\text{mol}/\text{L}$ of compound 1 for 24 or 48 h, respectively. After 24 or 48 h, the cells were fixed with ice-cold 100% methanol for 1 h. The slides were rinsed thrice with PBS and the cells permeabilized with 0.5% Triton X-100 in PBS for 15 min. The slides were washed thrice for 5 min each with PBS and blocked with 5% boiled donkey serum at room temperature for 30 min; all antibodies were diluted in 5% boiled donkey serum and incubated at room temperature. The slides were washed as before and incubated with a 1:200 dilution of lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The slides were washed as before and incubated with a 1:200 dilution of Cy3-conjugated donkey anti-mouse immunoglobulin G (H+L) secondary antibody (Jackson ImmunoResearch Laboratories) in the dark for 45 min. The slides were washed as before and incubated with a 1:50 dilution of monoclonal anti- β -tubulin TUB2.1 FITC antibody (Sigma) containing 4',6-diamidino-2-phenylindole at 0.5 $\mu\text{g}/\text{mL}$ in the dark for 45 min. After washing as before, coverslips were mounted on slides using Gel/Mount (Biomed, Foster City, CA) and examined under a Leica DM RA2 fluorescence microscope. Images were captured with a Hamamatsu charge-coupled device camera and Adobe Photoshop.

Results

Compound 1 Is a Potent and Selective Inhibitor of PLK1 and PLK3

Compound 1 is a novel thiophene benzimidazole inhibitor of PLK1 (structure in Table 1) and is a potent

inhibitor of both PLK1 and PLK3 enzymes, with IC_{50} values of 2.2 and 9.1 nmol/L, respectively. The IC_{50} for PLK1 and PLK3 is >1,000-fold lower than cell cycle [cyclin-dependent kinase (CDK)-2/cyclin A] and mitotic (CDK1/cyclin A and aurora A) kinases, as well as the additional kinases shown in Table 1. Compound 1 is also >100-fold selective against ~30 other kinases (data not shown).

Compound 1 Inhibits the Growth of Tumor Cells in Culture

The ability of compound 1 to inhibit proliferation in a variety of tumor cells grown in culture was evaluated. Compound 1 is a submicromolar inhibitor of proliferation of most tumor cells in culture, a notable exception being PC-3, a prostate carcinoma cell line (Table 2). Compound 1 is also selective for tumor cells compared with normal diploid fibroblasts (HDF), with >10-fold difference in potency. The activity of compound 1 was evaluated in a drug-resistant cell line and its nonresistant parental line. MES-SA/DX5 is a drug-resistant uterine sarcoma line that expresses high levels of *mdr-1* mRNA and its product, P-glycoprotein (27, 28). Compound 1 is equipotent (~200 nmol/L) against MES-SA/DX5 and the parental line MES-SA, suggesting that the compound is not effectively removed by the P-glycoprotein efflux pump in the cell.

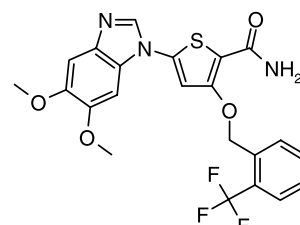
Mechanistic Assay to Measure Intracellular Inhibition of PLK1

A mechanistic assay was developed to measure the inhibition of PLK1 in the cell in response to drug treatment. Because PLK1 expression and activity is maximal during

Table 1. Structure of compound 1 and enzyme inhibition

Enzyme	IC_{50} (nmol/L)
PLK1	2.2
PLK3	9.1
PDGFR1 β	160
VEGFR2	360
Aurora A	4,800
CDK2/cyclin A	7,600
CDK1/cyclin A	>10,000
p38 α	>10,000
p38 β	>10,000
EGFR	>10,000
c-src	>10,000

Abbreviations: PDGFR1 β , platelet-derived growth factor receptor 1 β ; VEGFR2, vascular endothelial growth factor receptor 2.



Structure of Compound 1

Table 2. *In vitro* cell proliferation inhibition activity

Cell line	Tumor type	IC ₅₀ (μmol/L)	SD
A549	Lung adenocarcinoma	0.41	0.04
BT474	Breast ductal carcinoma	0.57	0.19
HeLa	Cervical adenocarcinoma	0.11	0.06
H460	Lung adenocarcinoma	0.38	0.18
HCT116	Colorectal carcinoma	0.70	0.37
HDF	Human diploid fibroblasts	6.14	4.35
HN5	Head and neck squamous cell carcinoma	0.68	0.18
MCF7	Breast adenocarcinoma	0.56	0.29
N87	Gastric carcinoma	0.60	0.05
PC-3	Prostate adenocarcinoma	6.82	3.75
RKO	Colon carcinoma	0.40	0.18
Drug-resistant cell line and nonresistant parental cell line			
MES-SA	Uterine sarcoma (parental)	0.21	0.02
MES-SA/DX5	Uterine sarcoma (resistant)	0.21	0.02

mitosis, it was desirable to identify a known substrate of PLK1 whose phosphorylation could be monitored during this short period of the cell cycle. Whereas there existed candidate substrates when the current studies were initiated [e.g., SCC1 (29) and nudC (30)], a *tet*-inducible HeLa cell line was developed that expressed a chimeric protein consisting of the complete p53 protein fused to the kinase domain of PLK1 (Fig. 1A). p53 was used as a substrate because it was reported to be phosphorylated by PLK1 *in vitro* (31) and phosphospecific antibodies for many sites on p53 are commercially available. We screened several of these antibodies and found that PLK1 can phosphorylate p53 on Ser¹⁵ *in vitro* in the chimeric protein (data not shown). The chimeric protein also has an NH₂-terminal eukaryotic biotinylation domain, allowing binding to streptavidin, and a flexible linker (GGGS) between the p53 and PLK1 sequences to facilitate intramolecular phosphorylation. The use of a *tet*-inducible protein was required because constitutive expression of the p53-PLK1 chimera inhibited growth of the HeLa cells (data not shown). Initial tests of the chimeric protein showed that it had very little activity above background controls. To increase the activity of the PLK1 kinase domain, T210 was mutated to aspartic acid, which mimics phosphorylation, has been reported to increase kinase activity significantly in human PLK1, and is the major phosphorylation site *in vivo* (32). In addition, the T210D mutation had no effect on potency of compound 1 or other related thiophene benzimidazoles in *in vitro* PLK1 kinase assays (data not shown). This mechanistic assay can thus serve as a very useful intracellular reporter assay for PLK1 activity. There was a dose-dependent inhibition of phosphorylation of Ser¹⁵-p53 by PLK1 by compound 1 (Fig. 1B). The *tet*-inducible HeLa cell line was used to develop an ELISA to test other PLK1 inhibitors of interest. Inhibition curves can be generated using this ELISA (Fig. 1C), and there was a dose-dependent inhibition of PLK1 phosphorylation of Ser¹⁵-p53 by compound 1, with an IC₅₀ of inhibition of

0.14 μmol/L. Lack of complete inhibition of Ser¹⁵-p53 phosphorylation is probably due to other kinases that can phosphorylate p53 in the cell. These data clearly showed that compound 1 was able to inhibit potently the p53-PLK1 chimeric protein in the cell. In addition to compound 1, many other related thiophene benzimidazole inhibitors of PLK1 were screened in the mechanistic assay, and a comparison of pIC₅₀ values in this assay and in the methylene blue cell proliferation assay in H460 cells shows a good correlation between the two assays (Fig. 2). These data show that PLK1 compounds that were potent against PLK1 in the cell also potently inhibit cell proliferation, as would be expected for a PLK1 inhibitor.

Outgrowth Assays

To determine concentrations of compound 1 required to induce irreversible growth arrest in HDF cells and H460 tumor cells, cells were exposed to the compound for 72 h,

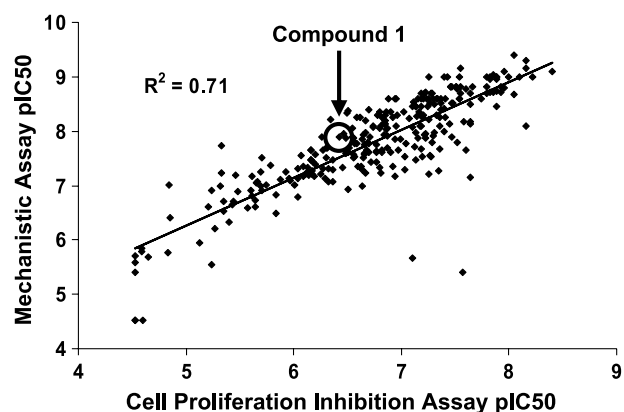


Figure 2. Graph showing the correlation between the pIC₅₀s of the methylene blue cell proliferation assay and the mechanistic assay of compounds screened in both assays. The data for the methylene blue assay are from H460 cells and the mechanistic assay data are from HeLa cells expressing the *tet*-inducible chimeric p53-PLK1 protein. Compound 1 is indicated on the plot. The pIC₅₀ is calculated as $-\log_{10}(\text{IC}_{50})$.

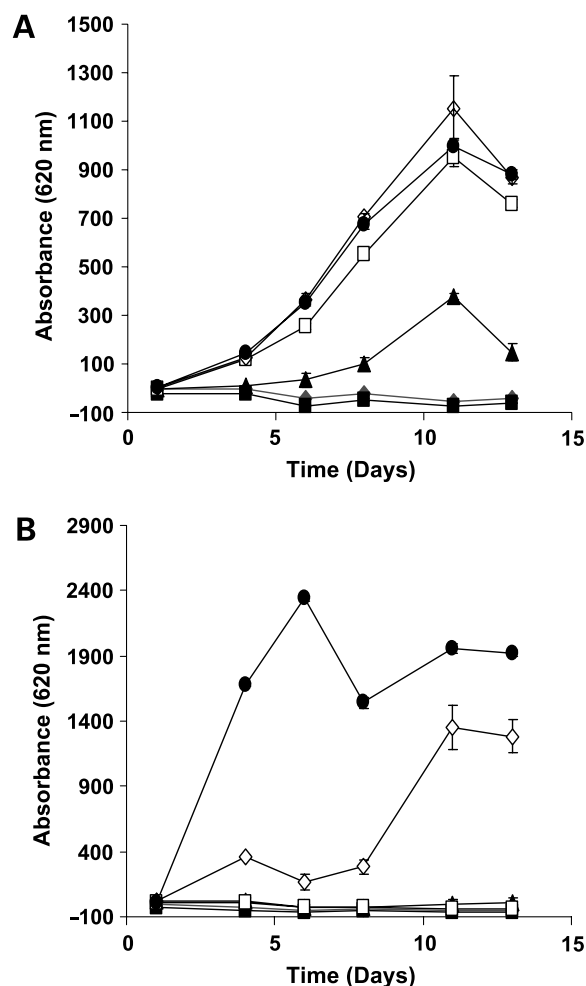


Figure 3. Outgrowth assays following treatment with compound 1. **A**, HDF cell outgrowth. **B**, H460 outgrowth. \blacklozenge , 30 $\mu\text{mol/L}$; \blacksquare , 10 $\mu\text{mol/L}$; \blacktriangle , 3.3 $\mu\text{mol/L}$; \circ , 1.1 $\mu\text{mol/L}$; \diamond , 0.37 $\mu\text{mol/L}$; \bullet , vehicle control.

medium was aspirated and replaced with fresh media to remove the compound, and the cells were incubated in the absence of compound. The time frame of the assay allows ample time for the cells to regrow following compound removal. HDF cells were able to regrow at all concentrations except 10 and 30 $\mu\text{mol/L}$, with $\sim 30\%$ regrowth observed at 3.3 $\mu\text{mol/L}$ (Fig. 3A). In contrast, H460 tumor cells were much more sensitive to the compound and were only able to regrow at the lowest concentration, 0.37 $\mu\text{mol/L}$ (Fig. 3B).

Cell Cycle Analysis and Measurement of Apoptosis

Because PLK1 expression and activity is tightly linked to mitosis, the effects on the cell cycle from treating HDF cells and H460 cells with compound 1 were analyzed. Treatment of HDF cells with 3 $\mu\text{mol/L}$ compound 1 for 24, 48, and 72 h resulted in a strong G₂-M arrest (Fig. 4A; Table 3); very little G₂-M arrest was observed at 1 $\mu\text{mol/L}$ compound 1. Significantly, there was very little cell death (sub-2N DNA) in the HDF cells treated with either 1 or

3 $\mu\text{mol/L}$ compound [none at 24 h; ~ 2 -fold at 1 $\mu\text{mol/L}$ and ~ 4 -fold at 3 $\mu\text{mol/L}$ at 48 h (Table 3; Fig. 4A)]; the increase at 72 h to 9% may be a result of the prolonged arrest at G₂-M. The H460 cells showed a different profile compared with the HDF cells (Fig. 4B). There was a G₂-M arrest at 1 and 3 $\mu\text{mol/L}$ of compound 1 at 24 h ($\sim 33\%$ and $\sim 38\%$, respectively, compared with 20% in controls, Table 3) but it was not as pronounced as that seen in the HDF cells at 3 $\mu\text{mol/L}$ at 24 h. However, H460 cells treated with 1 and 3 $\mu\text{mol/L}$ of compound 1 for 48 and 72 h showed a reduced G₂-M arrest, but there was an increase in sub-2N DNA content, a measure of apoptosis [3- and 6-fold at 24 h; 6- and 8-fold at 48 h; and 8- and 15-fold for 1 and 3 $\mu\text{mol/L}$ of compound 1, respectively, over controls (Table 3)] and a dramatic increase in $>4\text{N}$ DNA content at 48 and 72 h.

Because the fluorescence-activated cell sorting results showed that H460 cells treated with compound 1 have an increase in sub-2N DNA, two common markers for apoptotic cell death, caspase activation and cleavage of poly(ADP ribose) polymerase (PARP), were examined to verify that the cells were undergoing apoptosis. There was a strong increase in caspase activity in response to increasing time of compound exposure, peaking at

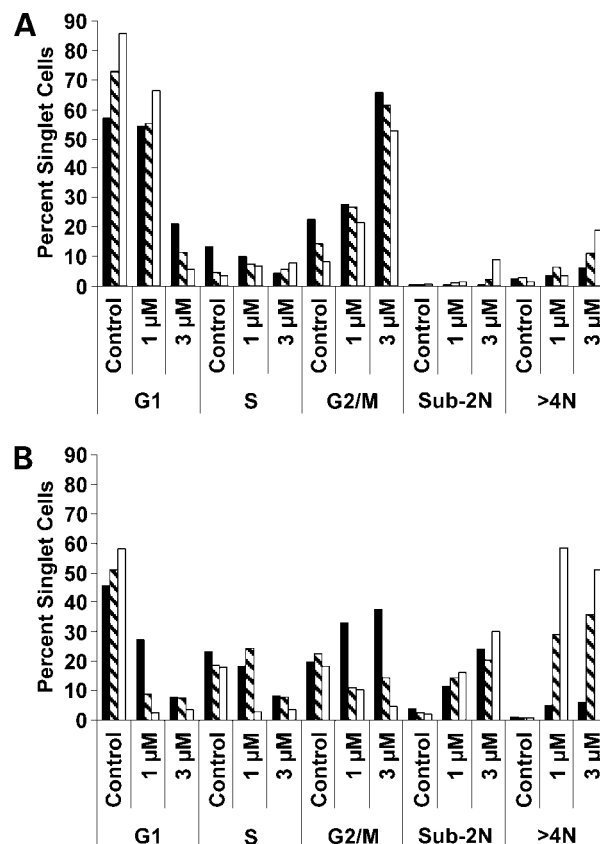


Figure 4. Cell cycle profiles of HDF (**A**) and H460 lung adenocarcinoma cells (**B**) treated with no compound and with 1 and 3 $\mu\text{mol/L}$ of compound 1 for 24 h (\blacksquare), 48 h (\square), or 72 h (\square).

Table 3. Fluorescence-activated cell sorting analysis of cell cycle phases in HDF and H460 lung adenocarcinoma cells treated with compound 1

Cell cycle phase	Treatment ($\mu\text{mol/L}$)	HDF (% singlet cells)			H460 (% singlet cells)		
		24 h	48 h	72 h	24 h	48 h	72 h
G ₁	Control	57.0	72.8	85.6	45.5	50.9	58.2
	1	54.2	55.3	66.2	27.3	8.9	2.5
	3	21.1	11.3	5.6	7.8	7.5	3.7
S	Control	13.2	4.7	3.6	23.2	18.5	17.8
	1	10.2	7.4	6.7	18.2	24.3	2.8
	3	4.2	5.9	8.0	8.4	8.0	3.6
G ₂ -M	Control	22.6	14.3	8.2	19.8	22.5	18.2
	1	27.6	27.0	21.6	33.0	11.2	10.6
	3	65.6	61.2	52.7	37.7	14.4	4.7
Sub-2N	Control	0.2	0.5	0.7	4.0	2.5	2.1
	1	0.3	0.9	1.3	11.6	14.4	16.0
	3	0.2	2.3	9.1	24.1	20.3	30.0
>4N	Control	2.4	3.0	1.4	1.2	0.9	0.9
	1	3.5	6.4	3.5	5.1	29.0	58.6
	3	6.0	11.2	19.1	6.2	35.9	51.0

46 h (Fig. 5A). The decrease in activity at later time points may be a result of massive cell death in the cell population. HDF cells showed very little (<50 relative fluorescent units) caspase activity (Fig. 5B). In PARP cleavage assays, the 116-kDa protein was cleaved to a 24-kDa DNA-binding fragment and an 85-kDa catalytic fragment; the anti-PARP antibody recognized the 116- and 85-kDa proteins. The presence of the 85-kDa cleavage fragment was evident at all time points whereas there was very little at the 72-h untreated control (Fig. 5C). Fluorescence-activated cell sorting analysis showed that there was 2.5% to 4% sub-2N DNA in untreated H460 cells (Table 3); thus, a small amount of apoptosis in untreated cells was expected. Taken together, these data suggested that whereas HDF cells were able to arrest at G₂-M with little cell death over 72 h, H460 cells transiently arrested at G₂-M, followed by increases in DNA content >4N and cell death.

Immunofluorescence Microscopy of H460 Cells Treated with Compound 1

Because compound 1 induced cell cycle effects and apoptosis in H460 cells, we investigated the cellular phenotypes resulting from treatment of H460 cells with compound 1. Untreated H460 cells at 24 h had a single nucleus and the mitoses appeared to be normal (Fig. 6A). However, treating H460 cells with 0.5 $\mu\text{mol/L}$ compound 1 for 24 h caused generation of cells with aberrant, multiple spindles (Fig. 6B, *arrowheads*). By 48 h of treatment with 1 $\mu\text{mol/L}$ compound 1, there were cells that were larger in size than the untreated H460 cells and had multiple nuclei (Fig. 6C and D, *open arrows*). These cells may result from abortive mitoses in response to PLK1 inhibition. Cell cycle analysis showed that the H460 cells also have an increase in >4N DNA content (Fig. 4B), and these multinucleate cells may represent the cells with this higher content due to aberrant mitoses.

Discussion

PLK1 is a member of an evolutionarily conserved family of serine/threonine kinases and plays key roles in the regulation of mitotic progression, including mitotic entry, spindle formation, chromosome segregation, and cytokinesis (3). PLK1 has been postulated as a potential cancer therapeutic target (4) due to its strong association with cell proliferation and elevated expression in a variety of tumors. Additionally, inhibition of PLK1 activity or interference with its function leads to mitotic arrest, spindle defects, and aberrant cytokinesis, resulting in growth arrest and/or apoptosis. As a result, we were interested in developing small-molecule inhibitors of PLK1 and identified a novel and selective thiophene benzimidazole ATP-competitive inhibitor of PLK1, compound 1. Although very few small-molecule inhibitors of PLK1 have been published to date, it is clear that development of such inhibitors is progressing (20).

Compound 1 is a potent inhibitor of the PLK1 enzyme, with an IC₅₀ of 2.2 nmol/L, and is also potent against PLK3 (IC₅₀, 9.1 nmol/L), another PLK family member. Compound 1 is ~100-fold more selective for the PLKs compared with the other enzymes screened, including cell cycle kinases like CDK1 and CDK2, which should reduce off-target effects. Compound 1 potently inhibits the proliferation of a wide variety of tumor cell lines and is ~10-fold less selective for HDF cells, suggesting that there will be the possibility of achieving a therapeutic window *in vivo*. Further corroborating the increased sensitivity of the tumor cell line to compound 1 compared with normal cells, lower concentrations of compound 1 were required to induce irreversible growth arrest following compound washout and regrowth in media without compound. Additionally, an MDR-positive cell line that was tested (MES-SA/DX5) was equally sensitive to compound 1 as its

nonresistant parental line, suggesting that compound 1 is not a substrate of the P-glycoprotein efflux pump (27, 28). A recently described non-ATP-competitive inhibitor of PLK1, ON01910, also showed potent inhibitory activity against a variety of cultured human tumor cell lines and drug-resistant cell lines (including MES-SA/DX5) and their normal counterparts (21). However, ON01910 is also a potent inhibitor of many other kinases, such as CDK1, Abl, FLT, platelet-derived growth factor receptor, and Met (21), in addition to PLK1. Inhibition of these other kinases may play a role in the phenotypes observed in cells treated with ON01910.

In addition to inhibiting tumor cell proliferation, compound 1 shows other characteristics expected for a PLK1 inhibitor. As originally reported in PLK1 antibody microinjection experiments by Lane and Nigg (6), interfering with PLK1 function in HeLa cells resulted in impairment

of mitotic progression and cell division. Additionally, HeLa cells showed either multinucleate or micronucleate phenotypes. However, Hs68 nonimmortalized cells showed a strong arrest in G₂ without any apparent defects, suggesting a distinct difference in the response of tumor cells and normal cells to PLK1 inhibition. Our fluorescence-activated cell sorting data showed that HDF cells arrested strongly at G₂-M following exposure to compound 1. In HDF cells, the G₂-M arrest was accompanied by very little cell death (sub-2N DNA) up to 48 h; increasing cell death at 72 h may be a result of the prolonged arrest at G₂-M. In contrast, H460 tumor cells seemed to arrest at G₂-M, but not as strongly as the HDF cells, followed by increasing cell death as measured by sub-2N DNA content. The cell death observed in the H460 cells was significantly greater than that observed in the HDF cells. Other studies using small interfering RNAs directed against PLK1 resulted in no changes in the cell cycle in normal MCF10A and hTERT-RPE1 cells (19) or normal human fibroblasts, IMR90 foreskin fibroblasts, and mammary gland epithelial cells (17). Because small interfering RNAs eliminate PLK1 protein whereas small-molecule inhibitors prevent kinase activity, there may be additional functions of PLK1 that are eliminated by completely ablating the protein rather than by inhibiting the kinase activity. For example, it has been noted that different methods used to interfere with the function and/or activity of aurora kinase, including small-molecule kinase inhibitors, can yield different phenotypes (33). Confirmation of apoptosis was shown by PARP cleavage and caspase activation. Interestingly, we also observed a dramatic increase in >4N DNA content in H460 cells, suggesting endoreduplication of the DNA. The increase in DNA content may also be indicated by the multinucleate phenotype we observed following exposure to compound 1 for 48 h. Immunofluorescence microscopy of H460 cells treated with compound 1 also showed phenotypes similar to those observed in tumor cells in which PLK1 function and activity were ablated. We observed multiple spindles in many cells that had been exposed to the compound. Similar spindle abnormalities were observed by Seong et al. (8) in U2OS (osteosarcoma) cells that overexpress a dominant-negative form of PLK1 missing the kinase domain but possessing the polo boxes. A recent study using the non-ATP-competitive inhibitor ON01910 also showed that HeLa cells treated with ON01910 exhibited multiple, disorganized spindles (21).

Whereas enzyme screening and inhibition of cell proliferation were used to identify potent PLK1 inhibitors, it was also important to show that the compound inhibits PLK1 in the cell. Because PLK1 expression and activity are highest during mitosis, it was desirable to identify an abundant PLK1 mitotic substrate whose phosphorylation could be monitored to show a reduction in phosphorylation in response to inhibition of PLK1. To circumvent the need for an abundant mitotic substrate of PLK1 whose phosphorylation could be easily monitored, a mechanistic assay was developed using a HeLa cell line that expresses a p53-PLK1 kinase domain fusion protein under the control of a

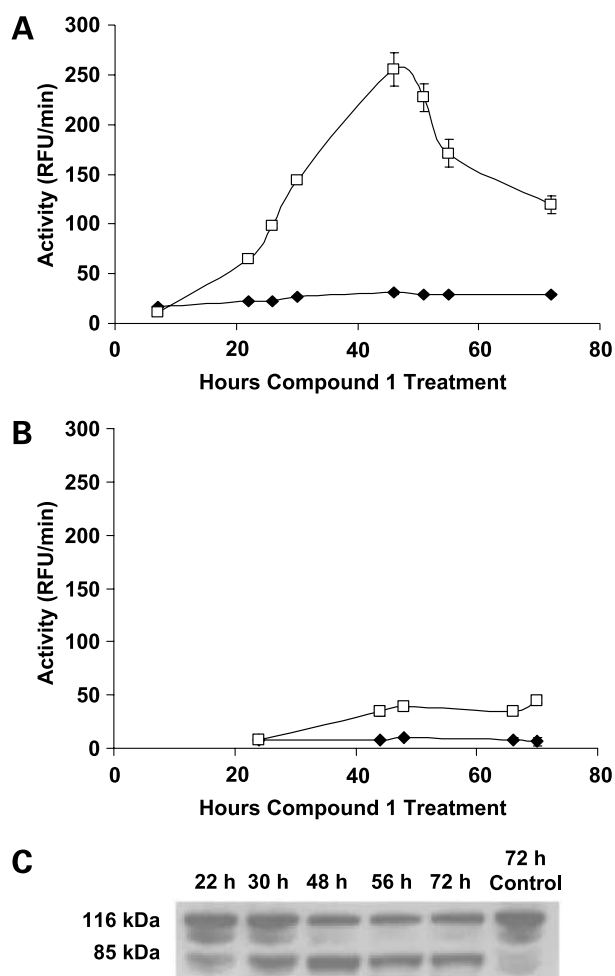


Figure 5. Compound 1 induces apoptosis in H460 cells but not in HDF cells. **A** and **B**, caspase activation in response to treatment with 5 μ mol/L compound 1 in H460 cells (**A**) and HDF cells (**B**). \blacklozenge , untreated cells; \square , treated cells. **C**, Western immunoblot of PARP cleavage in response to treatment with 5 μ mol/L compound 1 in H460 cells. Equal amounts of cellular protein were loaded in each lane.

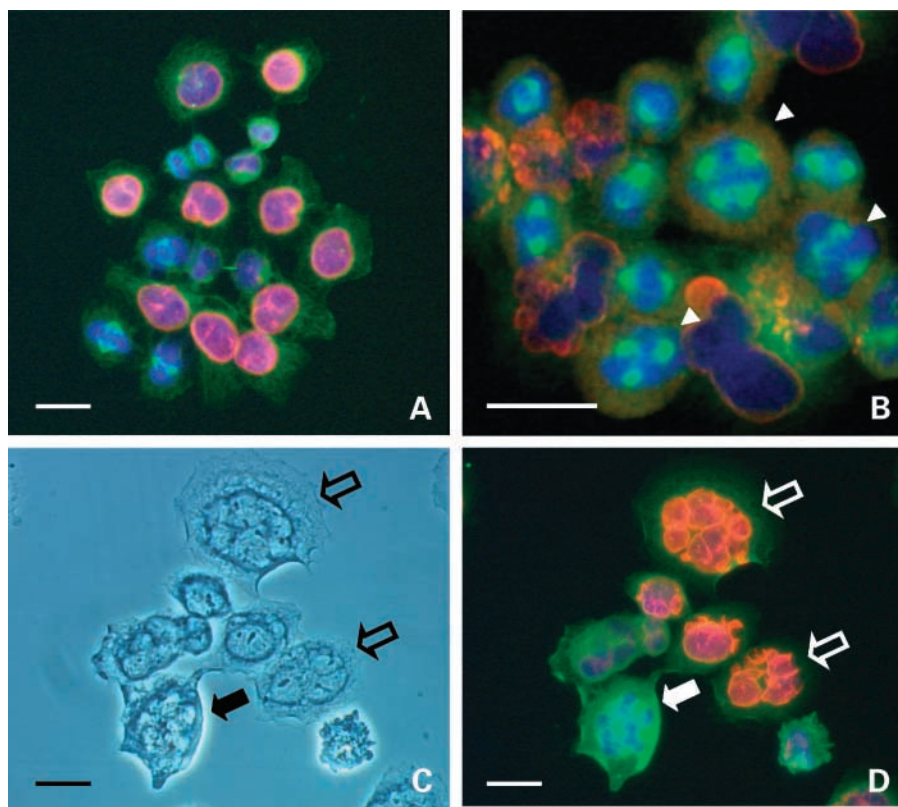


Figure 6. Immunofluorescence microscopy of H460 cells treated with compound 1. Cells were stained for α -tubulin (green); 4',6-diamidino-2-phenylindole (blue); and α -lamin A/C, a component of the nuclear membrane (orange). Bar, 20 μ m. **A**, untreated cells at 24 h. **B**, H460 cells treated with 0.5 μ mol/L compound 1 for 24 h. **Arrowheads**, cells with multiple spindles. **C** and **D**, phase-contrast and fluorescent micrographs of cells treated for 48 h with 1 μ mol/L compound 1, respectively. **Open arrows**, multinucleate cells; **closed arrow**, a cell with multiple spindles.

tet-inducible promoter. The PLK1 kinase domain was constitutively activated by mutating T210 to Asp (32), which mimics phosphorylation of T210 and results in robust kinase activity and phosphorylation of the p53 protein, and Xie et al. (31) showed that PLK1 can phosphorylate p53 *in vitro*. Antibodies for the phosphorylation site on p53 are also commercially available. It is important to note that although we were able to show that PLK1 can phosphorylate p53 in the context of the chimeric protein, it is not known if p53 is a substrate for PLK1 in the cell. The assay was adapted to an ELISA format, allowing for a higher-throughput screening format. Using a combination of this mechanistic assay and the cell proliferation inhibition assay, we identified a large number of cell-potent thiophene benzimidazole PLK1 inhibitors in addition to compound 1. This mechanistic assay can thus serve as a very useful reporter assay for PLK1 activity. More recently, another group showed that phosphorylation of Ser¹⁹⁸ on *cdc25C* can be used as a reliable marker for PLK1 activity in a cellular context (34). Reduction in phosphorylation of *cdc25C* resulted in a change in electrophoretic mobility of the protein. Inhibition of phosphorylation of *cdc25C* was also shown by Western immunoblotting of ectopically overexpressed *cdc25C* with a phospho-Ser¹⁹⁸ specific antibody (34).

Compound 1 is also a potent inhibitor of PLK3. Much less is known about the functions of PLK3 in the cell than PLK1; however, from a cancer therapeutic perspective, it has been

shown that overexpression of PLK3 in cells is toxic, resulting in loss of cellular adhesion and cell death (35). Further, PLK3 mRNA expression is down-regulated in 25 of 36 primary head and neck tumor samples compared with adjacent uninvolved tissue (36). It has been suggested that PLK3 expression decreases during tumor development (37), indicating that PLK1 is the more important target for therapeutic intervention.

In conclusion, we identified a novel and potent inhibitor of PLK1, compound 1. Our *in vitro* and cellular characterization of this thiophene benzimidazole showed that it potently and selectively inhibits PLK1 in cells and inhibits the proliferation of a wide variety of tumor cell lines of diverse origin. Compound 1 will serve to further anticancer therapy both as an important starting point in the development of new clinical agents and as a useful tool to probe the function of PLK1 in the cell.

Acknowledgments

We thank Warren Rocque (Department of Gene Expression and Protein Biochemistry) for purification of the proteins used in the *in vitro* kinase assays; Kendra Hightower (Screening and Compound Profiling) for her help with the kinase assays; and Nelson Rhodes, Gary Smith, and Jeff Jackson for their useful comments on the manuscript.

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