Molecular alterations after Polo-like kinase 1 mRNA suppression versus pharmacologic inhibition in cancer cells

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Abstract

Multiple roles within mitosis have been assigned to Polo-like kinase 1 (Plk1), making it an attractive candidate for mitotic targeting of cancer cells. We have employed chimeric antisense oligonucleotides to investigate the molecular alterations after targeted interference with Plk1 in RKO human colon adenocarcinoma and PC3 prostate cancer cells. Suppression of Plk1 mRNA resulted in a dramatic increase of the mitotic index followed by the onset of apoptosis. Mitotically arrested cells displayed randomly separated condensed chromosomes and the occurrence of multiple spindle poles with well-formed asters. Induction of apoptosis was strictly dependent on cell cycle progression: Genetically engineered RKO cells with inducible expression of the cyclin-dependent kinase inhibitor p27Kip1 were completely refractory to Plk1 depletion-induced apoptosis when they were arrested in the G1 phase of the cell cycle. Various mitotic markers, including MPN-2, cdc25c, cyclin B1, or phosphorylated histone H3, were investigated to explore the molecular consequences of Plk1 depletion. Whereas most marker proteins showed similar alterations compared with treatment with paclitaxel, cdc25c was fully phosphorylated in Paclitaxel-treated cells but only partially phosphorylated in Plk1-depleted cells, although both treatments caused a profound mitotic arrest. This differential phosphorylation of cdc25c was used to test whether a pharmacologic inhibitor of Plk1 would exert the same cellular effects as interference with Plk1 on a mRNA level. It was found that the differential electrophoretic mobility of cdc25c can serve as a reliable molecular marker to track inhibition of Plk1 by small-molecule inhibitors within a cell. [Mol Cancer Ther 2006;5(4):809–17]

Introduction

A stringent control of mitosis is mandatory to warrant the accurate segregation of sister chromatids in dividing cells. Eukaryotic cells have evolved sophisticated mechanisms to monitor faithful progression through each phase of mitosis to prevent the occurrence of aneuploid daughter cells. Polo-like kinase 1 (Plk1) has been identified to be a key player for G2-M transition and mitotic progression in both normal and tumor cells (1). Multiple roles have been assigned to Plk1 at the entry into M phase, mitotic spindle formation, condensation and separation of chromosomes, exit from mitosis by activation of the anaphase-promoting complex, and cytokinesis (reviewed in ref. 2). Moreover, recent reports implicated an involvement of Plk1 in the resumption of cell cycle reentry after checkpoint activation through DNA-damaging agents (3).

The earliest function attributed to Plk1 at the beginning of the M phase is the phosphorylation of cdc25c at Ser198 (4) and of Wee1 at multiple sites (5). The cyclin B1/cyclin-dependent kinase 1 complex is also reported to be a substrate of Plk1, most likely at Ser133 (6). The functional consequences of cyclin B1 phosphorylation awaited further clarification. Plk1 is shown to be involved in centrosome maturation by the recruitment of γ-tubulin to the centrosome and by the phosphorylation of Nlp1, which interacts with γ-tubulin ring complexes (7). Microtubule nucleation and dynamics may be influenced by Plk1 activity through phosphorylation of katanin, TCTP (8), stathmin, or α-tubulin itself (9). Besides being located at spindle poles, Plk1 is also detectable at kinetochores during mitosis (10). The onset of anaphase is intimately linked to the degradation of key regulatory elements by the anaphase-promoting complex. Plk1 and cyclin-dependent kinase 1 cooperatively phosphorylate subunits of the anaphase-promoting complex. It is speculated that cyclin-dependent kinase 1–phosphorylated sites thereby generate primed docking sites for the polobox of Plk1 (11). Moreover, Plk1 may regulate anaphase-promoting complex activation through degradation of the anaphase-promoting complex inhibitor Emi1 whose ubiquitination is stimulated by Plk1 (12). Plk1 may also participate in the regulation of anaphase onset by the phosphorylation of the cohesin subunit SCC1 (13). Finally, a function in cytokinesis has been ascribed to Plk1 through the phosphorylation of Mklp2 (14) and NudC (15).

Two independent observations highlight Plk1 as an attractive candidate molecule for targeted tumor therapy:
First, overexpression of Plk1 has been observed in a wide variety of carcinomas of different pathologic origin, including breast (16), ovary, colon, pancreas, lung, endometrium, brain, skin, head and neck, esophagus, gastric tract, and prostate (reviewed in ref. 17). Notably, in some instances, Plk1 expression has not only been confined to mitotic cells but also was overexpressed homogeneously independent of cell cycle stage. In ovarian cancer, Plk1 overexpression was established as an independent prognostic marker even in multivariate analyses (18).

Second, targeted interference with Plk1, primarily by antibodies, antisense, or small interfering RNA technology, has been reported to result in a blockade in mitosis with subsequent induction of cell death (19–22). Interestingly, some of these reports describe a differential response of nontransformed cells toward interference with Plk1 function compared with tumor cells. This intrinsic dependence of tumor cells on Plk1 activity in mitosis together with its overexpression in a variety of cancer types and thus a possible differential role of Plk1 in normal versus tumor cells makes this molecule an attractive candidate for molecular targeted cancer therapy. Various small-molecule inhibitors have been described in the literature (23–25). A prerequisite for the cellular profiling of potential pharmacologic inhibitors of Plk1 is the availability of a traceable target-related molecular alteration in a cell. However, many of the inhibitors described display only a poor selectivity toward Plk1, inhibit Plk1 via indirect mechanisms, or display antimitic activity via mechanisms unrelated to Plk1 inhibition, such as interference with microtubule polymerization.3 It is therefore difficult to judge whether the cellular phenotypes induced by Plk1 small-molecule inhibitors are intimately related to their ability to inhibit Plk1 kinase activity in a biochemical assay. Finding a reliable molecular surrogate for the specific cellular activity of a kinase inhibitor in general is hampered by the limited specificity of many kinase inhibitors (26) and by the observation that kinase cross-talk, feedback loops (27), and branching may substitute missing substrate phosphorylations if the kinase of interest is inhibited (28, 29).

In this study, we used chimeric antisense molecules specific for human Plk1 and analyzed the molecular alterations after depletion of Plk1. Many of them were strictly confined to mitosis as such and were also detected using the microtubule-targeting agent paclitaxel. One alteration we were able to attribute specifically to Plk1 knockdown was the differential electrophoretic mobility ofcdc25c. Although Plk1-depleted cells accumulate in mitosis, the electrophoretic mobility of cdc25c was between control cells and cells treated with paclitaxel, indicating that certain sites within cdc25c as a substrate of Plk1 were not phosphorylated. Using a modified benzoimidazole thiophene small-molecule Plk1 inhibitor (23), we observed the same altered electrophoretic mobility as seen after Plk1 suppression by antisense molecules, which is attributable to the missing phosphorylation of cdc25c at Ser198. These results show that molecular alterations identified after Plk1 depletion by RNA interfering strategies can be used to determine the specificity of Plk1 small-molecule inhibitors within a cellular context.

Materials and Methods

Materials and all other reagents were purchased from Sigma Chemical (Deisenhofen, Germany) unless otherwise specified. Paclitaxel (Taxol) was obtained from Bristol-Myers Squibb (Princeton, NJ). Antibodies were obtained from various suppliers: cdc25c (Santa Cruz Biotechnology, Santa Cruz, CA), Plk1 (Oncogene Research Products, San Diego, CA), β-actin, α-tubulin (Sigma), MPM-2 (WAK-Chemie, Steinbach, Germany), cyclin B1, and phosphorylated histone H3 (Cell Signaling Technology, Beverly, MA). Transfection lipids were provided by Atugen (Berlin-Buch, Germany).

Oligonucleotides and Taqman Analysis

Oligonucleotides were up to 20 bases in length containing a phosphorothioate backbone and 2′-alkoxy modifications on the ribose residue at bases 1 to 5 and 16 to 20 at the 5′ and 3′ ends, respectively. A panel of 17 oligonucleotides was screened for its antisense activity to reduce Plk1 expression. The most effective sequence was 5′-GGCTGTA-GAACCCACACC-3′ with 2′-alkoxy modifications at bases 1 to 4 and 15 to 18 at the 5′ and 3′ ends, respectively. The inverted sequence served as a control. For Taqman reverse transcription-PCR analysis of Plk1 mRNA suppression, total RNA (purified with RNeasy Mini kit, Qiagen, Hilden, Germany) was reverse transcribed for 1 hour at 37°C using random hexanucleotide primers and RAV-2 reverse transcriptase (Amersham Biosciences, Chalfont St. Giles, United Kingdom). Real-time Taqman PCR amplification of cDNA was done on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using AmpliTaq Gold DNA polymerase. For normalization of RNA concentration, Taqman PCR analysis of 18S rRNA was applied. Sequences used for Plk1 were forward primer 5′-TGCCCTACCTACGAGCTTG-3′, reverse primer 5′-AAGTGTATCGCCAGCTGCC-3′, and probe 5′-FAM-CGCACTACCTCGACTCAGCA-TAMRA-3′; sequences used for 18S rRNA were forward primer 5′-GGCTGTA-GAACCCACACC-3′, reverse primer 5′-GGCTGTA-GAACCCACACC-3′, and probe 5′-VIC-TGCTGCCACAGCCACGCTC-TAMRA-3′.

Cells and Cell Culture

RKO human colon cancer cells, PC3 human prostate cancer cells, and U2OS human osteosarcoma cells were obtained from LGC Promochem (Wesel, Germany) and maintained in DMEM supplemented with 10% FCS. RKOp27 cells (30) were cultured in double-selection culture medium containing 20 µg/mL zeocin and 500 µg/mL neomycin (G418).

Cell Transfection with Antisense Oligonucleotides

RKO or PC3 cells were seeded into 96-well plates at a density of 4,000 per well or into six-well plates at a density...
of $1 \times 10^5$ to $2 \times 10^6$ per well, respectively. Twenty-four hours later, medium was replaced with 80 µL fresh medium and the cells were transfected with 20 µL antisense-lipid mixture prepared as follows: The oligonucleotides were diluted as $10\times$ stock in DMEM and then mixed with the lipid to obtain a final concentration of 1 µg/mL lipid and a 5× concentration of the antisense oligonucleotide. After incubation of the mixture for 30 minutes at 37°C, 20 µL of each dilution as indicated were added to the wells containing the cells. Cell harvesting was done at the indicated time points. Eight wells were pooled for subsequent Taqman analysis to determine suppression of the target mRNA.

**Flow Cytometric Analysis**

After completion of the desired treatment, cells were harvested by trypsinization, and an aliquot of $1 \times 10^6$ cells was washed once with cold PBS and then fixed with cold 70% ethanol. The DNA was stained with a solution containing 25 µg/mL propidium iodide and 10 µg/mL RNase A in PBS for 6 hours. Cell cycle distribution was analyzed with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) at an excitation wavelength of 488 nm.

**Cell Staining**

On completion of the desired treatment, cells were harvested by trypsinization. An aliquot ($5 \times 10^3$ cells) was spun onto glass slides using a cytospin and subsequently fixed and stained with the Hemacolor kit (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. For the staining of microtubules, cells were fixed with paraformaldehyde in PBS for 5 minutes, washed with PBS, and permeabilized with 0.2% Triton X-100 for 5 minutes. After blocking with 2% bovine serum albumin for 30 minutes, the cells were incubated with an antibody against α-tubulin for 1 hour at 37°C. Binding was visualized by incubation with a Cy3-labeled antimouse antibody (Dianova, Hamburg, Germany). The slides were washed with PBS between each antibody incubation step and DNA was counterstained for 5 minutes with 100 ng/mL Hoechst 33342 (Invitrogen, Carlsbad, CA) followed by final rinsing with deionized water. The slides were covered with Vectashield and sealed before analysis by confocal microscopy.

**Quantitation of the Mitotic Index**

The mitotic index was quantitated using the mitotic index hit kit (Cellomics, Pittsburgh, NJ) according to the manufacturer’s instructions. Briefly, the cells were seeded into 96-well plate and treated as desired. Cells were fixed with fixative solution at the indicated time points and subsequently treated with 0.2× permeabilization buffer. The first and second antibody solutions containing Hoechst 33342 dye (50 µL) were kept on the cells for 1 hour at room temperature at a dilution of 1:400 for the antibodies. The second incubation step was conducted in the dark and the wells were washed in between each incubation step twice with 100 µL washing buffer. The mitotic index was then determined in 200 µL washing buffer per well using the Cellomics array scan.

**Immunoblot Analysis**

Cells were lysed in a lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 50 mmol/L NaF, 1 mmol/L Na3VO4, and 1 mmol/L phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation and the supernatants were collected. Equal amounts of lysate protein were separated by SDS-PAGE and subsequently blotted onto polyvinylidene difluoride membranes for incubation with antibodies used for Western blot analysis as indicated. Specific signals were visualized by use of the enhanced chemiluminescence detection kit (Amersham, Braunschweig, Germany).

**Generation of a cdc25c-Overexpressing U2OS Cell Line**

Human cdc25c cDNA was amplified by PCR techniques using RKO human colon cancer cell cDNA as a template and subcloned into pcDNA5/TO V5 HisA (Invitrogen) via restriction sites included in the cdc25c-specific oligonucleotide primers. pcDNA3-cdc25c vector was transfected with the Fugene-6 kit (Roche Diagnostics, Mannheim, Germany) into U2OS human osteosarcoma cells. Stable clones (U2OS-cdc25c) were kept in selection culture medium containing 200 µg/mL hygromycin. Expression of cdc25c was examined by Western blot analysis with specific antibodies (Invitrogen) against the V5 epitope included in the protein.

**Quantitation of Apoptosis**

To detect the onset of apoptosis in the target cells, an apoptosis detection ELISA kit (Roche) was used according to the manufacturer’s instructions. This photometric enzyme immunoassay quantitatively measures cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) that are characteristic of apoptotic cell death. Triplicate aliquots of $10^5$ cells per well were seeded in 96-well plates and treated as indicated. After incubation for 24 hours at 37°C in 5% CO2 and 95% humidified air, the apoptosis assay was then carried out according to the manufacturer’s instructions.

**Results**

**Suppression of Plk1 mRNA with Antisense Oligonucleotide Results in Mitotic Accumulation**

We have derived a 2′-alkoxy-modified chimeric phosphorothioate antisense oligonucleotide against the 3′-untranslated region of the Plk1 mRNA. To test its capacity to suppress Plk1 mRNA and consequently Plk1 protein, we transfected RKO human colon adenocarcinoma and PC3 prostate cancer cells with 25 nmol/L antisense oligonucleotide as described in Materials and Methods. The cells were harvested and analyzed by Taqman PCR analysis for suppression of Plk1 mRNA 24 and 36 hours after transfection, respectively. As shown in Fig. 1A, the oligonucleotide was very effective in suppressing Plk1 mRNA. A control oligonucleotide encoding the inverted sequence exerted only very little effect on the levels of Plk1 mRNA. Parallel samples were investigated for Plk1 protein depletion by Western blotting of the lysates with antibodies against Plk1. The antibody used displayed some cross-reactivity with a
protein of slightly retarded electrophoretic mobility than predicted for the molecular weight of Plk1. As shown in Fig. 1B, treatment of both RKO and PC3 cells with the Plk1-specific antisense oligonucleotide specifically depleted a protein whose electrophoretic mobility corresponds with the predicted molecular weight of Plk1 (arrow) but not the protein with a slightly higher molecular weight. The cell densities chosen in this experiment (1 × 10^5 per six-well plate) precluded Plk1 down-regulation by confluency.

It has been described that down-regulation of Plk1 by RNA interfering strategies results in mitotic accumulation with subsequent induction of apoptosis (20, 31, 32). To test whether treatment of RKO and PC3 cells with the antisense oligonucleotide used herein would also result in mitotic accumulation, we transfected both cell lines as described above and analyzed lysates for phosphorylation of histone H3 (33). As depicted in Fig. 2A, transfection with the antisense oligonucleotide led to a significant increase in the phosphorylated histone H3 content in both cell lines, similar to cells treated with the spindle poison paclitaxel. Kinetic experiments in PC3 cells (Fig. 2B) revealed that the mitotic accumulation increased already 15 hours and peaked 36 hours after transfection.

In-depth analysis of transfected RKO cells by flow cytometry (Fig. 3A) revealed mitotic accumulation and induction of apoptosis. Immunohistochemistry with antibodies against α-tubulin (Fig. 3B, top) obviated the occurrence of multiple spindle poles without perturbation of aster formation itself. Methylene blue/eosin staining of transfected cells again confirmed the increased mitotic index and a random distribution of condensed chromatin in mitotic cells (Fig. 3B, bottom). No such mitotic abnormalities were observed in untreated cells or cells transfected with the inverted sequence of the antisense oligonucleotide.

G1-Arrested Cells Are Refractory to Apoptosis on Suppression of Plk1 mRNA

We recently described the generation of an inducible expression system for the cyclin-dependent kinase inhibitor p27^Kip1 in RKO colon cancer cells (30). Inducible expression of p27^Kip1 results in a complete G1 arrest of the cells, which causes growth inhibition without any signs of toxicity. We used this cell system to explore the cell cycle dependence of apoptosis induction through suppression of Plk1. Twenty-four hours before transfection, the cells were treated with 10 μmol/L ponasterone A to arrest them in G1. Control cells were treated with DMSO carrier only. The cells were then transfected with various amounts of antisense oligonucleotide or inverted control as indicated in Fig. 4. Another 30 hours later, the cells were harvested and subjected to an apoptosis detection ELISA as described in Materials and Methods (Fig. 4A). Apoptosis was only detected in...
proliferating cells but not in cells arrested in G\textsubscript{1} or transfected with the inverted oligonucleotide. As a control for equal transfection efficiencies in arrested and cycling cells, we transfected both cell stages with FITC-labeled oligonucleotides. Subsequent fluorescence microscopy did not indicate any differences in oligonucleotide uptake of arrested versus proliferating cells (Fig. 4B). These data indicate that induction of apoptosis by interference with Plk1 is strictly dependent on active cell cycle progression and that the functionality of Plk1 is dispensable for survival of nonproliferating cells.

**Mitosis-Confined Molecular Alterations after Suppression of Plk1**

The data described above indicate that molecular alterations after Plk1 suppression are well confined to mitosis. We therefore analyzed multiple mitotic markers for changes in expression and/or phosphorylation as a consequence of Plk1 suppression, such as the expression of cyclin B1, which is up-regulated on entry into mitosis (reviewed in ref. 34), and reactivity with the MPM-2 antibody (35) that recognizes serine-proline epitopes specifically phosphorylated in mitosis (36). Finally, we analyzed the phosphorylation status of cdc25c, which is indicative for activation at the onset of mitosis (37). Moreover, cdc25c has been described to be phosphorylated by Plk1 at Ser\textsuperscript{198} (4) and therefore represents a direct substrate of Plk1. RKO and PC3 cells were transfected with the Plk1 antisense oligonucleotide as described above and harvested 24 or 36 hours after transfection, respectively. Lysates were immunoblotted with antibodies against MPM-2, cyclin B1, and cdc25c. The results are displayed in Fig. 5: antisense suppression of Plk1 resulted in a strong increase in reactivity with the MPM-2 epitope, similar in intensity and band reactivity to cells treated with 20 nmol/L paclitaxel for 24 hours. No consistent changes in the expression levels of cyclin B1 were observed. Extensive phosphorylation of cdc25c was visualized by a significant shift in its electrophoretic mobility in paclitaxel-treated versus untreated control cells. Depletion of Plk1 in both RKO and PC3 cells resulted in an electrophoretic mobility...
of cdc25c that was between the molecular weight of interphase and of mitotic cdc25c. From the previous experiments, it was obvious that cells treated with Plk1 antisense molecules arrest within mitosis before they undergo programmed cell death. We therefore concluded that the differential electrophoretic mobility of cdc25c in Plk1-depleted cells most likely is due to the missing phosphorylation of cdc25c Ser198 and that phosphorylation of cdc25c at this site is dispensable for mitotic entry at least in these two cancer cell lines.

To further substantiate the differential electrophoretic mobility, we treated PC3 cells as described above; however, 5 hours after transfection, 10 nmol/L paclitaxel was added into the medium and the cells were incubated for another 20 hours at 37°C to drive virtually all cells into mitosis. Cell lysates were then analyzed by immunoblotting to determine reactivity of the antibodies with the MPM-2 antibody, which specifically reacts with mitotic pSer-Pro epitopes, with an antibody against cyclin B1, or with an antibody against cdc25c as indicated. Arrows, different electrophoretic mobilities of cdc25c. Equal loading of lysates in each immunoblot was checked using an antibody against β-actin.

In this study, we investigated the molecular alterations of Plk1 inhibition in cancer cells using either RNA interfering molecules or a putative small-molecule benzoimidazole thiophene Plk1 inhibitor named herein compound 1 (cited in ref. 23) to investigate the effects on cdc25c electrophoretic mobility after pharmacologic inhibition of Plk1. In vitro kinase assays conducted with recombinant Plk1 and α-casein as substrate revealed that this compound inhibits Plk1 with submicromolar IC50 (data not shown). PC3 prostate cancer cells were incubated for 20 hours with various amounts of compound 1 and then subjected to cdc25c analysis by Western blotting. Paclitaxel-treated cells and cells treated with a less selective benzoimidazole thiophene carboxylic acid amide analogue (compound 2) were included as controls. As depicted in Fig. 6B, cdc25c from lysates of compound 1-treated cells displayed a migratory behavior between cdc25c of control cells and cdc25c of control cells and cells treated with paclitaxel exactly as observed after knockdown of Plk1 by antisense oligonucleotides. Because the phosphorylation of cdc25c at Ser198 by Plk1 has been described, we questioned whether the differential electrophoretic mobility is attributable to the lack of Ser198 phosphorylation. We generated a U2OS osteosarcoma cell line stably expressing V5-epitope-tagged cdc25c (U2OS-cdc25c), because the phosphospecific antibody available (see Materials and Methods) recognizes cellular Ser198-phosphorylated cdc25c only when it is ectopically overexpressed. These cells were treated with 40 ng/mL nocardazole or 2 μmol/L compound 1. In analogy to Fig. 6B, the electrophoretic mobility of cdc25c was analyzed. Additionally, phosphorylation of cdc25c at Ser198 was determined with a phosphospecific antibody. The results are shown in Fig. 6C: Treatment of U2OS-cdc25c cells with nocardazole caused a significant increase in the electrophoretic mobility and phosphorylation of cdc25c at Ser198. Treatment with compound 1 resulted in an electrophoretic mobility of cdc25c exactly as observed in Fig. 6A and B. Moreover, phosphorylation of Ser198 in cdc25c was abrogated in cells treated with compound 1. Therefore, it is highly likely that the differential electrophoretic mobility of cdc25c in cells treated with the putative Plk1 inhibitor is due to the lacking phosphorylation at Ser198. Methylene blue/eosin staining of U2OS cells after 20 hours of treatment with 1 μmol/L compound 1 (Fig. 6D) revealed that a very high fraction of cells (>80%) accumulated within mitosis. From these data, we conclude that phosphorylation at Ser198 can serve as a reliable marker to monitor Plk1 activity in a cellular context. Moreover, compound 1 induces a strong mitotic arrest and very specifically inhibits of Plk1 activity in the cell because other phosphorylation sites within cdc25c obviously do not seem to be influenced.

**Discussion**

In this study, we investigated the molecular alterations of Plk1 inhibition in cancer cells using either RNA interfering strategies or a pharmacologic inhibition of Plk1 kinase...
activity. Consistent with other reports, we found that suppression of Plk1 mRNA and protein by antisense oligonucleotides results in a significant mitotic accumulation and subsequent onset of apoptosis. Many molecular alterations observed, such as increase in phosphorylated histone H3 content or occurrence of MPM-2 specific epitopes, confirmed the mitosis-specific mode of action of Plk1. Using a genetically engineered cell line with inducible cell cycle arrest after ectopic expression of p27kip1, we were able to show that depletion of Plk1 is compatible with survival in arrested but not in actively cycling cells. The remarkable specificity of this approach was emphasized by the observation that a lack of a single phosphorylation in the Plk1 substrate cdc25c was detectable resulting in altered electrophoretic mobility of cdc25c. Moreover, these data imply that the phosphorylation of cdc25c by Plk1 obviously is not a prerequisite for the entry of cancer cells into mitosis. Cross-validating these data using a pharmacologic inhibitor of Plk1, we observed virtually the same effects on cdc25c phosphorylation and mitotic accumulation as with Plk1 antisense treatment. An in-depth characterization of this inhibitor will be described elsewhere.

Sequence specificity is of fundamental importance when antisense oligonucleotides are employed to study the function of a gene within a cell (38). To exclude non-sequence-specific effects of the chimeric oligonucleotides containing a phosphorothioate backbone and 2-alkoxy modifications, we tested an additional set of 16 different oligonucleotides. Whereas nine oligonucleotides suppressed the Plk1 mRNA <20% of control cells, three of these suppressed Plk1 mRNA <10% of control cells and were investigated in detail as were the corresponding inverted control oligonucleotides. The phenotypes induced by the use of these three different oligonucleotides was comparable with the one described herein and neither of the inverted controls induced apoptotic effects (data not shown). We attribute the high degree of specificity to the chemical modifications inserted in the oligonucleotides together with the use of an optimized lipid formulation (see Materials and Methods). It has to be noted that the lipid alone (without nucleic acid) had a minor effect on Plk1 protein expression in PC3 cells (Fig. 1), which might be explainable by a certain degree of adverse physicochemical properties. These were not seen with the lipid-nucleic acid mixture.

However, pharmacologic inhibition of a kinase is mechanistically different from suppression of target protein expression, especially for a kinase with many association partners and subcellular localizations, such as Plk1. Thus, the effects of a small interfering RNA or antisense oligonucleotide against the kinase of interest may or may not reflect the cellular activity of a specific kinase inhibitor that is developed within a certain disease context. In this study, we identified differential phosphorylation of cdc25c as marker to monitor a target-specific interference with Plk1 functionality. Most notably, the effects we observed on

Figure 6. Differential electrophoretic mobility of cdc25c after treatment with Plk1 inhibitors is attributable to inhibition of phosphorylation at Ser198. A, PC3 cells were treated as described in Fig. 5; however, 5 h after transfection, 10 nmol/L paclitaxel was added into the medium and the cells were incubated for another 25 h at 37°C to arrest virtually all cells within mitosis. Cell lysates were analyzed by Western blotting with an antibody against cdc25c. B, PC3 cells were treated for 20 h with either paclitaxel or the indicated amounts of compound 1 (cpd. 1). Control cells were left untreated. Lysates were subjected to immunoblotting with antibodies against cdc25c. Arrows, different mitotic phosphorylation forms. Note that cdc25c in lysates of compound 1 treated cells has a migratory behavior between cdc25c of control cells and cells treated with paclitaxel. C, U2OS cells overexpressing cdc25c were treated for 20 h with either nocodazole alone (Noc.) or 1 μmol/L compound 1. Lysates were analyzed by immunoblotting with antibodies against phosphorylated cdc25c Ser198 (top), V5, which recognizes ectopically expressed cdc25c (middle), and β-actin as a loading control (bottom). Note the differential electrophoretic mobility and missing phosphorylation of cdc25c Ser198 in cells treated with compound 1. D, the same cells were treated for 20 h with 2 μmol/L compound 1 (right) and subsequently stained with methylene blue/eosin to visualize mitotic cells. Control cells (left) were treated with DMSO only. Further abbreviations are described in Fig. 5 legend.
altered cdc25c phosphorylation on either Plk1 protein depletion or pharmacologic inhibition of the kinase activity are fully congruent and therefore represent to our knowledge the first example of an orthogonal target validation of Plk1 using these two different approaches. The data presented herein therefore strengthen the validity of Plk1 targeting as therapeutic concept together with the clinical observations of Plk1 overexpression in many tumor types and a potentially different role of Plk1 in normal versus tumor cells (19, 22).

A central question in the development of many kinase inhibitors is whether they can exploit their full therapeutic efficacy alone or only in combination with standard chemotherapy. Although the latter is certainly true for kinase inhibitors specific for signaling kinases, such as the epidermal growth factor receptor or B-raf (39, 40), it obviously does not seem to be the case for essential mitotic kinases whose function is mandatory for progression through mitosis. Another family of mitotic kinases, besides Plk1, fulfilling this paradigm of inhibition being cytotoxic on its own seems to be the aurora family, albeit the cellular phenotypes after kinase inhibition significantly differ between Plk1 and aurora A/B (41). Although a certain degree of cross-selectivity therefore seems to be desirable (or even a prerequisite) for the targeted interference with kinases involved in mitogenic and/or survival signaling, a high degree of specificity is desired for inhibitors addressing kinases whose inhibition on its own without concomitant chemotherapy should already result in a significant therapeutic benefit. A major hurdle in the identification of truly target-related readouts for the efficacy of kinase inhibitors in a cellular context is the observation that (a) many phosphorylations are only of transient nature, (b) they can be complemented by other kinases, (c) feedback mechanisms may exist, and (d) insufficient specificity of phosphospecific antibodies limits a specific detection of the signal (42). Therefore, despite the knowledge of many Plk1 substrates, there are only very few molecular alterations that seem to be suitable for the specific tracking of Plk1 interference within a cell. It will be interesting to explore whether other putative pharmacologic inhibitors of Plk1 (23–25) display the same degree of specificity in cellular systems as the molecule described herein. Moreover, we conclude that differential cdc25c phosphorylation might serve as a biomarker in patients treated with inhibitors of Plk1.

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References


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