Binding and Inhibition of Cdc25 Phosphatases by Vitamin K Analogues[†]

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ABSTRACT: A synthetic K vitamin analogue, 2-(2-mercaptothenol)-3-methyl-1,4-naphthoquinone or Cpd 5, was previously found to be a potent inhibitor of cell growth [Nishikawa et al., (1995) *J. Biol. Chem. 270*, 28304−28310]. The mechanisms of cell growth were hypothesized to include the inactivation of cellular protein tyrosine phosphatases, especially the Cdc25 family [Tamura et al. (2000) *Cancer Res. 60*, 1317−1325]. In this study, we synthesized PD 49, a new biotin containing Cpd 5 derivative, to search for evidence of direct interaction of these arylating analogues with Cdc25A, Cdc25B, and Cdc25C phosphatases. PD 49 was shown to directly bind to GST-Cdc25A, GST-Cdc25B, their catalytic fragments, and GST-Cdc25C. The binding could be competed with excess glutathione or Cpd 5, and a cysteine-to-serine mutation of the catalytic cysteine abolished binding. This was consistent with an involvement in binding of cysteine in the catalytic domain. This interaction between PD 49 and Cdc25 also occurred in lysates of treated cells. PD 49 also bound to protein phosphatases other than Cdc25. We found that the new analogue also inhibited Hep3B human hepatoma cell growth. This growth inhibition involved ERK1/2 phosphorylation and was inhibited by a MEK antagonist. The results demonstrate a direct interaction and binding between this growth-inhibiting K vitamin derivative with both purified as well as with cellular Cdc25A, Cdc25B, and Cdc25C.

Protein phosphorylation and dephosphorylation are essential mechanisms for intracellular control of protein function and signaling. Protein kinases are involved in phosphorylation, whereas dephosphorylation is catalyzed by protein phosphatases (*1*). Two types of mammalian protein phosphatases have been identified: S/T specific (protein serine/threonine) and Y specific (protein tyrosine, PTP). DSPs, which dephosphorylate both tyrosine and threonine/serine on the same protein, are a subclass of PTP (*2*). DSPs share the PTP active site sequence motif $HC(X)_5R$, where H is a highly conserved histidine, C is the catalytic cysteine, the five X residues form a loop in which all the amide nitrogens hydrogen bond to the phosphate of the substrate, and R is a highly conserved arginine that hydrogen bonds to the phosphorylated amino acid of the substrate. DSPs and PTPs also have a similar mechanism of reaction, although there is limited amino acid sequence identity beyond the active site region (*3*). The two important motifs for binding substrates in DSPs are defined by the VH1-like phosphatases (pTEpY) and the Cdc25-like phosphatases (pTpY). DSPs display a marked preference for cyclin-dependent kinases and MAP kinases, and they are being recognized as a family of important regulators of cell cycle control and mitogenic signal transduction (*3*−*6*).

Important members of the DSP family that control cell cycle progression include the Cdc25 family. Three homologues of Cdc25 exist in mammalian cells: Cdc25A, Cdc25B, and Cdc25C (*7*−*9*), and several splice variants of Cdc25 proteins have been reported (*10*, *11*). Cdc25s activate several cell cycle-dependent kinases (Cdks) by dephosphorylating an inhibitory phosphotyrosine (*12*). Cdc25A is important for entry into S-phase (*13*), although it appears to also have some role in mitotic progression (*14*). Both Cdc25B and Cdc25C are regulators of G2-M transition. Cdc25A and Cdc25B also have oncogenic properties and are overexpressed in several tumors, reflecting poor prognosis (*5*, *15*−*18*). Potent and selective inhibitors of Cdc25s would thus be attractive candidates as potential anticancer agents.

Several K vitamin analogues have been synthesized that inhibit cell growth in culture and in vivo (*19*, *20*−*24*). One of these analogues, Cpd 5, was postulated (the Dowd hypothesis) to be involved in addition−elimination reactions with cellular sulfhydryl groups (*25*) and was shown to induce protein tyrosine phosphorylation in treated cells, which could be antagonized by exogenous thiols. This suggested that PTPs were likely target proteins of Cpd 5. All PTPs contain an essential cysteine residue in the enzyme active site, which

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[1] Abbreviations: Cpd, compound; Cdk, cyclin-dependent kinase; PTP; protein-tyrosine phosphatase; DSP, dual specific phosphatase; ERK, extracellular signal regulated kinase; MEK, ERK kinase; GSH, glutathione; PD compounds, named after the late Dr. Paul Dowd. Cpd 5, 2-(2-mercaptoethanol-3-methyl-1,4-naphthoquinone; PD 49 is a biotinylated Cpd 5 derivative; PD 48 is the corresponding unbiotinylated Cpd 5 derivative; FACS, fluorescence activated cell sorter.

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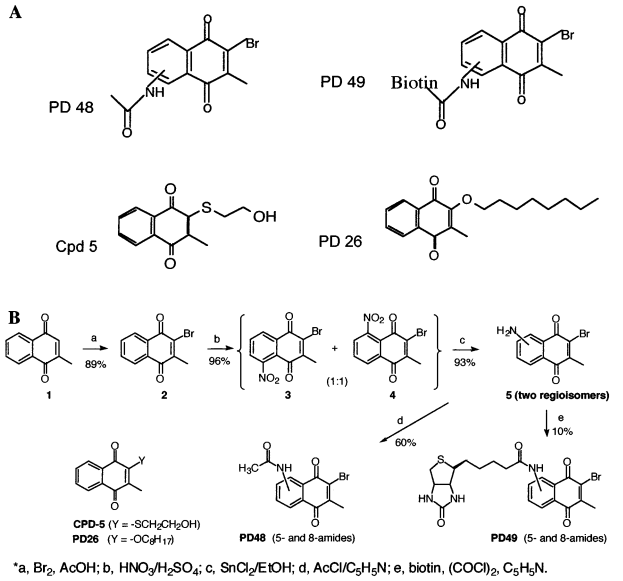

FIGURE 1: Chemical structure and synthesis of compounds. The structures of PD 48, PD 49, PD 26, Cpd 5 are shown in panel A. The scheme of synthesis of PD 49 from vitamin K_3 is shown in panel B.

is the target for specific modification by various sulfhydryl-alkylating reagents (*26*, *27*). This catalytic cysteine could also be a target of arylation by Cpd 5, which inhibits the activities of Cdc25A, Cdc25B, and Cdc25C (*28*), consistent with arylation of their catalytic cysteine. Furthermore, treatment of cells with Cpd 5 caused increased tyrosine phosphorylation of the Cdc25 substrates Cdk1, Cdk2, and Cdk4 (*28*, *29*). However, we have not yet shown a direct interaction of Cpd 5 or any other arylating K vitamin analogue with specific phosphatases such as Cdc25, although recently a quinone analogue was shown to bind to a serine residue in the catalytic domain of Cdc25A (*30*).

To approach this, we synthesized a biotin-labeled arylating K vitamin analogue (PD 49) for this study to directly examine binding of the K vitamin to Cdc25, which could be used for immunoprecipitation of itself and proteins bound to it. Characterization of the interaction of these arylating K vitamin analogues with Cdc25 and other DSPs will be invaluable for the future design of new inhibitors.

MATERIALS AND METHODS

Chemical Synthesis of Cpd 5, PD 48, and PD 49. Chemical synthesis of Cpd 5 has been described before (*21*). PD 48 and PD 49 were synthesized from vitamin K_3 as outlined in the scheme in Figure 1. PD 48 is a mixture of [*N*-(6-bromo-7-methyl-5,8-dioxo-5,8-dihydro-naphthalen-1-yl)-acetamide] and [*N*-(7-bromo-6-methyl-5,8-dioxo-5,8-dihydro-naphthalen-1-yl)-acetamide] and was synthesized from a mixture of 5 and 8-amino-2-bromo-3-methyl-[1,4]naphthoquinone by reaction with acetyl chloride in tetrahydrofuran. PD 49 is a mixture of [*N*-(6-bromo-7-methyl-5,8-dioxo-5,8-dihydro-naphthalen-1-yl)-biotinamide] and *N*-(7-bromo-6-methyl-5,8-dioxo-5,8-dihydro-naphthalen-1-yl)-biotinamide] and was synthesized from a mixture of 5- and 8-amino-2-bromo-3-methyl-[1,4]naphthoquinone by the reaction with biotin acid chloride and pyridine in tetrahydrofuran.

Cell Culture and Growth Inhibition Assay. Hep3B cells were cultured in minimum essential medium (MEM) (Life Technology, Gaithersberg, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The medium contained 10% fetal bovine serum. Cells were plated at 2×10^4 cells/well in 24-well dishes (Corning, Inc., Science Products Div., Corning, NY) for cell growth inhibition assays. After incubation in serum-free MEM for 24 h, the medium was replaced with a growth medium with or without the K vitamin analogue, for the indicated periods of time. The medium, with or without analogue, was replaced every day with fresh medium. The medium was removed, and the cells were immediately washed with ice-cold phosphate-buffered saline (PBS) to terminate the reaction. Cells were then harvested and stored at −80 °C until used. Cell number was measured by a DNA fluorometric assay with Hoechst 33258 as previously described (*42*).

To examine the effects of ERK1/2 inhibitor U0169 (Calbiochem, La Jolla, CA) or glutathione (GSH), we preincubated cells for 1 h with the inhibitor or antioxidant before adding Cpd 5.

Cell cycle analysis was done following the methods described by us before (*28*).

Western Blots and Immunoprecipitation. Western blot was done following our standard protocol (*22*). Immunoprecipitation of PD 49 bound proteins were performed as follows: We incubated 50 μL of 1% (w/v) protein A-Sepharose (Sigma, St. Louis, MO) with 5 μg of anti-biotin antibody (Sigma, St. Louis, MO) overnight at 4 °C. The excess antibody was removed by centrifugation of the antibody bound protein A-Sepharose three times with PBS. The resulting anti-biotin protein A-Sepharose was then incubated overnight at 4 °C with 50 μM of PD 49. The excess unbound PD 49 was washed away as above. The PD 49-anti-biotin antibody-protein A-Sepharose complex was then incubated with the target proteins overnight at 4 °C. The bound protein was centrifuged at 4000*g* and resuspended in SDS gel sample buffer. Proteins were separated on 10% SDS−PAGE and transferred to a nylon membrane and the specific phosphatases were probed using the corresponding antibody. Cdc25A, Cdc25B, Cdc25C, PTP1C, PTP1D, and MKP1 antibodies were all from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). GST-tagged Cdc25 proteins were from Upstate Biotechnologies Inc. (Lake Placid, NY).

Enzyme Assay of Cdc25A, VHR, and PTP1B. The GST-fusion protein phosphatases Cdc25A, VHR, as well as the soluble human recombinant PTP1B were purified, and their phosphatase activity was determined as described before (*28*).

Construction and Purification of Catalytic Fragments of Cdc25A, Cdc25B, and its Inactive (C446S) Form. Cdc25A catalytic fragment was purified from a plasmid construct described in ref *30*. Cdc25B catalytic fragment was constructed by fusion of an amino terminal His tag to a 275-amino acid carboxyl terminal fragment of the protein. The C-terminal part of Cdc25B2 beginning with Gln275, nucleotides 1045−1842, (Acc. no. NM 021872) was PCR amplified and subcloned into prokaryotic expression vector pET28a (Novagen, Madison, WI) using NdeI and XhoI recognition sites. This allowed high expression level of the last 265 amino acids of Cdc25B2, N-terminally tagged by His(6) and thrombin cleavage site. Cys446 was mutated by using QuikChange protocol (Stratagene, San Diego, CA) and the following primers: forward 5′-CACTCTGAATTCT-CATCTGAGCGTGG-3′ and reverse 5′-CCACGCTCAGAT-

GAGAATTCAGAGTG-3′. Nucleotide sequence of each construct was verified by dye termination sequencing of overlapping segments. We used the same protocol for expression of both recombinant proteins. Bacterial culture was harvested after 3−4 h IPTG induction, at $OD_{600} = 0.8$. His(6)-tagged recombinant proteins were further purified by affinity binding using Talon metal affinity resin (Clontech, Palo Alto, CA) and the protocol suggested by the manufacturer with the following modifications. The resuspension and binding buffers (calculated for 2 L of bacterial culture and 400 μL of beads) for all of the steps until thrombin cleavage contained 1 mM tris(2-carboxyethyl) phosphine (TCEP), (Sigma, St Louis, MO). After final washing of the sample in 50 mM sodium phosphate, 300 mM NaCl, 1 mM TCEP, 10% glycerol (pH 7.0), the buffer was changed to 50 mM Tris, 300 mM NaCl, 10% glycerol, 2.5 mM $CaCl_2$ (pH 7.0). A total of 1 mL of the buffer containing 10 U thrombin (Sigma) was added to the beads in a 4 mL tube, and thrombin cleavage was performed at room temperature for 2 h. At the end the beads were centrifuged, and the supernatant was aspirated and stored on ice. Gel filtration using Superdex 200 GL column (Pharmacia) and injection volumes of 500 μL was the final purification step. Elution fractions of 500 μL were collected using 50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, and 5 mM TCEP as elution buffer. The amount of the purified recombinant protein was determined by Bradford assay, the purity was verified by Coomassie staining followed by at least 24 h of distaining, and the phosphatase activity was measured by the standard for the laboratory assay.

Phospho-MBP Dephosphorylation Assay. Tyrosine phosphorylated myelin basic protein (MBP) was prepared from a kit (NEB, Waltham, MA) using Abelson tyrosine kinase to tyrosine phosphorylate MBP in vitro. Phospho-MBP was incubated for 60 min at 25 °C with Hep3B lysate in the presence or absence of 40 μM PD 49. MBP was then immunoprecipitated by an anti-MBP antibody (10 μg), resolved on a Western blot, and probed with phosphotyrosine antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA).

Competition of PD 49 and Cpd 5 Binding to Cdc25Bcat. A total of 180 ng of Cdc25Bcat was incubated in a 10 μL reaction volume for 18 h at 4 °C, with 0.1 mM PD 49 and Cpd 5 at a concentration of 0, 0.01, 0.1, 1, or 10 mM to give ratios of Cpd 5/PD 49 of 0, 0.1, 1, 10, and 100, respectively. PD 49 was immunoprecipitated with anti-biotin antibody and Cdc25Bcat bound to PD 49 in the immunoprecipitate was determined on a Western blot probed with anti-Cdc25B antibody. The amount of Cdc25Bcat on the Western blot was determined by scanning the band density.

RESULTS

Structures of Cpd 48 and Cpd 49 and Schema of their Synthesis. We prepared a labeled K vitamin (PD 49), using a biotin tag that could be readily detected with anti-biotin antibody. The structures of Cpds 49 and 48 (from which it was derived), and a scheme for their chemical synthesis from vitamin K_3, are shown in Figure 1. Cpd 5, our prototype arylating analogue, and PD 26 (a nonarylating analog) are also shown for comparison to the new compounds.

Growth Inhibition of Hep3B Cells. Hep3B cells in log phase growth were cultured with various concentrations of Cpd 5, PD 48, PD 49, or PD 26, and cell numbers were determined. All three analogues were found to inhibit cell growth in a concentration-dependent manner. The IC_{50} of the arylating analogues Cpd 5, PD 48, and PD 49 were 5, 10, and 20 μM, respectively, whereas the nonarylating analogue PD 26 was inactive (Figure 2).

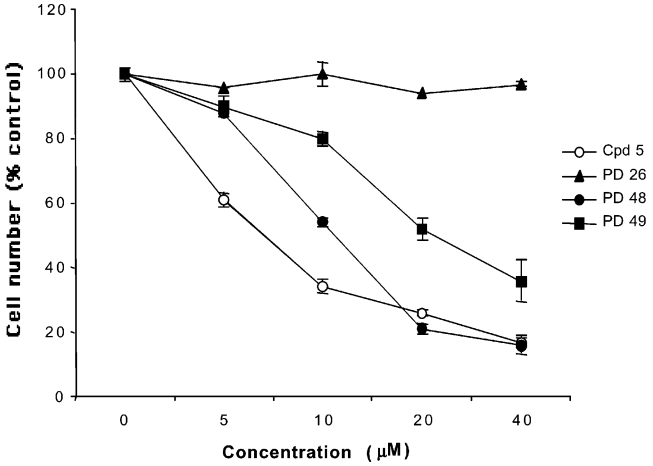

FIGURE 2: Growth inhibition of Hep3B cells by Cpd 5. PD 48, PD 49, and PD 26. Hep3B cells in log phase of growth were treated with various doses of the compounds and the surviving cell numbers were determined after 3 days of culture. The cell numbers were expressed as "%" of an untreated control and the bars represent standard deviation (SD).

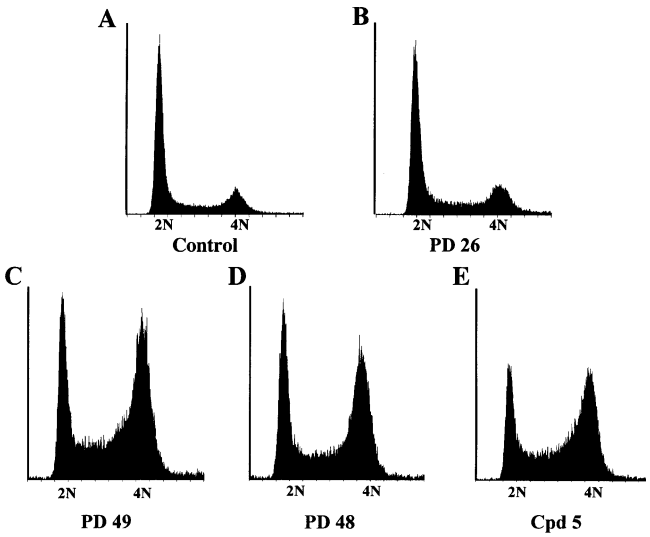

FIGURE 3: Cell cycle inhibition of Hep3B cells by Cpd 5, PD 48, PD 49, and PD 26. Hep3B cells growing exponentially were treated with 10 μM of the compounds for 24 h. Cells were fixed in methanol. Cell DNA was labeled with propidium iodide and the number of cells in different phases of the cell cycle was determined by FACS.

G_2-M Phase Cell Cycle Arrest. Cpd 5 was earlier found to arrest Hep3B cells at the G_2-M phase of the cell cycle (*28*). We analyzed the effect of the new analogues PD 48 and PD 49 on cell cycle progression. Cells growing exponentially in serum containing culture medium were treated with 10 μM PD 48, PD 49, Cpd 5, or Cpd 26 for 24 h. As seen in Figure 3, all of the analogues except PD 26, arrested cells at the G_2-M phase of the cell cycle. The G_2-M inhibition was similar to that seen with another structurally unrelated inhibitor of Cdc25 family of phosphatases, SC-$\alpha\alpha\delta$9 (*28*).

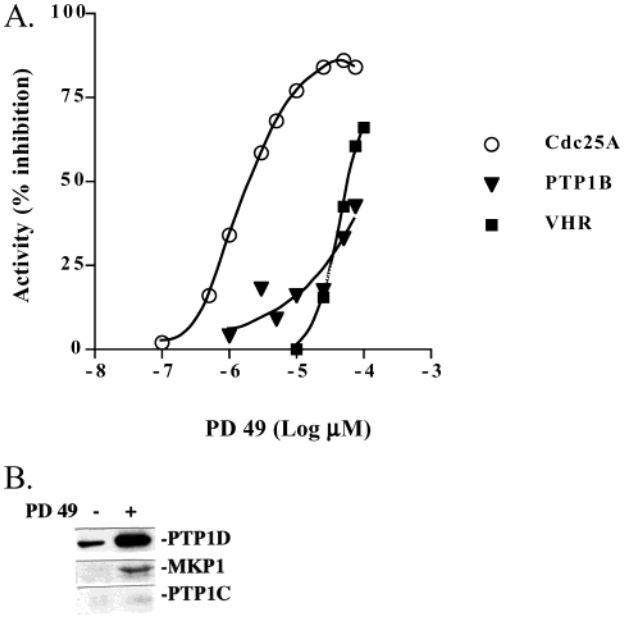

FIGURE 4: Specificity of binding and inhibition of phosphatases by PD 49. (A) Phosphatase activities of purified Cdc25A, VHR, and PTP1B were determined in absence or in the presence of different concentrations of PD 49 and were expressed as a "%" inhibition compared to control. (B) Binding of phosphatases (PTP1C, PTP1D, and MKP1) to PD 49 was detected by immunoprecipitation (anti-biotin antibody) of PD49 bound protein from Hep3B cell lysate, and probing for a specific phosphatase on a Western blot of the immunoprecipitated proteins with the corresponding phosphatase antibody.

PD 49 and Cpd 5 Are Selective Inhibitors of Cdc25. PD 49 arrested cells in G_2-M phase of the cell cycle. Since we had previously shown that the structurally similar Cpd 5 had phosphatase inhibitory activity, we examined the inhibitory activities of PD 49 against the purified PTPs, Cdc25A, VHR, and PTP1B. The apparent IC50 of PD 49 for recombinant Cdc25A was found to be about 1.5 μM, whereas the IC50 for VHR and PTP1B were 30 μM and more than 100 μM respectively (Figure 4A). PD 49 was also found to bind to the phosphatases MKP1 and PTP1D but not to PTP1C in Hep3B cell lysates (Figure 4B). PD 49 was thus found to be a selective PTP inhibitor. Cpd 5 was previously found to have similar specificity for Cdc25 (*28*), whereas identical concentrations of the related analogue Cpd 26 did not inhibit either of the phosphatases (*28*).

Association of PD 49 with Purified Cdc25s and their Catalytic Fragments. We used the biotin tagged PD 49 to investigate binding of the K vitamin analogues to purified GST-Cdc25A, GST-Cdc25B, and GST-Cdc25C. PD 49 was incubated with the GST-Cdc25 isoforms in cell-free conditions and PD 49 was immunoprecipitated with an anti-biotin antibody. The immunoprecipitated proteins were separated by electrophoresis, and the gels were probed with antibodies to Cdc25A, Cdc25B, or Cdc25C. All three Cdc25 isoforms were found to immunoprecipitate with PD 49 (Figure 5A), suggesting a direct interaction between them. This interaction could be antagonized by the presence of excess GSH (Figure 5B), suggesting the involvement of the catalytic cysteine of Cdc25 in the interaction. The purified Cdc25A and Cdc25B catalytic fragments also bound to PD 49 (Figure 5B), further supporting the idea that the cysteines at the catalytic site interact with PD 49.

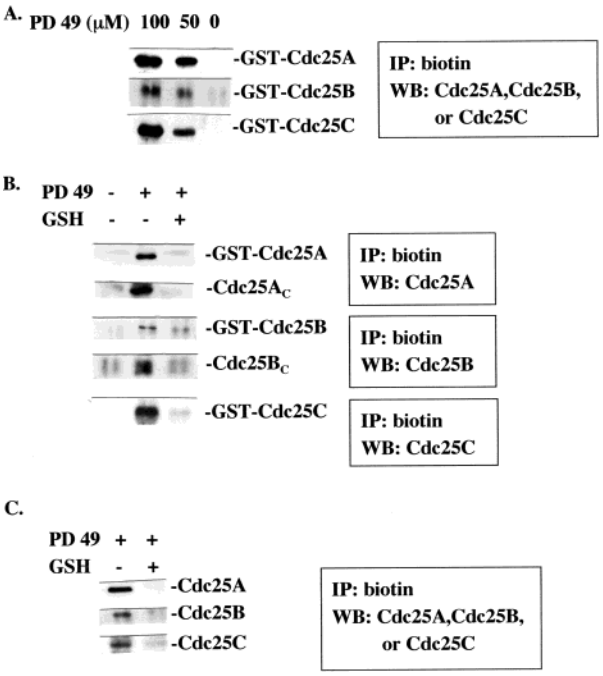

FIGURE 5: PD 49 binding to purified GST-Cdc25A, GST-Cdc25B, their catalytic fragments, GST-Cdc25C, and to Cdc25A, Cdc25B, and Cdc25C in PD 49-treated Hep3B cell lysate. (A) Purified GST-Cdc25A, GST-Cdc25B, or GST-Cdc25C was incubated with increasing concentrations of PD 49 in solution and the bound protein was immunoprecipitated with anti-biotin anibodies. The immunoprecipitated protein was visualized on a Western blot with anti-Cdc25A, Cdc25B, or anti-Cdc25C anibody. (B) Purified GST-Cdc25A, GST-Cdc25B, GST-Cdc25C, Cdc25A catalytic fragment or Cdc25B catalytic fragment was incubated with PD 49 (50 μM) in solution in the absence or presence of excess GSH, and the bound protein was detected on Western blots as described in panel A. (C) Hep3B cells in culture were treated with PD 49 (40 μM for 2 h) and Cdc25A, Cdc25B, or Cdc25C bound to PD 49 was immunoprecipiated by anti-biotin antibodies and detected on Western blot by anti-Cdc25A, B, or C antibody.

Construction of Cdc25B Catalytic Fragment with Mutation of Catalytic Cysteine to Serine and Its Binding to PD 49. To investigate the involvement of the catalytic cysteine, we mutated the catalytic cysteine to serine of Cdc25B by site-directed mutagenesis. The mutant protein was enzymatically inactive (data not shown). It was purified and then used to examine PD 49 binding. In contrast to the wild-type protein, we found very poor PD 49 binding to the mutant protein (Figure 6A).

PD 49 Binding to other Proteins in Hep3B Cell Lysate. We incubated proteins from Hep3B cell lysates with PD 49 in the presence or absence of excess arylator Cpd 5 or the nonarylator PD 26. The results showed many proteins specifically bound to PD 49 from the lysate (Figure 6B). The binding of a few of these proteins to PD 49 was abolished when incubated in the presence of excess nonarylator and was presumed to be binding through the naphthoquinone part of PD 49.

Competition for PD 49 Binding to Cdc25B Catalytic Fragment by Cpd 5. We explored whether PD 49 and Cpd 5 might bind to the same cysteines in the Cdc25B catalytic fragment (Cdc25Bcat). PD 49 was incubated with Cdc25Bcat in the presence of increasing concentrations of Cpd 5. The PD 49−Cdc25Bcat complex was immunoprecipitated with an anti-biotin antibody and visualized on Western blot, using

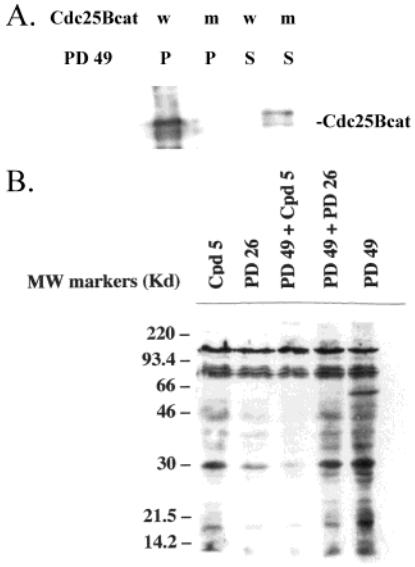

FIGURE 6: PD 49 binding to Cdc25B catalytic cysteine to serine mutant protein, and to Hep3B cell lysate proteins. (A) Purified Cdc25Bcat (w) and its mutant (cysteine to serine) derivative (m) were incubated with 50 μM PD 49 and the PD 49-bound proteins were immunoprecipitated with anti-biotin antibody. The immunoprecipitate (P) and supernatants (S) were resolved on a Western blot and was probed using anti-Cdc25B antibody. (B) Hep3B cell lysate proteins were incubated with PD 49 (50 μM), both in the presence and absence of an excess (1 mM) of the competing arylator Cpd 5 or the nonarylator PD 26. The PD 49-bound proteins were resolved on a Western blot with anti-biotin antibody. Incubation of lysate proteins with either PD 26 or Cpd 5 alone was done for nonspecific binding.

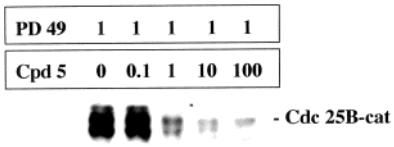

FIGURE 7: Competition of binding to Cdc25B catalytic fragment between Cpd 5 and PD 49. Cdc25B catalytic fragment (Cdc25Bcat, 180 ng) was incubated with increasing ratio of Cpd 5/PD 49 for overnight at 4 °C. PD 49 was used at a concentration of 0.1 mM and Cpd 5 concentrations were varied from 0 to 10 mM. The enzyme bound to PD 49 was immunoprecipitated with anti-biotin antibody.

anti-Cdc25B antibody. Cpd 5 was found to reduce the binding of PD 49 to Cdc25Bcat. We varied the concentration of PD 5, while keeping the concentration of PD 49 at 0.1 mM. By interpolation of the data, we determined that 0.02 mM Cpd 5 was sufficient to cause a 50% reduction of biotinylation (Figure 7).

Association of PD 49 with Cellular Cdc25A, Cdc25B, and Cdc25C. We next examined whether the interaction between PD 49 and Cdc25s seen in vitro also occurred in intact Hep3B cells. Cells were incubated in culture with PD 49, then lysed, and PD 49 was immunoprecipitated from the cell lysates using anti-biotin antibody. The immunoprecipitates were subjected to electrophoresis on a SDS−polyacrylamide gel and the proteins were transferred to membranes, which were probed with an anti-Cdc25A, Cdc25B or Cdc25C antibody. All three Cdc25 isoforms were found to co-immunoprecipitate with PD 49 (Figure 5C), thus showing a likely interaction within the cells, and under our experimental conditions immunoprecipitation was quantitative (data not shown).

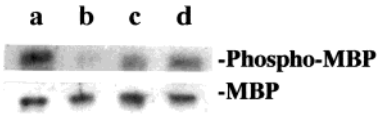

FIGURE 8: Inhibition of PTP activities in Hep3B lysates by PD 49. Phospho-MBP (lane a) was incubated with Hep3B lysate (lane b), lysate depleted of Cdc25A and Cdc25B (lane c), or 40 μM PD 49 treated (60 min at 25 °C) lysate (lane d). Phospho-MBP and MBP were detected on Western blot with anti phosphotyrosine and anti-MBP antibodies.

PD 49 Inhibited Cellular PTP Activity. We previously showed that tyrosine phosphorylated myelin basic protein can be used as a Cdc25A substrate in vitro (*38*). We used this assay to extend our observations on the inhibitory properties of PD 49 against purified Cdc25A to the PTPs in Hep3B cell lysates. Hep3B cell lysates were incubated in the presence (Figure 8, lane d) or absence (Figure 8, lane b) of PD 49, and its PTP activity for dephosphorylating tyrosine phosphorylated myelin basic protein (phospho-MBP) (Figure 8, lane a) was measured. PD 49 (at 10 μM) was found to partially inhibit this PTP activity (Figure 8, lane d). Moreover, when the Hep3B cell lysates were cleared of Cdc25A and Cdc25B proteins by immunoprecipitation, the lysate PTP activity was partially lost (Figure 8, lane c), suggesting the presence of these and other phosphatases in the Hep3B lysates.

ERK1/2 Phosphorylation Induced by PD 49 Was Involved in Growth Inhibition. We have previously found that Cpd 5-induced growth inhibition of different cell types was accompanied by phosphorylation of ERK1/2, and this ERK activation was due to PTP inhibition (*31*, *32*). We therefore examined whether PD 49 had a similar effect on ERK1/2. Hep3B cells were treated with growth inhibitory concentrations of PD 49 and ERK1/2 phosphorylation status was probed with a dual phospho-ERK1/2 specific antibody and was found to be dually phosphorylated by the action of PD 49. We found treatment with PD 49, GSH or, U0126 did not alter total ERK1/2 levels (Figure 9A).

Growth inhibition of various cell lines by Cpd 5 strongly correlated with its ability to induce ERK phosphorylation and inhibit phosphatase activity (*31*, *33*). We explored this relationship using PD 49 and found that when PD 49-induced ERK1/2 phosphorylation was antagonized by GSH or by the MEK1/2 inhibitor U0169, growth inhibition was concomitantly antagonized (Figure 9B). Thus, PD 49-induced ERK1/2 phosphorylation was likely involved in the mechanism of its growth inhibitory effect and GSH might form a sulfhydryl adduct with PD 49 to neutralize its action.

DISCUSSION

We previously synthesized and characterized Cpd 5, an arylating thioalkyl K vitamin analogue, which has potent growth inhibitory activity and also induces cellular protein tyrosine phosphorylation (*21*, *22*, *31*). This suggested that Cpd 5 likely inhibited cellular protein tyrosine phosphatases. The antiproliferative and antiphosphatase activities were found to be antagonized by exogenous GSH, suggesting a mechanism of sulfhydryl arylation of the phosphatases. Dowd hypothesized (*25*) that Cpd 5 inhibited phosphatases by binding to their active site cysteine residue in an addition−elimination reaction. Subsequently, Cpd 5 was found to

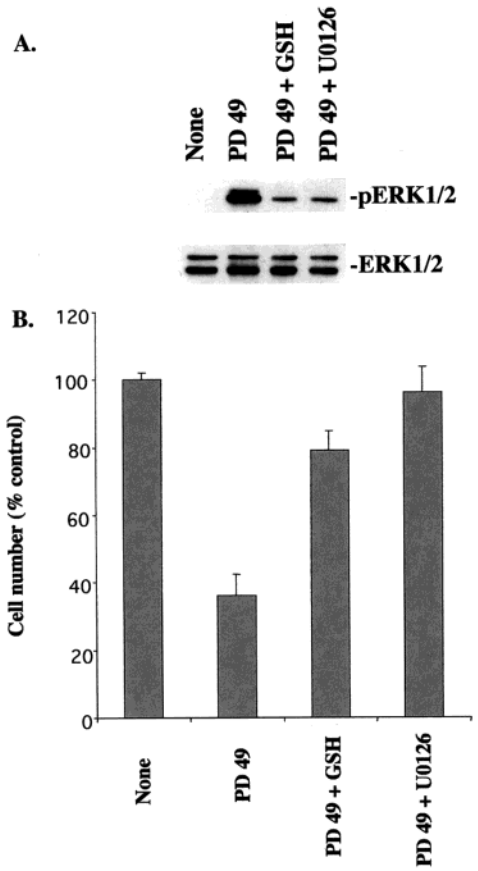

FIGURE 9: Involvement of ERK1/2 phosphorylation in growth inhibition. Hep3B cells were treated with 40 μM PD 49 for 2 h either in the presence or absence of 1 mM GSH or 20 μM U0169 and the Western blotted cell lysate proteins were probed with an anti-dual-phospho-ERK1/2 antibody or a ERK2 antibody, which was used as a loading control (A). The mean cell numbers ($\pm$SD) after 3 days of the above treatments were determined (B).

inhibit the activities of purified protein tyrosine phosphatases, especially the Cdc25 class (*28*, *34*). Several substrates of Cdc25 were also found to be phosphorylated by the action of Cpd 5 on cultured cells (*28*, *29*), suggesting inactivation of Cdc25 by Cpd 5.

However, we lacked evidence for a direct binding and interaction between Cpd 5 and Cdc25. Therefore, PD 49, a biotin labeled derivative of Cpd 5, was synthesized. The biotin tag was used to immunoprecipitate proteins that bind to and interact with PD 49. We found that GST-Cdc25A, GST-Cdc25B, or GST-Cdc25C could be immunoprecipitated after incubation with PD 49, suggesting a direct binding between the two molecules.

In addition to binding to full-length human Cdc25, PD 49 also bound to a purified carboxyl terminal catalytic fragment, which contains amino acids 336 to 523 of Cdc25A or 306 to 580 of Cdc25B. These Cdc25 fragments contain the signature catalytic sequence HCEFSSER, which also has the catalytic cysteine. This result further localized the site of interaction of Cdc25 with PD49 to a carboxyl terminal region of the phosphatase surrounding the catalytic site.

Binding of PD 49 to full-length Cdc25 or its catalytic fragment was blocked by the presence of excess GSH. This also supported the idea of a likely sulfhydryl reaction between PD 49 and a cysteine in the catalytic fragment region. Four other cysteine residues are in the catalytic fragment beside the cysteine at the catalytic site, which could undergo sulfhydryl arylation with PD 49. However, not all five cysteines might be available for binding, because of the secondary structure of the region and disulfide bonds between the cysteines. The crystal structure of the Cdc25A catalytic fragment (*35*, *36*) suggests the catalytic cysteine is free to interact with the phosphotyrosine of the phosphatase substrate protein. Hence, we speculate that this cysteine is a likely candidate to also interact with PD 49. We therefore mutated this cysteine to serine by site-directed mutagenesis and found the mutated Cdc25B catalytic fragment bound very poorly to PD 49, thus demonstrating its importance in binding to PD 49. We also found evidence of interaction of PD 49 with Cdc25 in Hep3B cells. When PD 49 was immunoprecipitated from lysates of Hep3B cells that had been treated with PD 49 in culture, Cdc25 was also co-immunoprecipitated. This binding was also competed by GSH, similar to our findings in a cell-free system. In the cell lysates, however, the binding of Cdc25 and PD 49 could also possibly be indirect.

PD 49 not only inhibited Cdc25 activity in vitro, but also partially inhibited PTP activity of Hep3B cell lysates, suggesting the presence of other, PD 49-resistant phosphatases in the lysate. Cell lysates, cleared of Cdc25A and Cdc25B proteins, partially lost phosphatase activity. This also suggested the presence of phosphatases other than Cdc25A and Cdc25B with myelin basic protein phosphatase activity. We also found that the phosphatases PTP1B and VHR were much less sensitive to inactivation by PD 49 than Cdc25A (Figure 4A) and PD 49 bound to the phosphatases MKP1 and PTP1D but not to PTP1C in Hep3B cell lysates (Figure 4B). These results suggested that there was some specificity of PD 49 binding to various phosphatases and other PD 49-resistant phosphatases were present in the Hep3B cell lysates. We found that PD 49 bound to many proteins in Hep3B cell lsates (Figure 6B). These proteins might be various other cellular protein phosphatases or other nonphosphatse proteins.

Cpd 5 was found to compete with PD 49 for binding to Cdc25Bcat. The result suggested that Cpd 5 was capable of interfering with the process through which PD 49 induced biotinylation of Cdc25Bcat. The most straightforward interpretations of this observation would assume that Cpd 5 and PD 49 interacted with Cdc25Bcat at the same site and by the same mechanism. The similar structure and chemical properties of the two ligands encouraged this expectation. We varied the initial concentration of Cpd 5 while keeping initial concentration of PD 49 at 0.1 mM. By interpolation of the data, we determined that 0.02 mM Cpd 5 is sufficient to cause a 50% diminution of biotinylation. At these concentrations the two inhibitors were both well below the concentrations required for saturation. This allowed the tentative conclusion that $k_2/(k_2 + k_{-1})/k_1$ (where k_2 is the rate of reaction between the bound inhibitor and the protein and k_1 and k_{-1} are the forward and reverse rate of binding of inhibitor to protein) for Cpd 5 is about five times greater that the corresponding activity of PD 49. This is consistent with our more direct measurement of the activity of these inhibitors.

PD 49 induced ERK1/2 phosphorylation. This was similar to the effect of Cpd 5 (*29*). We showed previously that Cdc25A regulates endogenous ERK1/2 phosphorylation status in cultured cells (*32*). Thus, binding of PD 49 to

Cdc25A likely caused its inactivation and consequent phosphorylation of ERK1/2. This ERK1/2 phosphorylation correlated with growth inhibition. When ERK1/2 phosphorylation was inhibited by MEK1/2 inhibitor U0169 in PD 49 treated cells, the growth inhibitory effect of PD 49 was almost completely antagonized (Figure 9). Conversely, when the growth inhibition by PD 49 was antagonized by GSH, ERK1/2 phosphorylation was also antagonized (Figure 9). ERK1/2 is activated in response to proliferative factors, such as EGF, as well as in response to differentiation factors, such as nerve growth factor (NGF) (*39*). Although ERK is thought to play a key role in the proliferative process, it has been suggested that persistent activation of ERK might also mediate cell cycle arrest and differentiation. For instance, in rat PC12 cells, both EGF and NGF trigger the activation of ERK, but only NGF is able to induce PC12 cell differentiation. The important difference between these two growth factors is that NGF induces sustained ERK phosphorylation and nuclear translocation, whereas EGF only induces transient ERK phosphorylation and no significant nuclear translocation (*39*, *40*, *41*). However, the mechanisms by which persistent ERK phosphorylation induces cell growth inhibition or differentiation have not been completely elucidated.

The biotin labeled K vitamin analogue PD 49 thus provided evidence for binding to Cdc25 phosphatases both in cell free conditions and within Hep3B cells. This binding localized to a carboxyl terminal region of Cdc25 surrounding the catalytic site, although we cannot exclude binding to other sites in the amino terminal domain. The mechanism of binding was probably through sulfhydryl arylation of the cysteine residues in this region, supporting for Dowd hypothesis for K vitamin analogues. The binding was probably mediated by the catalytic cysteine, since the Cdc25B catalytic fragment with the catalytic cysteine to serine mutation, abolished PD 49 binding. Although we have found evidence for binding of PD 49 to Cdc25 with purified reactants, the co-immunoprecipitation of the two from PD 49 treated Hep3B cell lysates might have an alternative interpretation. In intact cells, PD 49 could bind to Cdc25 indirectly through another molecule. In this model, PD 49 would bind directly to an unknown protein or proteins in the cells, which in turn could interact with Cdc25A.

Our previous studies on molecular modeling of binding of these K-vitamin derivatives to Cdc25B catalytic domain suggested that the ligand carbonyl oxygens interact with both R482 and R544 near the catalytic cysteine C473 (*37*). Minimal substitutions at positions 2 and 3 of the *para*-quinones, revealed the importance of these positions. A bis-thiohydroxylethyl moiety produced the most favorable interaction. Our proposed model of interaction at the neighboring site in the Cdc25 catalytic domain could be validated by mutating the R482 and R544 residues of Cdc25B. Once validated, the model would be useful in the design of new and better Cdc25 inhibitors.

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