Involvement of Cdc25A Phosphatase in Hep3B Hepatoma Cell Growth Inhibition Induced by Novel K Vitamin Analogs

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ABSTRACT

We previously found that K vitamin analogues caused cell growth inhibition in Hep3B hepatoma cells in vitro, which was associated with their inhibitory effects on protein tyrosine-phosphatases. In this study, we show that Cdc25A, a protein phosphatase, was inactivated by novel arylating K vitamin analogues. The inactivation of Cdc25A correlated with their effects on cell growth inhibition. Cyclin-dependent kinase (Cdk) 4, an important regulator for G1 progression, was found to be tyrosine-phosphorylated by the arylating analogues, and this phosphorylation was correlated with the inhibitory effects of the analogues on Cdc25A activity. Furthermore, Cdk4 dephosphorylation experiments showed that Compound (Cpd) 5, a prototype arylating analogue, inhibited Cdc25A-mediated Cdk4 dephosphorylation, whereas Cpd 26, a nonarylating vitamin K analogue, had no effect on this event. We also examined Cdk4 kinase activity using retinoblastoma protein as a substrate and found that Cpd 5 inhibited retinoblastoma protein phosphorylation in a concentration-dependent manner, indicating that Cdk4 activity was inhibited by Cpd 5 treatment. Moreover, the thiol-antioxidants glutathione and N-acetyl-L-cysteine antagonized the Cpd 5-induced Cdk4 tyrosine phosphorylation, whereas the nonthiol-antioxidants catalase and superoxide dismutase did not. These results suggest that Hep3B cell growth inhibition by these K vitamin analogues may be related in part to inactivation of Cdc25A activity and support the hypothesis that Cdc25A is an attractive target for drugs designed to inhibit cancer cell growth.

INTRODUCTION

The growth of eukaryotic cells is a consequence of their consecutive progression through four distinct cell cycle phases called G1, S, G2, and M. This process is regulated by various phosphorylation and dephosphorylation events (1–4). Key phosphorylation events during the cell cycle include those carried out by Cdkss, which are positively dephosphorylated by Cdk-inhibitory PTPases (15). Key phosphorylation events during the cell cycle include those carried out by Cdkss, which are positively dephosphorylated by Cdk-inhibitory PTPases (15). Key phosphorylation events during the cell cycle include those carried out by Cdkss, which are positively dephosphorylated by Cdk-inhibitory PTPases (15). Key phosphorylation events during the cell cycle include those carried out by Cdkss, which are positively dephosphorylated by Cdk-inhibitory PTPases (15).

In this study, we provide evidence showing that arylating K-vitamin analogues inhibit tyrosine-phosphatases and subsequent tyrosine dephosphorylation (22, 23), which are correlated with cell growth inhibition, the target PTases are not yet clear. We examined SH-PTP1 and SH-PTP2, two prototype PTases, for epidermal growth factor receptor dephosphorylation and mitogen-activated protein kinase phosphatase 1, a dual-specificity phosphatase for extracellular signal response kinase, and found that their activity was not altered by arylating K-vitamin analogues (22). There is growing evidence demonstrating that the dual-specificity phosphatase Cdc25 plays an important role in regulating signal transduction pathways and cell growth, and some recent reports (24–26) show that vitamin K and Cpd 5 can inhibit Cdc25 activity in SiHa and fibroblastic cells. Therefore, we focused on the study of K-vitamin analogue effects on Cdc25A and the subsequent effects on hepatoma Hep3B cell growth inhibition. In this study, we provide evidence showing that arylating K-vitamin analogues inhibited Cdc25A activity, and this was correlated with their activity as inhibitors of the growth of asynchronous Hep3B population. We found that treatment of these asynchronous cells with the arylating Cpd 5 induced Cdk4 tyrosine-phosphorylation, inhibited Cdc25A-induced Cdk4 dephosphorylation, and subsequently induced Rb protein hypophosphorylation. These effects were blocked by thiol-antioxidants GSH and NAC, but not by nonthiol-antioxidants catalase and SOD. The data demonstrate that Cdc25A is likely one of the phosphatase targets of arylating analogues and may play an important role in K-vitamin analogue-induced cell growth inhibition.

MATERIALS AND METHODS

Synthesis of Vitamin-K Analogs. Cpd 1, 5, and 26 were synthesized as described previously (20). Cpd 37 and 42 were synthesized from 2-methyl-1,4-naphthoquinone, which was first brominated and then nitrated to form a mixture of the 5-, 6-, 7-, and 8-isomers. The 5- and 8-isomers of the nitrate product were separated from the 6- and 7-isomers via recrystallization. The thiol side chain was attached via nucleophilic substitution to form Cpd 37. For Cpd 42, the 5- and 8-isomers of the nitrate product were reduced and...
protected as the acetamide, and then the thiol side chain was added via nucleophilic substitution.

**Cell Culture.** Cells of the human hepatoma cell line Hep3B were maintained in Eagle's MEM supplemented with 10% fetal bovine serum. For the growth inhibition assay, Hep3B cells were plated at a concentration of 5 × 10^4 cells/well in 12-well plates. Twenty-four h after plating, the medium was changed with fresh medium containing test compounds. Three days after culture, the medium was removed and the cell number was estimated by a DNA fluorometric assay using the fluorochrome Hoechst 33258 (27). Flow cytometry studies were conducted with asynchronous Hep3B cells plated at 5 × 10^5 cells/ml and treated for 24 or 48 h with Cpd 5 or vehicle alone. Cell cycle distribution was determined using our methods previously (26) with a Beckman Counter Epics XL (Fullerton, CA).

**Cdc25A Activity Assay.** The preparation of plasmid DNA and GST-fusion protein has been described previously (25). The activity of GST-Cdc25A was measured in a 96-well microtiter plate using the substrate o-methyl fluorescein phosphate (Molecular Probes, Inc., Eugene, OR), which is readily metabolized to the fluorescent o-methyl fluorescein. The K-vitamin analogues were resuspended in DMSO, and all of the reactions including controls were performed at a final concentration of 7% DMSO. The final incubation mixture (150 μl) was optimized for enzyme activity and comprised of 30 mM Tris (pH 8.5), 75 mM NaCl, 1 mM EDTA, 0.033% BSA and 1 mM DTT. Reactions were initiated by addition of 1 μg of Cdc25A phosphatase. Fluorescence emission from the product was measured over a 10–60 min reaction period at ambient temperature with a multiwell plate reader. The reaction was linear over the time used in the experiments and was directly proportional to both the enzyme and substrate concentration. Half-maximal inhibition constants were calculated by nonlinear regression analysis, fit to an equation for sigmoidal dose response using GraphPad Prism 3.00 (GraphPad Software, Inc., San Diego, CA).

**Immunoprecipitation and Western Blot Assay.** Hep3B cells were plated in 100-mm tissue culture dishes and treated with different K-vitamin analogues for the indicated times. After treatment, the cells were washed twice with cold PBS and then lysed in 100 μl of immunoprecipitation buffer (50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotime). After removing the cell debris, the supernatants were used for Western blotting with antiphosphotyrosine antibody (New England BioLabs). After removing the cell debris, the supernatants were used for Western blotting with antiphosphotyrosine antibody (New England BioLabs).

**RESULTS**

**Growth Inhibitory Effects of K-vitamin Analogues.** To evaluate the cell growth inhibitory effects of K-vitamin analogues, we treated Hep3B cells with various concentrations of analogues for 3 days, and the cell numbers were estimated by DNA fluorometric assay. The thiol-analogues showed strong growth inhibitory activity, with IC_{50} less than 20 μM. Analogues with a hydroxy group at the end of the side chain (Cpds 5, 37, and 42) were more potent growth inhibitors than that without it (Cpd 1). Cpd 26, the octyl ether analogue, was the least potent growth inhibitor with an IC_{50} of 140 μM (Figs. 1 and 2). We examined the cell cycle distribution of Hep3B cells treated with Cpd 5 and Cpd 26. Twenty-four h after treatment with 5 μM Cpd 5, cells accumulated in G2-M phase (Cpd 5, 48.5 ± 0.5% versus control, 16.5 ± 0.3%) with a concomitant decrease in G1 (Cpd 5, 28.5 ± 0.5% versus control, 67.9 ± 0.3%). Thus, the G2-M checkpoint appeared to be more sensitive to Cpd 5. After 48 h, considerable apoptosis was seen, making flow cytometry analysis problematic (data not shown). Treatment with 20 μM Cpd 26 for 24 hr caused no change in cell cycle distribution.

**Inhibition of Cdc25A Activity in Vitro by K-vitamin Analogues.** We reported previously (23) that thiol-containing K-vitamin analogues induced protein-tyrosine phosphorylation in Hep3B cells, and this induction was caused by inhibition of PTPase activity. Because Cdc25A is a dual-specificity phosphatase containing a cysteine residue in the active site and is a main regulator of the G1-S transition of Hep3B cultures were treated with or without Cpd 5 or Cpd 26 for 24 h, lysed with immunoprecipitation buffer, and cleared of Cdk4 using anti-Cdk4 antibody. The Cdk4-cleared cell lysates were immunoprecipitated with Cdc25A antibody, and the immunocomplexes were incubated with equal amounts of activated Cdk4-containing cell lysates (200 μg) in phosphatase buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM DTT] for 15 min at 30°C. After centrifugation, the supernatant containing dephosphorylated Cdk4 was immunoprecipitated again with anti-Cdk4 antibody. The immunocomplexes were resolved and separated by 12% SDS-PAGE. The dephosphorylation of Cdk4 was monitored by Western blotting using antiphosphotyrosine antibody (Oncogene Science).

![Fig. 1. Chemical structures of K vitamins and median growth inhibitory concentrations.](cancerres.aacrjournals.org)
the cell cycle and possibly of G2-M phase, we examined whether our K-vitamin analogues inhibited its activity. The activity of Cdc25A was measured by incubating GST-Cdc25A fusion protein with different K-vitamin analogues at various concentrations using O-methyl fluorescein phosphate as the substrate (see “Materials and Methods”). Fig. 3 shows that all of the thiol-analogues inhibited Cdc25A activity, whereas nonthiol-analogue Cpd 26 had no inhibitory effects on Cdc25A activity.

**Thiol-analogues Induce Cdk4 Phosphorylation in Hep3B Cells.**
One of the putative cellular substrates for Cdc25A activity is the cyclin D-dependent kinase Cdk4, which must be dephosphorylated to allow cells to pass through the G1-S transition. We reasoned that inhibition of Cdc25A by K-vitamin thiol-analogues would prevent Cdk4 dephosphorylation. Therefore, we measured Cdk4 phosphorylation levels in asynchronous growing Hep3B cells that had been treated with various vitamin-K analogues. Analogue-treated Hep3B cell lysates were immunoprecipitated with anti-Cdk4 antibody, followed by immunoblotting with antiphosphotyrosine antibody. Fig. 4 shows that the thiol-analogues strongly induced Cdk4 tyrosine-phosphorylation, but the nonthiol-analogue Cpd 26 had almost no effect. We also examined the effects of K-vitamin analogues on Cdk1 and Cdk2 tyrosine-phosphorylation but found that there were no significant changes in their phosphorylation levels after treatment with thiol-analogues (Fig. 4). We cannot, however, exclude the possibility that the antibodies used in these studies lacked the sensitivity required for detection. Nonetheless, our results clearly demonstrate reduced dephosphorylation of Cdk4.

**Cpd 5 Inhibits Cdk4p Dephosphorylation by Cellular Cdc25A.**
It has been shown previously that Cdk4 phosphorylation at its conserved threonine and tyrosine residues is an important mechanism for keeping Cdk4 inactive. On the other hand, Cdc25A, the only known Cdk4 phosphatase, removes the inhibitory phosphate groups, resulting in activation of Cdk4 kinase. Because we found that thiol-analogues inhibited Cdc25A activity and induced Cdk4 tyrosine-phosphorylation, we speculated that Cdk4 phosphorylation might be caused by inhibition of Cdc25A-mediated Cdk4 dephosphorylation. To test this, we measured the dephosphorylation of Cdk4p by cellular Cdc25A in the presence of the prototype thiol-analogue Cpd 5 or the nonthiol-analogue Cpd 26. Phosphorylated Cdk4 protein containing cell lysates were prepared from Hep3B cells treated with Cpd 5 and used as a target for Cdc25A dephosphorylation. Cdc25A immunocomplexes were obtained from Hep3B cells treated with Cpd 5 or Cpd 26 at concentrations from 0 to 80 μM. After phosphorylated Cdk4-containing Hep3B cell lysates were incubated with Cdc25A immunocomplexes in phosphatase buffer at 30°C for 15 min, the cell lysates were reimmunoprecipitated with anti-Cdk4 antibody and then immunoblotted with antiphosphotyrosine antibody (see “Materials and Methods”). Fig. 5A shows that Cdk4 dephosphorylation was inhibited by Cdc25A immunocomplexes from Cpd 5-treated Hep3B cells, whereas Cdc25A immunocomplexes from untreated Hep3B cells had most of the Cdk4 phosphorylation removed. By contrast, Cpd 26-treated Hep3B cells did not show inhibitory effects on Cdk4 dephosphorylation (Fig. 5B).
Thiol-antioxidants GSH and NAC Antagonize Cpd 5-induced Cdk4 Phosphorylation. If thiol-analogues of vitamin K cause arylation of Cdc25A, then we reasoned that exogenous thiols should antagonize this effect. We tested the effects of the thiol-antioxidants GSH and NAC on Cdk4 phosphorylation induced by Cpd 5. The presence of GSH or NAC completely antagonized the Cdk4 phosphorylation induced by Cpd 5, whereas nonthiol-antioxidants catalase and SOD had no antagonistic effects (Fig. 6A). To further confirm these findings, we used purified GST-Cdc25A protein to dephosphorylate Cdk4 in vitro. As shown in Fig. 6B, Cpd 5 inhibited the dephosphorylation activity of GST-Cdc25A, and thiol-antioxidants GSH and NAC abolished this inhibitory effect of Cpd 5. Nonthiol-antioxidants catalase and SOD had no antagonistic effects.

Cpd 5 Inhibits Cdk4 Kinase Activity. A well-studied function of cyclin D-dependent kinases is to phosphorylate Rb protein, which in turn activates gene transcription. Phosphorylation of Thr-14 and Tyr-15 of Cdk4 by either activation of wee 1/mik 1 protein kinases or inhibition of Cdc25A phosphatase can inactivate Cdk4 kinase activity, resulting in Rb protein hypo-phosphorylation and thus in cell growth arrest. Because we showed that thiol-analogues inhibited Cdc25A activity and induced Cdk4 tyrosine-phosphorylation, we wished to confirm that the phosphorylation of Cdk4 resulted in its inactivation. This was approached in two ways. Firstly, we examined the effects of K-vitamin analogues on Rb phosphorylation levels. Hep3B cells were treated with K-vitamin analogues, and whole cell lysates were extracted for Western blot analysis using specific anti-phosphorylated Rb antibody. Fig. 7A shows that thiol-analogues significantly inhibited Rb phosphorylation, whereas nonthiol-analogue Cpd 26 had no such inhibitory effects. Secondly, Cdk4 kinase activity was assayed directly by using GST-Rb protein as the substrate for Cdk4 immunocomplexes from Hep3B cells treated with or without Cpd 5. As shown in Fig. 7B, Cpd 5 treatment resulted in decreased phosphorylation of GST-Rb protein in a concentration-dependent manner.

K-vitamin Analogues Do Not Alter the Expression of Cdk4 Inhibitors. Cdk4 activity can also be regulated by Cdk4 inhibitors, such as p15, 16, 21, and 27. To examine this, these protein levels were measured, and no changes of the expression were found (Fig. 8).

DISCUSSION

Our previous studies showed that Cpd 5, a thiol-analogue of vitamin K, is a potent growth inhibitor for Hep3B hepatoma cells and for DNA synthesis in normal rat hepatocytes in culture (22, 23). We proposed and provided some evidence that this growth inhibitory effect is probably attributable to antagonism of PTPases by sulfhydryl arylation (21). Recently, our own and other groups found that the Cdc25 phosphatase family of PTPases may be target proteins of vitamin K3 and its analogues (24, 26, 28). We have extended our previous studies to explore the mechanism(s) and consequence(s) of Cdc25 inhibition by K-vitamin analogues and the relationship between Cdc25 activity and Hep3B cell growth inhibition. Cdc25A has been shown to be an important regulator of the G1-S cell cycle transition, and we found previously (22) that normal rat hepatocytes, which only exist in G0, G1, and S phase of the cell cycle in vitro, were

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Fig. 5. Effects of Cpd 5 (A) and Cpd26 (B) on Cdk4 dephosphorylation. Hep3B cells were treated with 40 µM Cpd5 for 6 h, and the whole cell lysates were used as the target protein of dephosphorylation. A parallel group of Hep3B cells was treated with Cpd5 or Cpd26 at the concentrations from 0 to 80 µM for 6 h, and the cell lysates were immunoprecipitated with anti-Cdc25A antibody. After phosphorylated-Cdk4 containing Hep3B cell lysates were incubated with Cdc25A immunoprecipitates in phosphatase buffer at 30°C for 15 min, the cell lysates were reimmunoprecipitated with anti-Cdk4 antibody and immunblotted with antiphosphotyrosine antibody. The same blots were stripped and probed with anti-Cdk4 antibody as the protein-loading control.

Fig. 6. Thiol-antioxidants antagonize the effects of Cpd 5 on Cdk4 phosphorylation. A. Hep3B cells were treated with Cpd 5 or Cpd 5 plus GSH (1 mM), NAC (1 mM), SOD (500 units/ml), or catalase (25 µg/ml) for 6 h, and then the cell lysates were immunoprecipitated with anti-Cdk4 antibody and blotted with antiphosphotyrosine antibody as described in Materials and Methods. B. GST-Cdc25A-kd4 dephosphorylation. Phosphorylated Cdk4 immunocomplexes were prepared as described in “Materials and Methods.” Equal amounts of phosphorylated Cdk4 immunocomplexes were incubated with GST-Cdc25A (1 µg) alone, GST-Cdc25A plus Cpd 5, or GST-Cdc25A, Cpd 5, plus antioxidants GSH, NAC, catalase, or SOD in phosphatase buffer for 15 min at 30°C. The dephosphorylation of Cdk4 was confirmed by Western blotting using antiphosphotyrosine antibody. The blot was subsequently stripped and probed with anti-Cdk4 antibody as a protein-loading control.
factor β increased Cdk4/6 tyrosine phosphorylation and inhibited their kinase activities. These changes were correlated with the transforming growth factor β inhibitory effect on Cdc25A expression (32). Similar findings were also shown for UV treatment of NRK cells (33). On the other hand, Cyclin A- and Cyclin E-dependent kinases are also reported as direct targets of Cdc25A in different cell lines (26, 34). Recent work (35) even indicates that arylating K-vitamin analogues strongly induced Cdk4 tyrosine phosphorylation, which was correlated with their inhibitory effects on cell growth (Fig. 2 and 4).

In this report, we have used a small group of novel and structurally similar vitamin-K analogues to provide evidence that Cdc25A is involved in Hep3B growth inhibition. We hypothesized previously (29) that Cpd 5 and similar structures would be involved in addition-elimination reactions, involving proteins with an SH-group in the catalytic domain. It was predicted that a likely target group of proteins would include PTPases, which play a central role in signal transduction and cell cycle control events. The inhibition of Cdc25A activity by thiol-analogues lends support to this hypothesis (30, 31). This conclusion is based on our findings that (a) all of the thiol-analogues of vitamin K that we examined inhibited GST-Cdc25A activity in vitro, but nonthiol-analogue Cpd 26 did not; (b) inhibition of GST-Cdc25A by thiol-analogues correlated with their inhibitory effects on Hep3B cell growth; and (c) thiol-antioxidants GSH and NAC completely abrogated Cpd 5-induced Cdk4 tyrosine phosphorylation and subsequent cell growth inhibition, but nonthiol-antioxidants catalase and SOD did not show this antagonistic effect. These data suggest that the inhibitory effects of K-vitamin analogues on Hep3B cell growth may be attributable at least in part to arylation of the protein phosphatase Cdc25A and with inhibition of its activity. This is also supported by experiments showing that the thiol-antioxidants abrogated the effects of Cpd 5 on GST-Cdc25A-mediated dephosphorylation of tyrosine-phosphorylated Cdk4.

Cdc25A is known to play a critical role at the G1-S-phase transition and also may assist in G2-M transition. Our flow cytometry results with asynchronous Hep3B cells are consistent with blocks at multiple checkpoints. The Cdc25A substrate(s) are still being resolved. Likely substrates for Cdc25A are cyclin-Cdk complexes that are activated during G1 phase, which include Cyclin A or E/Cdk2 and Cyclin D/Cdk4 or Cyclin D/Cdk6 complexes. It has been reported that in human epithelial MCF-10A breast cancer cells, transforming growth also inhibited by K-vitamin analogues. Recent results (15) suggest Cdc25A may also have a role in inhibiting mitosis. Thus, we focused on Cdc25A in this study to determine whether it is a target protein of K-vitamin analogue in Hep3B hepatoma cells.

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![Fig. 7. A, the effects of K-vitamin analogues on Rb phosphorylation. Hep3B cells were treated with K-vitamin analogues for 8 h, and whole cell lysates were extracted for Western blot analysis by using specific antiphosphorylated Rb antibody. B, Cdk4 kinase activity assay. Hep3B cells were treated with Cpd5 at concentrations from 0 to 30 µM for 6 h, and whole cell lysates were immunoprecipitated with anti-Cdk4 antibody. The immunoprecipitates were then incubated with equal amounts of GST-Rb protein in kinase buffer for 30 min at 30°C. The GST-Rb phosphorylation status was confirmed by Western blotting using specific antiphosphorylated Rb antibody.](image)

![Fig. 8. Western blot of p15, p16, p21, and p27. Hep3B cells were cultured with K-vitamin analogues for 6 h, and cell lysates were analyzed by Western blotting using anti-p15, p16, p21, and p27 antibodies. The first lane (C) is control, untreated Hep3B cells.](image)
caused by the antagonism of Cdc25A activity. It remains possible, of course, that some other not yet defined phosphatases for Cdk4 could also be affected by Cpd 5. Nonetheless, these results support our recent findings (28) that Cpd 5 can block cellular Cdc25A activity.

In summary, we demonstrate here that K-vitamin thiol-analogues are a group of compounds possessing the ability to inhibit the activity of an important cell growth regulator, Cdc25A. The inhibition of Cdc25A activity in Hep3B cells by thiol-analogues of K vitamin induces Cdk4 tyrosine-phosphorylation and subsequent Rb hypophosphorylation and cell growth arrest. On the basis of these findings, we suggest that Cdc25A may play a central role in K-vitamin analogue-induced Hep3B cell growth inhibition, and further understanding of Cdc25A in regulating signal transduction pathways may contribute to development of novel anticancer agents, based on selective interaction with Cdc25A phosphatase.

REFERENCES

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