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Original paper

Vitamin K_3 Induces Cell Cycle Arrest and Cell Death by Inhibiting Cdc25 Phosphatase

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Our early reports have indicated that vitamin K_3 (VK_3) exerts antitumour activity by inhibiting Cdk1 activity and overexpressing the *c*-myc gene to induce an apoptotic cell death. In the present study, we investigated the effect of VK_3 on Cdc25 phosphatase, a Cdk1 activator and c-Myc-downstream protein. Increased protein level but decreased activity of Cdc25A phosphatase was found in cervical carcinoma SiHa cells treated with VK_3 for 1 h and allowed to recover for 8, 24, 30 or 45 h. The binding of VK_3 to Cdc25 phosphatase was proven by incubating [methyl-^{3}H]-VK_3 with the 27 kDa-catalytic domain of Cdc25A phosphatase at 35°C for 2 h. We found that VK_3 inhibited cyclin E expression at late G1 phase and cyclin A at G1/S transition of the aphidicolin-synchronised SiHa cells, but had no effect on Cdk2 and Cdk4. The inhibition of cyclins E and A expression was associated with cell cycle progression delay in the S phase. These results indicate that binding of VK_3 to the catalytic domain of Cdc25 phosphatase results in the formation of inactive, hyperphosphorylated Cdk1 that subsequently induces cell cycle arrest, leading to cell death. These findings suggest a possible therapeutic strategy, with VK_3 serving as a potential antagonist to tumours expressing high levels of proteins containing cysteine such as oncogenic Cdc25A phosphatase. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: vitamin K_3, Cdc25 phosphatase, dual specificity phosphatases, cyclin E, cyclin A, human cervical carcinoma SiHa cells
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INTRODUCTION

CYCLINS AND the cyclin-dependent kinases (Cdks) are the key regulators of the eukaryotic cell cycle. The activity of Cdks is tightly regulated by an intricate system of kinase–cyclin interaction and phosphorylation. Cdk1, associated with the B-type cyclins, regulates the M phase [1]. Cdk2, associated with the A- and E-type cyclins, controls the S phase and G1/S transition, respectively [2, 3]. Cdk4, associated with the D-type cyclins, is important for G1 progression [3]. The activity of the kinase holoenzyme is inhibited by phosphorylation of Cdks at Thr-14 and Tyr-15, whose phosphorylation status is controlled by the antagonistic action of Wee1/Myt1 kinases and Cdc25 phosphatases [4]. Three *cdc*25 genes, *cdc*25*A*, *B* and *C*, have been identified in human cells [5–7]. These gene products are dual specificity phosphatases (DSPases), which possess a highly conserved cysteine residue at their active sites [8] and function at different phases of the cell cycle. Cdc25A and Cdc25B are expressed throughout the cell cycle with a peak expression of Cdc25A in the G1 phase and peak expression of Cdc25B in both the G1/S and G2 phases [6, 9]. Cdc25C is predominantly expressed in the G2 phase and regulates the timing of cells to enter into mitosis [7, 10].

Vitamin K_3 (VK_3 or menadione; 2-methyl-1,4-naphthoquinone), a synthetic compound, exhibits a broad spectrum of antitumour activity against several cell types both *in vitro* [11–13] and *in vivo* [14, 15]. VK_3 induces DNA damage [16] and exerts a greater toxic effect on rapidly growing cells than on stationary cells [17]. We found that VK3 disturbs the cell cycle progression, which is correlated with the accumulation of hypophosphorylated retinoblastoma (RB) protein [18, 19] and inactive hyperphosphorylated Cdk1 [17, 20]. VK_3 also induces apoptosis [21] as indicated by cell bleb formation, chromatin condensation, DNA fragmentation and alteration in the expression of *c*-myc [22].

Two primary possible mechanisms of VK_3's cytotoxicity have been proposed: oxidative stress through the redox

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cycling of the quinone structure to generate toxic oxygen species [23]; and direct arylation of cellular thiols resulting in depletion of glutathione (GSH) and inhibition of sulphydryl-dependent proteins [24–27]. The generated toxic oxygen species induced by VK_3 should undergo a Fenton reaction (iron-dependent) to induce DNA damage in cells. However, the fact that the toxic oxygen species, inducing most of the DNA breakages in VK_3-treated Chinese hamster ovary cells, are not responsible for VK_3's cytotoxicity was indicated by the co-incubation of iron-chelator [28]. The propensity of VK_3 quinones to undergo arylation of cellular thiols has been explored by a series of substituted derivatives of vitamin K and vitamin K_3 oxide [26, 27]. Recently, it has also been shown that human recombinant Cdc25A phosphatase can be inactivated by VK_3 *in vitro* [29]. Thus, whether VK_3 can bind to the active site of Cdc25 phosphatase and subsequently affect the cell cycle progression was the focus of our study.

MATERIALS AND METHODS

Chemicals and antibodies

The water-soluble form of VK_3 (menadione sodium bisulphite), aphidicolin and sulphorhodamine B (SRB) were obtained from Sigma (St Louis, Missouri, U.S.A.). Tissue culture supplies were purchased from Life Technologies (Gaithersburg, Maryland, U.S.A.). [Methyl-^{3}H]-vitamin K_3 (^{3}H-VK_3, 1.1 Ci/mmol) was synthesised by Moravek Biochemicals (Brea, California, U.S.A.). The bicinchoninic acid (BCA) protein determination kit was otained from Pierce (Rockford, Illinois, U.S.A.).

Human recombinant Ras and antibodies against Cdk1 (rabbit polyclonal IgG), cyclin A (rabbit polyclone), cyclin E (mouse monoclonal IgG1) or actin (mouse monoclonal IgM) were purchased from CN Biosciences Company (La Jolla, California, U.S.A.). Rabbit polyclonal antibody against Cdk4 was obtained from Upstate Biotechnology (Lake Placid, New York, U.S.A.). Cdc25A antigenic peptide and antibodies against Cdc25A, Cdc25B, Cdc25C (rabbit polyclonal IgG) or cyclin D1 (rabbit polyclone) were obtained from Santa Cruz Biotechnology (California, U.S.A.). Antibody against Cdk2 (mouse IgG2a) was obtained from Transduction Laboratories (Lexington, Kentucky, U.S.A.).

Cell culture and synchronisation

Human cervical carcinoma SiHa cells (American Type Culture Collection, Rockville, Maryland, U.S.A.) were cultured in Dulbecco's Modified Eagle Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 units/ml of penicillin and 100 μg/ml streptomycin) at 37°C in a 5% CO_2 humidified incubator. Subconfluent cells (1×10^6/100-mm dish) were treated with various concentrations of VK_3 for 1 h and allowed to recover for 8, 24, 30 or 45 h in asynchronous experiments. Cells were synchronised at the G1/S boundary by 48 h serum starvation followed by 24 h treatment with aphidicolin (2 μg/ml) (Sigma). Cells were then treated with 50 μM VK_3 for 1 h at the appropriate phase as indicated.

Cytotoxicity assay

The cytotoxicity of VK_3 was determined by the SRB–protein binding assay [13]. SiHa cells were seeded at a density of 1×10^4 cells/well and incubated at 37°C for 24 h, and then treated with VK_3 for 48 h. Cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. Absorbance at 515 nm was measured in a microtitre plate reader (Model EL340, Bio-Tec Inc., Vermont, U.S.A.).

Flow cytometric analysis

Cell cycle distribution of the cells after drug treatment or synchronisation was analysed by flow cytometry (Becton Dickinson, Florida, U.S.A.), using propidium iodide (PI) as a fluorescent dye. Cells were trypsinised, pelleted and then fixed overnight in 5 ml of 70% ethanol. After precipitation, the cell pellet was stained with PI solution containing phosphate-buffered saline (PBS), 1% Triton X-100, 75 μg/ml PI and 0.5 mg/ml RNase A, and analysed in a Becton Dickinson flow cytometer.

Protein concentration determination and immunoblotting

Extracts of SiHa cells were prepared by scraping cells into PBS, then lysed with sample loading buffer containing 125 mM Tris (pH 6.8), 20% glycerol, 1% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol and 0.01% bromophenol blue. The protein concentration was determined by BCA protein assay [30]. Cell extracts (80–100 μg) were boiled, electrophoresed in 10% polyacrylamide–SDS gels and electrophoretically transferred to Immobilon membrane (Millipore, Massachusetts, U.S.A.). Proper antibodies were used as probes to detect protein expression. After incubation with alkaline phosphatase-conjugated secondary antibodies, the membrane was developed in a substrate-chromogen solution containing 100 mM Tris (pH 9.5), 100 mM $MgCl_2$, 33 μg/ml nitroblue tetrazolium (NBT) and 66 μg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

Cdc25 phosphatase activity assay

SiHa cells with or without VK_3 (100 μM) treatment followed by a 24 h recovery were lysed by adding 3–5 volumes of lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 20 mM EGTA) containing protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Cell extracts containing 800 μg total protein were then immunoprecipitated with Cdc25A antibody in the presence or absence of Cdc25A antigenic peptide and assayed for phosphatase ability to hydrolyse p-nitrophenol phosphate (pNPP) [31]. The phosphatase activities were assayed in a volume of 200 μl containing 20 mM pNPP (Sigma), 50 mM imidazol (pH 7.5), and 1% 2-mercaptoethanol at 35°C for 1 h. At the end of the incubation, the reaction was terminated by the addition of 4 μl of 10 M NaOH, and absorbance at 410 nm was measured by a spectrophotometer.

Binding of VK_3 to the catalytic domain of Cdc25 phosphatase

Twenty μg of the catalytic domain (a.a. residues Nos 317–523) of Cdc25A phosphatase were incubated with 30 μCi of [methyl-^{3}H]-vitamin K_3 ([^{3}H]VK_3, 100 μM) at 35°C for 2 h. The reaction mixture was then eluted from a microconcentrator (10 kDa cut-off, Amicon, Massachusetts, U.S.A.) and washed with PBS three times to remove unbound [^{3}H]VK_3. The filters containing [^{3}H]VK_3–Cdc25A complex were dried overnight and the radioactivity of the complexes was counted in a Beckman scintillation counter.

RESULTS

Cytotoxicity of VK_3 against SiHa cells

Cells in the exponential phase were used to determine the IC_{50} value of VK_3 by the SRB-microculture assay. SRB binds

to the basic amino acids of cellular macromolecules, then the solubilised stain is measured spectrophotometrically to determine the relative cell growth in the drug-treated and -untreated cells. The estimated IC_{50} value was 37 μM (data not shown).

Effects of VK_3 on cell cycle progression and on cell cycle related proteins in asynchronous SiHa cells

SiHa cells treated with 0, 10, 25, 50, 75 or 100 μM of VK_3 for 1 h followed by 15, 30 or 45 h recovery were subjected to flow cytometric analysis of total DNA content and Western blot analysis of Cdk1 protein. Figure 1a showed different cell cycle phase distribution in cells treated with varying concentrations (>25 μM) of VK_3 followed by 15, 30 or 45 h recovery. With increasing VK_3 concentrations, the population of cells in the S phase progressively increased after 15 h recovery (25 and 50 μM VK_3), whilst the percentage of cells in the G2/M phase increased after 30 h recovery (25, 50 and 75 μM VK_3). After 45 h recovery, part of the cells traversed through mitosis and progressed into the G1 phase (25, 50 and 75 μM VK_3). However, cells exhibited a different cell cycle distribution when treated with toxic 100 μM of VK_3 followed by 15 h recovery. In summary, VK_3 induced a delay in cell cycle progression in a dose- and recovery time-dependent manner. The flow cytometric results showed that VK_3 might exert toxic effects on the cell cycle regulatory proteins.

In Figure 1b, the intensity of the hyperphosphorylated upper (U) and middle (M) bands of Cdk1 gradually increased in VK_3-treated cells whilst that of the hypophosphorylated lower (L) bands of Cdk1 decreased. Compared with the VK_3-treated cells, the control SiHa cells showed a hypophosphorylated pattern of Cdk1. Therefore, the extent of the cell cycle block at the G2 phase by VK_3 was positively associated with the accumulation of the hyperphosphorylated form of Cdk1. The activity of the immunoprecipitated Cdk1 kinase was measured by the kinase's ability to phosphorylate added exogenous H1 histone substrate. The activity was inhibited by VK_3 pretreatment (data not shown).

The expression of Cdc25 phosphatase in SiHa cells treated with 50 μM VK_3 and allowed to recover in a drug-free medium for 8, 24, 30 or 45 h was sequentially determined. As shown in Figure 2, Cdc25A phosphatase showed double bands, and the density of the upper hyperphosphorylated band was more intense than that of the lower hypophosphorylated band in untreated cells. The intensity of the upper and lower bands in treated cells increased by 0.5–1 fold and 2–3.5 fold, respectively, as compared with the corresponding bands in untreated cells after 8, 24, 30 or 45 h recovery. These results indicate that cells overproduced Cdc25A protein and that both hypo- and hyperphosphorylated forms of Cdc25A phosphatase increased with different intensity upon VK_3 treatment. No detectable change was observed for Cdc25C phosphatase. The activity of Cdc25 phosphatase is positively modulated by the phosphorylation of protein at its N-terminal [32] and overproduction of this protein leads cells to prematurely enter into mitosis. Whether VK3 inhibits the activity of Cdc25 phosphatase in cells was further confirmed.

Effect of VK_3 on G1/S transition and S phase progression in synchronised SiHa cells

To study the effect of VK_3 on G1 and S phase progression, SiHa cells were synchronised at the G1/S boundary by consecutive serum starvation followed by aphidicolin treatment. Synchrony of the cultures was monitored by flow cytometric analysis (Figure 3a). Those cells harvested immediately after their release from aphidicolin block (0 h) showed a G1 peak at the relative DNA content of 2N. Subsequently, this peak shifted to the relative DNA content of 4N at 8 h and back to the position of 2N at 18 h after release from aphidicolin block. Thus, the time point for each phase was assigned as follows: 0 h, G1- and S-phase boundary (G1/S); 2 h, early S phase (SE); 4 h, middle S phase (SM); 6 h, late S phase (SL); 8 h, major G2 phase and start of M phase (G2); 12 h, late M phase and early G1 phase (G1E); 18 h, G1 phase.

SiHa cells synchronised at late G1 (G1L, 1 h before aphidicolin removal), G1/S and SE phases were treated with VK_3

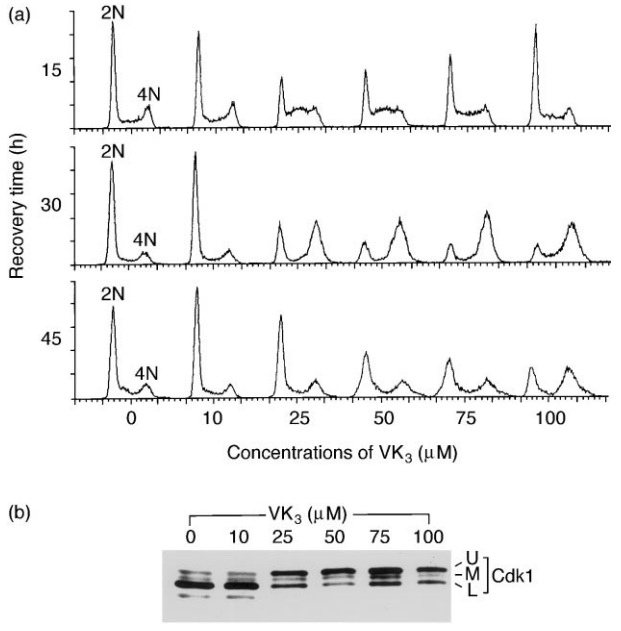

Figure 1. Effect of VK_3 on cell cycle progression and phosphorylation status of Cdk1 in SiHa cells. (a) DNA histograms from flow cytometric analyses of the control (0) and the VK_3 (10, 25, 50, 75 or 100 μM)-treated cells are shown. (b) Cells after 1 h VK_3 treatment and 30 h recovery were lysed, electrophoresed by 10% SDS–PAGE and probed with rabbit antiserum against C-terminal octameric antigenic peptide of Cdk1 (U, M and L represent three phosphorylated forms of Cdk1). U, Upper; M, Middle; L, Lower.

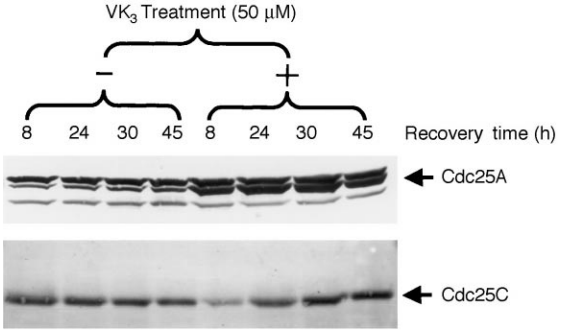

Figure 2. Effect of VK_3 on the protein levels of Cdc25 phosphatases A and C in SiHa cells. Cells (1×10^6) were treated with 50 μM VK_3 for 1 h and allowed to recover in a drug-free medium for 8, 24, 30 or 45 h. Cells were lysed, electrophoresed by 10% SDS-PAGE and probed with rabbit polyclonal antibodies against Cdc25A or Cdc25C.

(50 μM) for 1 h and harvested immediately for analyses of the expression of Cdk2, Cdk4, cyclins D, E and A (Figure 3b and 3c). There were no detectable changes for Cdk2 and Cdk4 after VK_3 treatment (Figure 3b). Cyclin D is synthesised sequentially during the G1 interval upon regulation by extracellular signals [33]. VK_3 did not affect cyclin D expression as compared with control cells (Figure 3c). Cyclin E is expressed periodically at maximum levels near the G1/S phase transition and is essential for completion of the G1 and entry into the S phase [33]. In Figure 3c, the expression of cyclin E was inhibited at both the late G1 (3-fold less, compare lane 2 with lane 1) and G1/S phase transition (2-fold less, compare lane 4 with lane 3). Cyclin A was expressed and accumulated after cells entered into the S phase, but the expression was inhibited at the G1/S phase transition upon VK_3 treatment (Figure 3c, compare lane 3 with lane 4). These data indicated that VK_3 interfered with the expression and/or accumulation of cyclins E and A at the late G1 and G1/S transition, as well as the phosphorylation status of Cdk1 at the G2/M phase.

Cdc25 phosphatase activity decreases in VK_3 treated cells

To study the effect of VK_3 on Cdc25A phosphatase, the catalytic activity of phosphatase was determined in SiHa cells treated with VK_3 for 1 h and allowed to recover for 24 h. Cdc25A was immunoprecipitated from the same amount of protein lysate (800 μg) and tested for its ability to hydrolyse substrate pNPP. The activity of Cdc25A phosphatase decreased by 4-fold in VK_3-treated cells as compared with that in untreated cells (Figure 4). Both VK_3-treated and -untreated cells exerted low or background activity when the activity assay was performed in the presence of Cdc25A antigenic peptide as a negative control (Figure 4).

VK_3 binds to Cdc25 phosphatase in vitro

To demonstrate the addition of thiols to VK_3, the binding of [^{3}H]VK_3 to the catalytic domain of Cdc25A phosphatase which contains one cysteine residue was performed. [^{3}H]VK_3 bound only to the catalytic domain of Cdc25 phosphatase but not to bovine serum albumin or to human recombinant Ras protein (Figure 5).

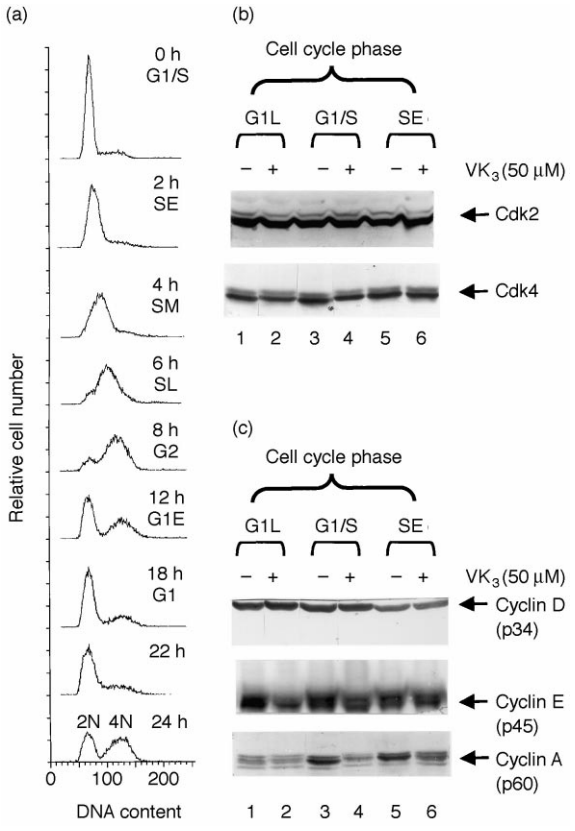

Figure 3. Effect of VK_3 on the protein levels of Cdks and cyclins in different cell cycle phases of SiHa cells synchronised by aphidicolin. (a) Flow cytometric analysis of untreated cells after release from the G1/S phase (aphidicolin) synchronisation. DNA histograms of cells were made at 0 h and at indicated times after release from synchronisation. (b) and (c) Cells (1×10^6) were treated with 50 μM VK_3 for 1 h at different cell cycle phases as indicated. Cells were lysed, electrophoresed by 10% SDS–PAGE and probed with antibodies against Cdk2 or Cdk4 (as shown in b), or against cyclin D, cyclin E or cyclin A (as shown in c).

DISCUSSION

We previously found a correlation between VK_3-induced cell growth inhibition and cell cycle perturbation at the S and G2/M phases [17]. These effects were accompanied by the decreased activities of Cdk1 and protein tyrosine phosphatases (PTPases), and finally led to apoptotic cell death [20]. The major protein phosphatase that is capable of dephosphorylating and reactivating Cdk1 *in vivo* and therefore activating Cdk1 is Cdc25 phosphatase—a DSPase. In the present study, we demonstrate that Cdc25 phosphatase is involved in VK_3-induced Cdk1 hyperphosphorylation, cell cycle arrest and cell death. This is the first report of the binding of radiolabelled [^{3}H]VK_3 to the catalytic domain of Cdc25 phosphatase being implicated in the mechanism of VK_3's action.

Three forms (A, B and C) of Cdc25 phosphatase were all detected in asynchronous SiHa cells (Figure 2). The A form is relatively abundant, whilst the C form is scarce and the B form (data not shown) barely detectable. The different

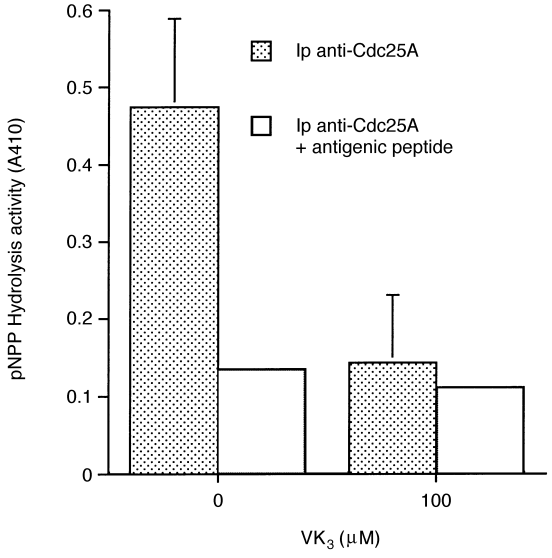

Figure 4. Inhibition of Cdc25A phosphatase activity by VK_3 treatment in SiHa cells. Cdc25A phosphatase protein was immunoprecipitated from lysate (800 μg) of VK_3-treated SiHa cells in the presence or absence of Cdc25 antigenic peptide. The precipitated proteins were then analysed for their ability to hydrolyse p-nitrophenol phosphate (pNPP).

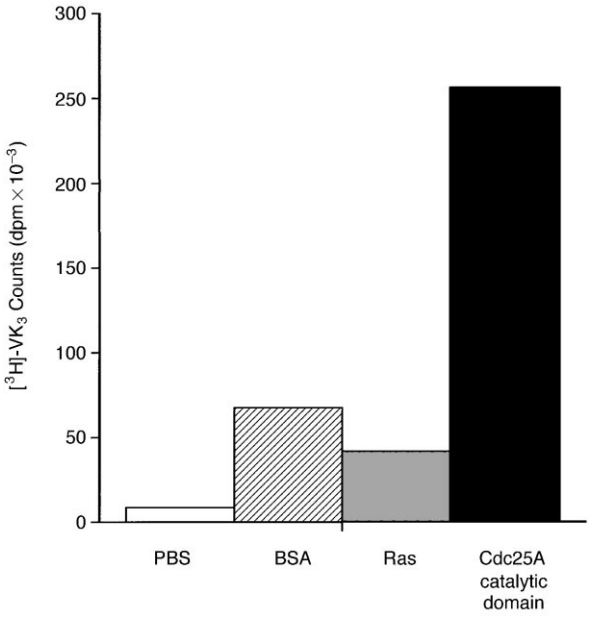

Figure 5. *In vitro* binding assay of [^{3}H]VK_3 to the catalytic domain (a.a. residues 317–523) of Cdc25A phosphatase. 100 μM of VK_3 (30 μCi) was incubated with 20 μg of Cdc25A catalytic domain bovine serum albumin (BSA) (50 μg) or human recombinant Ras (50 μg) at 35°C for 2 h. The reaction mixtures were then eluted from a microconcentrator (10 kDa cut-off). The filters were dried overnight and the retained complexes were counted using a Beckman scintillation counter.

proportions of G1, S and G2/M phases in asynchronous SiHa cells provide clues for the different compositions of the three forms of Cdc25 phosphatase (Figure 1). As reported in HeLa cells, Cdc25A phosphatase regulates S phase entry and remains active during the G2 and M phases, it also acts on the Cdk2–cyclin A and/or Cdk2–cyclin E complex at the S phase and also the Cdk1–cyclin B complex at the G2/M phase [31]. Together with our observations, these findings suggest that VK_3 can affect Cdk1 activity via inhibition of Cdc25A phosphatase (Figure 2). Since Cdc25C is predominantly expressed in the G2 phase and regulates the timing of cells to enter into mitosis, we do not exclude the possibility that VK_3 might affect the activity of Cdc25C phosphatase.

In cells synchronised at the G1/S phase, VK_3 did not alter the phosphorylation status of either Cdk2 or Cdk4, but altered those of cyclins A and E (Figure 3). Therefore, we postulate that in the exponentially growing cells, VK_3 may first affect the expression of cyclins E and A at the late G1 and G1/S transition phases, and delay the cell cycle progression through the S phase (Figure 3). In addition, the inhibition of Cdc25A phosphatase activity by VK_3 significantly retarded the delayed S phase cells at the G2/M phase (Figures 1 and 4).

We have recently reported that VK_3 binds to the peptide with a sequence motif—(I/V)HCX_5R(S/T)G—of all PTPases and DSPases but not to the mutated one containing a serine residue instead of cysteine according to SDS–PAGE analysis [34]. The wild-type sequence motif contains a conserved cysteinyl residue that is essential for the activity of all PTPases, including DSPases such as Cdc25 phosphatase. In the present study, we further demonstrated that VK_3 binds to Cdc25 phosphatase. [^{3}H]VK_3 bound to the catalytic domain of Cdc25A phosphatase (a.a. residues 317-523) containing the PTPase and DSPase sequence motif, as shown by the complex formation retained on the 10-kDa cut-off membrane, but did not bind to the non-PTPase and non-DSPase proteins such as Ras or bovine serum albumin (BSA) (Figure 5). Together, our data further confirm the arylation of target proteins by VK_3 [26] and suggest that VK_3 may covalently bind to the enzyme, alter the enzyme's conformation, and finally decrease its activity. More biochemical and biophysical studies on the interaction of VK_3 with Cdc25A will be published elsewhere. Our findings suggest a possible therapeutic strategy, with VK_3 serving as an antagonist to the highly expressed proteins in tumours containing conserved cysteine(s) at their active site, such as PTPases and DSPases, especially oncogenic Cdc25A phosphatase.

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