Cell Growth Inhibition by a Novel Vitamin K Is Associated with Induction of Protein Tyrosine Phosphorylation*

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We have shown that a synthetic vitamin K analog, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone or compound 5 (Cpd 5), potently inhibits cell growth and suggested that the analog exerts its effects mainly via sulfhydryl arylation rather than redox cycling. Since protein-tyrosine phosphatases (PTPases), which have pivotal roles in many cellular functions, have a critical cysteine in their active site, we have proposed PTPases as likely targets for Cpd 5. To test this hypothesis, we examined the effects of Cpd 5 on protein tyrosine phosphorylation of cellular proteins and on the activity of PTPases. We found that Cpd 5 rapidly induced protein tyrosine phosphorylation in a human hepatocellular carcinoma cell line (Hep3B) at growth inhibitory doses, and the effect was blocked by thiols but not by non-thiol antioxidants or tyrosine kinase inhibitors. Cpd 5 inhibited PTPase activity, which was also significantly antagonized by reduced glutathione. Furthermore, the well studied PTPase inhibitor orthovanadate also induced protein tyrosine phosphorylation and growth inhibition in Hep3B cells. These results suggest that inhibition of cellular PTPases by sulfhydryl arylation and subsequent perturbation of protein tyrosine phosphorylation may be involved in the mechanisms of Cpd 5-induced cell growth inhibition.

The vitamin K family of molecules contains both natural and synthetic forms, the former including vitamin K1 (phyloquinone) and vitamin K2 (menaquinones) and the latter including vitamin K3 (menadione). The physiological function of natural vitamin K is to act as a cofactor of γ-glutamylcarboxylase, which catalyzes the carboxylation of glutamate residues into γ-carboxyglutamate in prothrombin and the vitamin K-dependent coagulation factors VII, IX, and X; proteins C and S; as well as several other proteins (1). K vitamins, sharing a naphthoquinone nucleus, also inhibit cell growth both in vitro and in vivo, with vitamin K3 being more potent than vitamin K1 and K2 (2–6). The cell growth inhibitory effects of vitamin K3 have been ascribed to both sulfhydryl arylation (7–9) and oxidative stress due to its redox cycling capacity (10–13). This mixed nature of vitamin K action has also been suggested by our previous data showing that vitamin K3-induced growth inhibition in Hep3B cells, derived from human hepatocellular carcinoma, is prevented by both exogenous thiols and non-thiol antioxidants (catalase and deferoxamine mesylate) (14, 15).

We recently synthesized several vitamin K analogs and found that 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone or compound 5 (Cpd 5) is a more potent growth inhibitor and apoptosis inducer than K vitamins for Hep3B cells (15). From its chemical structure, Cpd 5 is predicted to exert its action through arylation of cellular thiols or thiol-dependent proteins by an addition-elimination mechanism (16). In fact, the Cpd 5-mediated cell growth inhibition has been shown to be antagonized only by thiols but not by non-thiol antioxidants in Hep3B cells, suggesting that the major mechanism of growth inhibition is sulfhydryl arylation rather than oxidative stress (15).

It is not clear which cellular proteins are inactivated by the sulfhydryl arylation. In a human hepatocellular carcinoma cell line (HepG2), vitamin K3 was found to induce a hyperphosphorylated state of p34cdc2 kinase and decrease the activity of cell lysates to hydrolyze p-nitrophenyl phosphate, indicating alteration of protein tyrosine phosphorylation (17). Since the active site of protein-tyrosine phosphatases (PTPases) contains a cysteine residue, which is known to play an essential role in the catalytic process of tyrosine dephosphorylation (18, 19), we have proposed that Cpd 5 may arylate the critical cysteine and inactivate PTPases, causing perturbed status of protein tyrosine phosphorylation (16). Protein tyrosine phosphorylation/dephosphorylation processes are involved in the regulation of cell growth and many other cellular functions (20, 21).

In the present study, we examined whether there was a relationship between growth inhibition and induction of tyrosine phosphorylation of cellular proteins in Hep3B cells by Cpd 5. We also compared the effects of Cpd 5 with those of orthovanadate, a well studied PTPase inhibitor. Our results suggest that PTPase inhibition and subsequent perturbation of protein tyrosine phosphorylation may have important role in Cpd 5-mediated growth inhibition.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Growth Inhibition Assay—Cpd 5 was synthesized as described previously (16). The Hep3B cells (American Type Culture Collection, Rockville, MD) were maintained in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. For the growth inhibition assay, the cells were plated at 2 × 10⁴ cells/well in 24-well plates (Corning, Inc., Science Products Div., Corning, NY). Twenty-four hours after plating, the medium was replaced with a medium containing Cpd 5 or orthovanadate at various concentrations. After treatment, plates were emptied and stored at −80 °C. Cell number was counted daily by a DNA fluorometric assay (22). On the day of assay, 250 μl of distilled water were added to each well, and plates were incubated for 1 h at room temperature. After the
incubation, the plates were frozen at ~80 °C and then thawed at room temperature. The fluorochrome Hoechst 33258 (20 μg/ml) in TNE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM NaCl) was added to each well (250 μl/well), and fluorescence was measured (excitation at 360 nm, emission at 460 nm). Another assay was employed to examine the effects of various antioxidants on growth inhibition by Cpd 5. Hep3B cells were plated at 5 × 10^4 cells/well in 5-plate wells (Corning, Inc.). After overnight incubation, the cells were treated with Cpd 5 (20 μM) with or without thiols and non-thiol oxidants and further cultured for 48 h. Then the medium was replaced with phosphate-buffered saline containing 0.1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and incubated for 3 h in a CO2 incubator. Converted dye was then dissolved in acid isopropanol (0.08 N HCl in isopropanol), and absorbance was measured at a wavelength of 570 nm with background subtraction at 660 nm.

Immunoblotting—Hep3B cells were plated at 5 × 10^5 cells/dish in 6-well plates with Eagle’s MEM supplemented with 10% fetal bovine serum. Twenty-four hours after plating, the cells were exposed to various agents at different concentrations. After the exposure, the cells were washed with phosphate-buffered saline and then lysed in 100 μl of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 ng/ml leupeptin, 10 ng/ml aprotinin). Insoluble material was removed by microcentrifugation at 12,000 rpm for 5 min. Aliquots of samples were used for measurement of protein concentration using the Bio-Rad assay (Bio-Rad). Treated cell lysates (40 μg of protein/fane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels. Then proteins were transferred to polyvinylidene difluoride membranes (NEN Life Science Products). After blocking the membrane with Tris-buffered saline with Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 1% bovine serum albumin, the membrane was incubated with anti-phosphotyrosine monoclonal antibody (Ab-2, Oncogene Research Products, Cambridge, MA), and then washed with the Tris buffer and incubated with anti-mouse IgG coupled to horseradish peroxidase. Detection was performed with enhanced chemiluminescence reagents (NEN Life Science Products).

RESULTS

Cpd 5 Inhibits Cell Growth and Induces Protein Tyrosine Phosphorylation in Hep3B Cells—The effect of Cpd 5 on Hep3B cell growth was first examined. It showed potent growth inhibition at micromolar concentrations (Fig. 1). The phosphotyrosine immunoblotting technique was then applied, to examine whether Cpd 5 affected tyrosine phosphorylation status of cellular proteins in Hep3B cells. Subconfluent Hep3B cells were treated with Cpd 5 at various times. There was a dramatic increase in protein tyrosine phosphorylation within a few minutes (Fig. 2A). The peak of the induction varied slightly in different experiments, in the range of 1 to 10 min. Cpd 5 appeared to be a specific inducer of tyrosine phosphorylation, since no significant induction was found for serine phosphorylation (Fig. 2B) or threonine phosphorylation (Fig. 2C). Anti-actin antibody was used as a control for protein loading (Fig. 2A, bottom). The induction of protein tyrosine phosphorylation was dose-dependent (Fig. 3), and broadly paralleled the doses that were found to be growth inhibitory (Fig. 1).

Thiols Antagonize Growth Inhibition and Induction of Protein Tyrosine Phosphorylation by Cpd 5—The effects of various thiols and non-thiol antioxidants on Cpd 5-induced growth inhibition and protein tyrosine phosphorylation were then examined. When Hep3B cells were treated with Cpd 5 (20 μM) in the presence of 2 mM of L-cysteine (Cys), GSH, and N-acetyl-L-cysteine, the growth inhibitory effect by Cpd 5 was completely abrogated (Fig. 4A). In contrast, non-thiol antioxidants, such as catalase, deferoxamine mesylate, butylated hydroxyanisole, and superoxide dismutase, had no antagonistic effects on the growth inhibition (Fig. 4A). Protein tyrosine phosphorylation was examined after pretreatment with above antioxidants for 5 min, followed by addition of Cpd 5 (20 μM). The induction of protein tyrosine phosphorylation by Cpd 5 was also completely abrogated by exogenous thiols, but not by any of non-thiol antioxidants (Fig. 4B), suggesting sulhydryl arylation is related to both growth inhibition and increased protein tyrosine phosphorylation in Hep3B cells.

Protein-tyrosine Kinase Inhibitors Do Not Antagonize Cpd 5-induced Tyrosine Phosphorylation—Since protein tyrosine phosphorylation is considered to be regulated by the balance of PTases and protein-tyrosine kinases (PTKs), either activation of PTKs or inhibition of PTases could result in net increase in tyrosine phosphorylation of cellular proteins. To test whether the activation of PTK is involved in Cpd 5-induced protein tyrosine phosphorylation, Hep3B cells were treated with genistein or tyrphostin, potent inhibitors of PTKs (23, 24), prior to addition of Cpd 5. Since epidermal growth factor (EGF) is known to activate PTKs, including EGF receptor kinase, EGF was added to parallel cultures. As shown in Fig. 5, the induction of protein tyrosine phosphorylation by Cpd 5 was affected neither by genistein nor tyrphostin 23, while EGF-induced protein tyrosine phosphorylation was completely blocked by these PTK inhibitors. The results suggest that the induction of protein tyrosine phosphorylation by Cpd 5 is not likely due to PTK activation.
approximately 40% by 40 m scans was also inhibited, but the inhibition was partial (approx.). The effects of Cpd 5 on PTPase activity in Hep3B cell significantly antagonized the PTPase inhibition by Cpd 5 (Fig. 6A). The presence of GSH in the reaction demonstrated by anti-phosphotyrosine antibody (A). The same membrane was repeatedly stripped and incubated with anti-actin antibody (loading control), with anti-phosphoserine antibody (B), and with anti-phosphothreonine antibody (C).

Cpd 5 Inhibits PTPase Activity—We previously proposed that PTPases containing a critical cysteine residue in their active site might be the targets of Cpd 5 (16). Therefore the ability of Cpd 5 to inactivate purified PTPase (T cell PTPase) was examined. It almost completely inhibited the PTPase activity at 40 μM (Fig. 6A). The presence of GSH in the reaction significantly antagonized the PTPase inhibition by Cpd 5 (Fig. 5A). The effects of Cpd 5 on PTPase activity in Hep3B cell lysates were subsequently tested. PTPase activity in cell lysates was also inhibited, but the inhibition was partial (approximately 40%) by 40 μM Cpd 5, suggesting that some PTPases in cell lysates may be resistant to the action of exogenously added Cpd 5, or the presence of protective substances in cell lysates (Fig. 6B). Preincubation of the cell lysates with GSH also effectively antagonized the Cpd 5-induced PTPase inhibition (Fig. 6B).

Orthovanadate Induces Protein Tyrosine Phosphorylation

In the present study, we investigated possible mechanisms for the growth inhibition by Cpd 5, a thioether vitamin K analog, which was generated by addition of 2-mercaptoethanol to vitamin K₃ (16). Although vitamin K₃ is known to inhibit growth of many cell types (2–6, 14), the growth inhibitory activity of Cpd 5 has been found to surpass that of vitamin K₃ (15). We previously suggested that Cpd 5 and other thioether analogs efficiently arylate cellular thiols by an addition-elimination mechanism (16) and provided evidence that the sulhydryl arylation mediates growth inhibitory effects (15). We also proposed that PTPases, which contain a critical cysteine in their active site (18, 19), might be likely targets for the sulhydryl arylation (16). In the experiments reported here, we found that Cpd 5 induced protein tyrosine phosphorylation in Hep3B cells and indeed inhibited PTPase activity. Furthermore, we found that exogenous thiols completely blocked these effects of Cpd 5. The results support our hypothesis.

The regulation of tyrosine phosphorylation in cellular proteins is implicated in many biological processes, including cell growth, cell differentiation, and cell cycle control (20, 21). Thus, it is conceivable that Cpd 5-induced rapid and potent tyrosine phosphorylation of many proteins in Hep3B cells may be responsible for its potent growth inhibitory effect. The close relationship between these two processes is supported by the fact that exogenous thiols that abrogated growth inhibition by Cpd 5 also inhibited the induction of protein tyrosine phosphorylation by Cpd 5. Although the ligand binding-mediated tyrosine phosphorylation of substrate proteins is thought to activate signal transduction by growth factor receptors with tyrosine kinase activity, such as EGF, platelet-derived growth factor, insulin, and insulin-like growth factor-1 receptors (27), the functional outcomes of tyrosine phosphorylation may vary among proteins (28, 29). Furthermore, it has been suggested

FIG. 2. Effects of Cpd 5 on protein tyrosine, serine, and threonine phosphorylation in Hep3B cells. Cells were exposed to 20 μM Cpd 5 for the indicated times. The cells were then lysed and cellular proteins (40 μg/lane) were resolved by SDS-PAGE under reducing conditions. The fractionated proteins were transferred to a polyvinylidene difluoride membrane and tyrosine-phosphorylated proteins were demonstrated by anti-phosphotyrosine antibody (A). The same membrane was repeatedly stripped and incubated with anti-actin antibody (loading control), with anti-phosphoserine antibody (B), and with anti-phosphothreonine antibody (C).

Fig. 3. Effects of Cpd 5 on protein tyrosine phosphorylation in Hep3B cells (dose response). Cells were plated in 6-well plates at 5 × 10⁴ cells/well and cultured for 24 h. Then, the cells were exposed to Cpd 5 at various concentrations for 3 min. The cells were then lysed and subjected to phosphotyrosine immunoblotting after SDS-PAGE as described under “Experimental Procedures.”

and Inhibits Cell Growth in Hep3B Cells—The results described above suggested that inhibition of PTPase and increase in protein tyrosine phosphorylation may play a role in Cpd 5-induced cell growth inhibition. Thus, the effects of orthovanadate, a well studied PTPase inhibitor (25, 26), on cell growth and protein tyrosine phosphorylation were investigated in Hep3B cells. First, Hep3B cells were cultured with orthovanadate at various concentrations as in the experiment shown in Fig. 1. Orthovanadate was also found to be a potent growth inhibitor (Fig. 7). It showed a significant effect on cell growth at 2.5 μM and completely inhibited cell growth at 5 μM. Orthovanadate also induced protein tyrosine phosphorylation in a dose-dependent fashion (Fig. 8), consistent with its known effect as a PTPase inhibitor.

DISCUSSION

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that duration (30) and extent (31) of protein tyrosine phosphorylation is also a determinant of consequences of receptor tyrosine kinase signaling. Interestingly, increased protein tyrosine phosphorylation has also been reported to be associated with growth inhibition and apoptosis (31–35).

Given that phosphorylation status of tyrosine residues in cellular proteins is jointly governed by PTPases and PTKs (20, 29), either activation of PTK or inhibition of PTPases could cause increased protein tyrosine phosphorylation. If PTK activation is the main cause, the induction of tyrosine phosphorylation should be inhibited by PTK inhibitors, such as genistein and tyrphostin 23 (23, 24, 35, 36). In fact, in our study, EGF-induced protein tyrosine phosphorylation in Hep3B cells was completely prevented by the pretreatment with genistein or tyrphostin 23. A similar approach has been used to elucidate the role of PTK activation in the increased protein tyrosine phosphorylation induced by other reagents (32, 33, 37). However, our results showed that Cpd 5-induced protein tyrosine phosphorylation was not affected either by genistein or tyrphostin 23, suggesting that PTK activation is not the main mechanism.

We demonstrated that Cpd 5 inhibited PTPase activity at micromolar concentrations. It completely inhibited the activity of T cell PTPase, a widely expressed intracellular PTPase originally cloned from a human peripheral T cell cDNA library (38). In contrast to T cell PTPase, PTPase activity in crude Hep3B cell lysates was only partially inhibited by Cpd 5 even at high concentrations. Similar results were obtained with orthovanadate. Although the reasons for the partial inhibition are not clear, it suggests that the cell lysates may contain inhibitors of the Cpd 5-PTPase interactions, that some PTPases are more resistant than others to inhibition by Cpd 5, or that some cellular PTPases are not accessible to the action of Cpd 5, possibly due to cellular compartmentation. The latter two possibilities might be more likely, since orthovanadate has been reported to inhibit cytosolic PTPase activity 10 times more effectively than particulate fraction PTPase activity purified from a rat hepatocellular carcinoma cell line (39). Importantly, we also found significant antagonistic effects of GSH on the PTPase inhibition by Cpd 5, implying that sulfhydryl reactions were involved in the inhibition of enzyme activity. Taken together, our results strongly suggest that inhibition of PTPases by Cpd 5 and subsequent perturbation of protein tyrosine phosphorylation are involved in its potent growth inhibition.

PTPases are tightly regulated in many cellular functions, including growth factor and cytokine signaling pathways (40–42). The tyrosine phosphatase superfamily represents a large family of enzymes, including tyrosine-specific phosphatases, VH1 ( vaccinia virus late H1 gene)-like dual specificity, Cdc25, and low molecular weight phosphatases (19). While tyrosine-specific phosphatases, intracellular (PTP1B, PTP1D, TC-PTPase, and so forth) and receptor-like (PTPα, PTPβ, CD45, and so forth), and low molecular weight phosphatases react with phosphotyrosine alone, VH1-like dual specificity phosphatases (VH1, VHR, PAC-1, MKP1, and so forth) and Cdc25 (Cdc25A, Cdc25B, and Cdc25C) hydrolyze both phosphotyrosine and phosphothreonine (phosphoserine) residues (18–20). All of these phosphatases share a motif consisting of a cysteine and an arginine, separated by five residues in their active site (18, 19). The cysteine functions as an essential nucleophile to accept the PO3 moiety and the mutation of this residue results in an almost total loss of the enzyme activity (43, 44). Since quinone sulfides, such as Cpd 5, can effectively arylate thiols by...
an addition-elimination mechanism, Cpd 5 may form a covalent bond with the critical cysteine in the active site of PTPases, thereby inhibiting their activity (16). Vitamin K₃ can also arylate cellular thiols by a simple addition mechanism (7–9). In fact, vitamin K₃ has been shown to inhibit the activity of Cdc25 (45, 46), consistent with the vitamin K₃-induced hyperphosphorylated state of p34CDC2 that is dephosphorylated by Cdc25 (17).

Vitamin K₃ also inhibits T cell PTPase activity, but the effect is less potent than Cpd 5. Interestingly, a benzoquinoid compound with likely arylation capacity (dephostatin) has been reported to be a potent PTPase inhibitor (47). In addition, halomethyl derivatives of vitamin K₃, being more sulfhydryl-reactive than original vitamin K₃, have been reported to inhibit thiol-containing NADPH-cytochrome c reductase (48). Sulfhydryl arylation has also been proposed for the mechanism of inhibition of pp60src PTK by quinone-containing herbimycin A (49) and a polyketide (50). It will be intriguing to know whether Cpd 5 may have inhibitory effects on these enzymes.

Orthovanadate is a general PTPase inhibitor (25, 26), although it has been reported to exert other diverse effects, including Na⁺,K⁺-ATPase inhibition and direct stimulation of PTKs (51, 52). As a phosphate analog, orthovanadate behaves as a reversible, competitive inhibitor of PTPases (53). Crystal structures of PTPases complexed with orthovanadate have revealed that vanadate ion forms a covalent linkage with the cysteine in the active site (54, 55). In the present study, we demonstrated that orthovanadate had similar, but slightly more potent cell growth inhibitory effects than Cpd 5, as well as induction of protein tyrosine phosphorylation and PTPase inhibition. Phenylarsine oxide, a PTPase inhibitor, has also been reported to induce cell death (apoptosis) and protein tyrosine phosphorylation in a murine T cell hybridoma (56) and in human leukocytes (31). Recently, another PTPase inhibitor, RK-682, has been demonstrated to inhibit cell growth and to induce protein tyrosine phosphorylation in a human B cell leukemia cell line (34). These data support the close relation-

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ship between PTPase inhibition, induction of protein tyrosine phosphorylation, and cell growth inhibition or apoptosis.

Commonly used cancer cell growth inhibitory drugs, such as doxorubicin, cis-platinum, and vinblastine, do not have PTPase inhibitory activity (57). Thus, Cpd 5, as a unique PTPase inhibitor and a potent cell growth inhibitor, may represent a novel class of cancer cell growth inhibitors. Our preliminary experiments have demonstrated that subcutaneous injection of Cpd 5 in rats almost completely inhibits liver DNA synthesis after a partial hepatectomy without causing significant systemic toxicity (58), indicating the effectiveness of the compound in vivo. It is encouraging that vanadate has been found to reverse multidrug resistance (58), indicating the effectiveness of the compound.

Cpd 5 in rats almost completely inhibits liver DNA synthesis ex vivo. It is encouraging that vanadate has been found to reverse multidrug resistance (58), indicating the effectiveness of the compound.

In summary, the present study demonstrated that the growth inhibitory effect of Cpd 5 is closely associated with induction of protein tyrosine phosphorylation, which may be due to the inhibition of PTPases by Cpd 5 through arylating the cysteine in the active site of the enzymes. Studies designed to elucidate the signaling pathways relevant to growth inhibition are under way in our laboratory.

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