Inhibition of Hepatoma Cell Growth in Vitro by Arylating and Non-arylating K Vitamin Analogs

SIGNIFICANCE OF PROTEIN TYROSINE PHOSPHATASE INHIBITION* 

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We recently found that a thioether analog of K vitamin (Cpd 5) inhibited the activity of protein-tyrosine phosphatases (PTPases) and induced protein-tyrosine phosphorylation in a human hepatoma cell line (Hep3B). We have now examined the structural requirements for induction of protein-tyrosine phosphorylation and PTPase inhibition by several K vitamin analogs. Thioether analogs with sulfhydryl arylation capacity, especially those with a hydroxy (Cpd 5) or a methoxy group at the end of the side chain, induced protein-tyrosine phosphorylation, but non-arylating analogs, such as those with an all-carbon or O-ether side chain, did not. Among the receptor-tyrosine kinases, epidermal growth factor receptor analogs were tyrosine-phosphorylated by treatment with thioether analogs, whereas insulin and hepatocyte growth factor receptor analogs were not. An increase in tyrosine-phosphorylated ERK2 mitogen-activated protein kinase was also observed. The activity of purified T cell PTPase was inhibited only by the thioether analogs, but not by non-arylating analogs. Furthermore, the epidermal growth factor receptor dephosphorylation activity of Hep3B cell lysates was inhibited by Cpd 5 treatment. A similar induction of protein-tyrosine phosphorylation by Cpd 5 was seen in other human hepatoma cell lines together with growth inhibition. However, one cell line (HepG2), which was relatively resistant to growth inhibition by Cpd 5, did not increase its phosphorylation levels upon Cpd 5 treatment. These results suggest that cell growth inhibition by thioether analogs is closely associated with inhibition of PTPases by sulfhydryl arylation and with tyrosine phosphorylation of selected proteins.

The synthetic vitamin K₃ (2-methyl-1,4-naphthoquinone or menadione) has been reported to inhibit growth of many cell types both in vitro and in vivo (1–5). The mechanisms of this growth inhibition have been ascribed to both oxidative stress due to its redox cycling activity (6–8) and arylation of cellular thiols at position 3 of the naphthoquinone nucleus (9–12). To further investigate the mechanisms and to generate more potent growth inhibitory compounds, we previously synthesized several K vitamin analogs with a thioether, O-ether, or all-carbon side chain at position 3 of the naphthoquinone nucleus of vitamin K₃ and found that thioether analogs, particularly a thioethanol analog (Cpd₁ ₅), are potent growth inhibitors for Hep3B cells derived from a human hepatocellular carcinoma (13, 14). Cell growth inhibition by the thioether analogs in vitro was almost completely antagonized by exogenous thiols, but not by any non-thiol antioxidants tested, suggesting the importance of sulfhydryl arylation rather than oxidative stress in mediating the growth inhibition (14).

Because the active site of protein-tyrosine phosphatases (PTPases) contains a cysteine residue, which is essential in the catalytic process of tyrosine dephosphorylation (15, 16), we have proposed that Cpd 5 might arylate the critical cysteine and inactivate PTPases (13), perturbing protein-tyrosine phosphorylation, which is known to play a crucial role in many cellular processes (17, 18). We recently found that Cpd 5 indeed inhibited the activity of purified T cell PTPase and induced protein-tyrosine phosphorylation in Hep3B cells (19), as well as in cultured rat hepatocytes (20). It was thought to be unlikely that Cpd 5 induced protein-tyrosine phosphorylation by activation of tyrosine kinases, because the increased protein-tyrosine phosphorylation was not affected by treatment with tyrphostin 23 or genistein, well described tyrosine kinase inhibitors (19).

In this study, we have examined the structural requirements for induction of protein-tyrosine phosphorylation and inhibition of PTPase using several vitamin K analogs (Fig. 1) and have compared the patterns of protein-tyrosine phosphorylation induced by vitamin K analogs with those of a well studied PTPase inhibitor, sodium orthovanadate. Our results demonstrate a close relationship between growth inhibition and induction of tyrosine phosphorylation of selected proteins in human hepatoma cell lines, as well as with inhibition of PTPase activity in vitro by the thioether analogs. Thus, cell growth inhibition by thioether analogs is closely associated with protein-tyrosine phosphorylation.

MATERIALS AND METHODS

Synthesis of Vitamin K Analogs—Cpds 1, 2, 3, and 5 were synthesized as described previously (13). Cpd 34 (2-(2-ethanolamine)-3-methyl-1,4-naphthoquinone) was generated by addition of ethanolamine to menadione in ethanol. Cpd 27 (2-hydroxy-3-methyl-1,4-naphthoquinone, phthioeol) was generated from vitamin K₃ by the reaction with Na₂CO₃ and hydrogen peroxide. Cpd 38 (2-(2-mercaptoethanol-O-ethylthio)-5-methyl-1,4-naphthoquinone) was generated from the mercaptoether methylether and menadione oxide (21) by addition of tributyolphosphate to a solution of menadione oxide and 2-methoxyethyl

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The abbreviations used are: Cpd or Cpds, compound or compounds; PTPase, protein-tyrosine phosphatase; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween 20; EGF, epidermal growth factor; EGFR, EGF receptor; HGF, hepatocyte growth factor; PLC-γ, phospholipase-Cγ.
disulfide in methylene chloride. Purification was achieved by gel chromatography. The structures are shown in Fig. 1.

**Cell Culture and Cell Growth Inhibition Assay**—Human hepatoma cell lines (HepG2, Hep3B, Huh7, Hep40, PLC/PRF/5, and HepG2) were maintained in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum. For the growth inhibition assay, the cells were plated at approximately 2 × 10^4 cells/well in 24-well plates. 24 h after plating, the medium was replaced with a medium containing test compounds. Two days after treatment, the medium was removed and the plates were stored at −80 °C until the day of assay. Cell number was estimated by a DNA fluorometric assay using the fluorochrome Hoechst 33258 (22).

**Western Blotting and Immunoprecipitation**—Confluent cells on 12-well plates were treated with various compounds or cytokines. The cells were then washed with phosphate-buffered saline and lysed with 50 μl RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM sodium chloride in 10 mM Tris-HCl buffer (pH 7.5)) containing protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, antipain, benzamide hydrochloride, and aprozin). Aliquots of samples were used for measurement of protein concentration using the Bio-Rad protein assay. Treated cell lysates (20 or 40 μg of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels. For two-dimensional electrophoresis, cells were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels. For two-dimensional electrophoresis, cells were lysed with urea solubilization buffer and subjected to isoelectric focusing before SDS-PAGE. For immunoprecipitation, cell lysates (300 μg containing 125 or 250 μg protein) were incubated with antibodies (250 or 500 ng) for 1 h on ice and immunoprecipitated with 15 μl of protein A/G plus agarose slurry. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. After blocking the membrane with Tris-buffered saline with Tween 20 (TBST; 150 mM NaCl and 0.05% Tween 20 in 10 mM Tris-HCl buffer (pH 8.0)) containing 1% bovine serum albumin (for the detection of tyrosine-phosphorylated proteins) or 5% skim milk (for the detection of other proteins), the membrane was incubated with various primary antibodies, then washed with TBST, and incubated with anti-mouse or rabbit IgG coupled to horseradish peroxidase (1:5,000; Amersham Pharmacia Biotech). Primary antibodies used were anti-phosphotyrosine antibody (1:200, Oncogene Research Products, Calbiochem), an anti-epidermal growth factor (EGF) receptor antibody (1:200, Santa Cruz), anti-insulin receptor antibody (1:200, Santa Cruz), anti-hepatecto grow factor (HGF) receptor (c-Met) antibody (1:200, Santa Cruz), anti-ERK2 antibody (1:5,000; Transduction Laboratories), and anti-phospholipase C-γ (PLC-γ) antibody (1:200, Santa Cruz). Detection was performed with enhanced chemiluminescence reagents (NEN Life Science Products). Membranes were treated with stripping buffer (100 mM 2-mercaptoethanol, 5% SDS in 62.5 mM Tris-HCl buffer (pH 6.8)) at 50 °C for 30 min before sequential reprobing with different antibodies.

**Assay of PTPase Activity**—PTPase activities were measured using a PTPase assay kit from New England Biolabs, Inc. Myelin basic protein, a substrate for PTPase, was phosphorylated on multiple tyrosine residues with Abl protein-tyrosine kinase in the presence of [γ-32P]ATP and then dialyzed overnight to remove residual ATP. The assay was done in 25-μl reactions. Prior to adding the substrate, purified T cell PTPase (5 ng/reaction) was preincubated with compounds at 30 °C for 30 min. They were then incubated with γ-32P-labeled myelin basic protein (1.5 μg/reaction) at 30 °C for 20 min. The reaction was stopped by addition of 200 μl of 20% trichloroacetic acid. The samples were then centrifuged, and the radioactivity in the supernatants was counted using a scintillation counter.

**EGF Receptor (EGFR) Dephosphorylation Assay**—Activated (tyrosine-phosphorylated) EGFR was prepared by incubating Hep3B cells with 1 mM sodium orthovanadate 30 min before the addition of 20 ng/ml EGF for 5 min. Whole cell lysates were immunoprecipitated with anti-EGFR antibody, and the EGFR immunoprecipitates were used as substrates for EGFR dephosphorylation assays. Hep3B cell lysates were pre-cleared of EGFR by incubation with anti-EGFR antibody. Equal amounts of EGFR immunoprecipitates were incubated with pre-cleared Hep3B cell lysates, treated with or without Cpd 5 for 60 min, in 1× phosphatase buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM dithiothreitol) for 15 min at 30 °C. The reaction was terminated by the addition of an equal volume of 2× sample buffer, and separated by SDS-PAGE. The dephosphorylation of EGFR was examined by Western blotting using the anti-phosphotyrosine antibody.

**RESULTS**

**Thioether Analogs**—Are More Potent Cell Growth Inhibitors Than Non-arylaters—The cell growth inhibitory activity of various K vitamin analogs (Fig. 1) was evaluated (Fig. 2). Hep3B cells were treated with various concentrations of analogs for 48 h, and cell numbers were estimated by the DNA fluorometric assay. Thioether analogs showed strong growth inhibitory activity, with 50% inhibitory doses (ID50) less than 20 μM (Fig. 2A). Analogs with a hydroxy group (Cpd 5) or methoxy group (Cpd 38) at the end of the side chain were more potent growth inhibitors than those without (Cpds 2 and 3). As compared with thioether analogs, the analogs with an all-carbon side chain (Cpd 1), O-ether side chain (Cpd 6), hydroxy group (Cpd 27), ethanol amine adduct (Cpd 34), were less potent in cell growth inhibition, with ID50 22.4 μM to >80 μM (Fig. 2B). To test possible cytotoxicity of the compounds, we performed a lactate dehydrogenase assay on supernatants of Cpd 5-treated Hep3B cells and found that lactate dehydrogenase release was minimum, indicating that cell growth inhibition was not due only to cytotoxic effects of the compounds (data not shown).

**Thioether Analogs Induce Tyrosine Phosphorylation of Cellular Proteins**—We reported that a thioether derivative (Cpd 5) induced protein-tyrosine phosphorylation in Hep3B cells (19). To examine the significance of sulfhydryl arylation in the induction of protein-tyrosine phosphorylation, we treated Hep3B cells with various analogs and analyzed tyrosine-phosphorylated proteins by Western blotting (Fig. 3). In control (nontreated) cells, there were several tyrosine-phosphorylated proteins, among which a group of ~100 kDa proteins were most prominent (Fig. 3A). However, treatment with thioether analogs (Cpd 2, 3, 5, and 38) induced tyrosine phosphorylation of many proteins, especially, approximately 170-, 75-, and 55-kDa proteins (Fig. 3A). Thioether analogs with a hydroxy (Cpd 5) or methoxy (Cpd 38) group at the end of the side chain had the most potent action on protein-tyrosine phosphorylation, whereas those without such modifications (Cpds 2 and 3) were much less potent (Fig. 3A). Although Cpd 3, which has a longer side chain than Cpd 2, did not induce protein-tyrosine phosphorylation at 50 μM for 30 min (Fig. 3A), it did induce protein-tyrosine phosphorylation at more than 100 μM (Fig. 3B). In contrast to thioether analogs, Cpd 1, 6, 27, and 34 did not induce protein-tyrosine phosphorylation (Fig. 3A), even when longer treatments at higher concentrations (Fig. 3B), suggesting that sulfhydryl arylation is crucial in inducing protein-tyrosine phosphorylation.

**Comparison of Tyrosine Phosphorylation Induced by Cpd 5, EGF, and Sodium Orthovanadate**—We compared protein-ty-
rosine phosphorylation induced by the thioether analog, Cpd 5, with that induced by EGF, which is known to induce autophosphorylation of EGFR (170 kDa), as well as with that induced by sodium orthovanadate, which is known to increase tyrosine phosphorylation of cellular proteins by inhibition of PTPases (Fig. 4A). EGF induced tyrosine phosphorylation of several proteins, including 170-, 75-, and 55-kDa proteins, which were also hyperphosphorylated following treatment with thioether analogs, such as Cpd 5 (Fig. 4A). Sodium orthovanadate strongly induced tyrosine phosphorylation of many proteins (Fig. 4A). We then compared the time course of protein-tyrosine phosphorylation induced by treatment with Cpd 5, EGF, and sodium orthovanadate by a densitometric analysis of the 170-kDa band on phosphotyrosine Western blots (Fig. 4B). Cpd 5-induced protein-tyrosine phosphorylation started within 10 min, peaked after 120 min, and then decreased to baseline by 360 min. By contrast, EGF-induced protein-tyrosine phosphorylation peaked after 1 min and then rapidly decreased. Sodium orthovanadate induced protein-tyrosine phosphorylation steadily over a period of hours (Fig. 4B).

To examine the profile of tyrosine-phosphorylated proteins induced by Cpd 5 and sodium orthovanadate, we performed phosphotyrosine Western blot analysis on two-dimensional electrophoresis gels (Fig. 5). Although several proteins, including the 170-kDa protein, were phosphorylated by both Cpd 5 and sodium orthovanadate, there were striking differences in the intensity and pattern of protein-tyrosine phosphorylation induced by these agents (Fig. 5).

Selectivity of Protein-tyrosine Phosphorylation Induced by Cpd 5—To examine whether the 170-kDa protein is EGFR, we performed EGFR Western blot analysis using the same blot used in Fig. 5 after stripping of the antibodies. The protein spots recognized by anti-EGFR antibody exactly corresponded to the tyrosine-phosphorylated spots in the phosphotyrosine Western blot in Cpd 5-treated cells (Fig. 6A, upper panels). In contrast, tyrosine-phosphorylated EGFR was not present in nontreated control cells (Fig. 6A, upper panels). This tyrosine phosphorylation of EGFR was confirmed by EGFR immunoprecipitation and subsequent probing with phosphotyrosine or EGFR antibodies (Fig. 6B, top panel). Sodium orthovanadate also induced EGFR phosphorylation, but Cpd 1 (all-carbon), a non-arylator, did not (Fig. 6B).

To examine tyrosine phosphorylation of other receptor pro-
tein-tyrosine kinases, we performed immunoprecipitation analysis of insulin receptors and HGF receptors (c-Met). Neither Cpd 5 nor Cpd 1 affected the tyrosine-phosphorylation status of these receptors, but sodium orthovanadate induced tyrosine phosphorylation of both, although the effect on insulin receptors was slight (Fig. 6B, middle and lower panels).

We then examined the effects of Cpd 5 on tyrosine phosphorylation of ERK2 (mitogen-activated protein kinase) which is phosphorylated on both tyrosine and threonine residues when activated by mitogen-activated protein kinase kinase (MEK), and has been shown to have a key role in EGF signal transduction. The two-dimensional gel blots used in Figs. 5 and 6A were again probed with anti-ERK2 antibody (Fig. 7A) on phosphotyrosine Western blots is shown.

Fig. 4. Effects of a thioether K vitamin analog (Cpd 5), EGF, and sodium orthovanadate (OV) on protein-tyrosine phosphorylation in Hep3B cells. A, induction of protein-tyrosine phosphorylation by Cpd 5, EGF, and sodium orthovanadate. Confluent cells grown on 12-well plates were exposed to 50 μM Cpd 5 for 1 h, 10 ng/ml EGF for 5 min, or 50 μM orthovanadate (OV) for 1 h. 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. B, time courses of induction of protein-tyrosine phosphorylation of a 170-kDa band. Confluent cells were treated with Cpd 5 (50 μM), EGF (10 μg/ml), and sodium orthovanadate (OV) (50 μM). A densitometric analysis of the 170-kDa band (identified as EGF receptor proteins in Fig. 6A) on phosphotyrosine Western blots is shown.

Fig. 5. Two-dimensional analysis of tyrosine-phosphorylated proteins induced by Cpd 5 and sodium orthovanadate in Hep3B cells. Confluent cells were treated with 50 μM of Cpd 5 or sodium orthovanadate for 1 h. The cells then were lysed and subjected to phosphotyrosine Western blotting after two-dimensional electrophoresis as described under “Materials and Methods”. Panel A, control; panel B, Cpd 5; panel C, sodium orthovanadate. Triangles in panel B indicate the tyrosine-phosphorylated EGF receptor proteins identified in Fig. 6A. Arrows in panels A and B indicate tyrosine-phosphorylated ERK2 proteins identified in Fig. 7A.
Confluent cells were treated with 50 μM Cpd 5 for 1 h. Parts of the phosphotyrosine Western blots shown in Fig. 5A are enlarged here. The EGFR Western blots were done on the same blots after stripping procedures. Arrows show tyrosine-phosphorylated EGFR proteins in Cpd 5-treated cells. B, immunoprecipitation analyses of tyrosine phosphorylation of several receptor tyrosine kinases. Confluent Hep3B cells were treated with 50 μM Cpd 1, Cpd 5, or sodium orthovanadate (OV) for 30 min. Cells were also treated with receptor ligands (EGF, 10 ng/ml; insulin, 10^{-4} M; HGF, 10 ng/ml) for 10 min. After treatment, cells were lysed, and protein samples were subjected to immunoprecipitation (IP) with anti-PLC-γ or anti-HGF receptor (c-Met) antibodies, followed by Western blot analyses (WB) of phosphotyrosine and each receptor protein.

Well as Cpd 5-treated cells, PLC-γ was barely phosphorylated, whereas it was strongly tyrosine-phosphorylated by sodium orthovanadate treatment (Fig. 7B).

**Thioether Analogs Inhibit the Activity of Purified PT-Pase**—We previously reported that Cpd 5 inhibited the activity of purified T cell PT-Pase (19). To examine the significance of thiol arylation in the inhibition of PT-Pase activity, we compared the effects of arylation and non-arylation on the inhibition of T cell PT-Pase in vitro. All the thioether analogs inhibited PT-Pase activity in a dose-dependent manner (Fig. 8A). Cpd 5 and Cpd 3B, which were potent growth inhibitors and tyrosine phosphorylation inducers, were also particularly potent inhibitors of PT-Pase activity. In contrast, non-arylation analogs did not inhibit PT-Pase activity (Fig. 8B), just as they did not induce protein-tyrosine phosphorylation (Fig. 3, A and B).

**Cpd 5 Inhibits EGFR Dephosphorylation**—Because it has been shown that Cpd 5 inhibits the activity of PT-Pases, we considered the possibility that EGFR phosphorylation by Cpd 5 was caused by PT-Pase inhibition. To examine this hypothesis, we measured the dephosphorylation of EGFR by Cpd 5. Activated EGFR was prepared from Hep3B cells treated with 20 ng/ml EGF for 5 min, and whole cell lysates were immunoprecipitated with anti-EGFR antibody. Separately, other Hep3B cell lysates were immunoprecipitated with EGFR antibody to remove the endogenous EGFR and treated with Cpd 5 in doses from 0 to 80 μM for 1 h. When activated EGFR was incubated with Hep3B cell lysates, which were precleared with anti-EGFR antibody, we found that EGFR was completely dephosphorylated by untreated cell lysates, and this dephosphorylation was inhibited by lysates that had been treated with Cpd 5 in a dose-dependent manner (Fig. 9).

**Effects of Cpd 5 on Cell Growth and Protein-tyrosine Phosphorylation in Various Human Hepatoma Cell Lines**—To study how the effects of Cpd 5 on cell growth inhibition and protein-tyrosine phosphorylation are cell-type-dependent, we also examined other human hepatoma cell lines, Huh7, Hep40, PLC/PRF/5, and HepG2 cells. Although Cpd 5 exerted growth inhibitory effects for these cell lines, HepG2 cells were more resistant to Cpd 5 than other cell lines (Fig. 10A). There were significant (p < 0.01) differences of ID_{50} between HepG2 and other cell lines by analysis of variance. Cpd 5 (50 μM) induced tyrosine phosphorylation of several proteins in Huh7, Hep40, and PLC/PRF/5 cells, but the effect was not seen in HepG2 cells (Fig. 10B). Cpd 5 did not induce protein-tyrosine phosphorylation in HepG2 cells even at 100 μM (data not shown).

**DISCUSSION**

We have previously shown that thioether analogs of vitamin K were potent growth inhibitors for Hep3B cells (13, 14), and the most potent growth inhibitory analog, a thioethanol derivative, named Cpd 5, induced protein-tyrosine phosphorylation, probably due to the inhibition of PT-Pases by sulfhydryl arylation (19). To further investigate the significance of sulfhydryl arylation in the induction of protein-tyrosine phosphorylation and PT-Pase inhibition by K vitamin analogs, in this study, we have compared thioether analogs (arylators) with several analogs that do not have the site for sulfhydryl arylation (non-arylators) and found that only arylation induce protein-tyrosine phosphorylation in Hep3B cells and inhibit the activity of purified PT-Pase.

Our data suggest that sulfhydryl interaction of the thioether
various concentrations, and then PTPase activity was assayed using cell PTPase. Purified T cell PTPase was preincubated with analogs at activity. Sodium orthovanadate acts as a competitive inhibitor of T cell PTPase (24, 25), has been shown to interact with the cysteine residue within the active site pocket. Addition of a methoxy group at the end of the side chain generated an analog, Cpd 5, that has a hydroxy group at the end of the side chain. Because both hydroxy and methoxy groups are known to act as hydrogen bond acceptors, they may interact with hydrogen bonds present among the amino acid residues in the active site. This suggests that the interaction of inhibitors with non-cysteine residues is also important. Interestingly, a structure designed to bind to both active site and non-catalytic site of a PTPase has been demonstrated to be a very effective inhibitor (30).

EGFR was strongly tyrosine-phosphorylated by arylating K vitamin analogs. After ligand binding, EGFR molecules are dimerized, autophosphorylate their tyrosine residues, and are thereby activated for subsequent signal transduction (18). The effects of EGFR phosphorylation has also been shown to occur by inhibition of dephosphorylation by radiation, oxidants, and alkylating agents (34). Other examples of the association of protein-tyrosine kinases and PTPases have also been demonstrated, such as the interactions between insulin receptor and PTP1B (35), c-Src and PTP1C (36), and JAK tyrosine kinases and SHP-2 (37).

The two-dimensional gel analysis showed differences in the profiles of tyrosine-phosphorylated proteins between Cpd 5- and orthovanadate-treated cells. Cpd 5 induced tyrosine phosphorylation of EGFR, but not insulin receptor or HGF receptor (c-Met), whereas sodium orthovanadate induced phosphorylation of all of these receptor tyrosine kinases. Furthermore, PLC-γ was phosphorylated by treatment with sodium orthovanadate, but not with Cpd 5. These results suggest that Cpd 5 is more selective in phosphorylation of cellular proteins than sodium orthovanadate. Peroxovanadate has also been

analogs with the critical cysteine residue of PTPases (15, 16) caused inhibition of the enzyme activity and resulted in an increased level of protein-tyrosine phosphorylation. An addition-elimination mechanism has been proposed for the interaction of the thioether analogs with thiols (13). The substitution of the sulfur atom of Cpd 5 with a nitrogen atom (Cpd 34) resulted in a loss of effects on protein-tyrosine phosphorylation and PTPase inhibition, indicating the importance of the sulfur atom for the interaction with thiols (Figs. 1 and 3). Sodium orthovanadate, the prototype PTPase inhibitor (24, 25), has also been shown to interact with the cysteine residue within covalent bond distance (26). Peroxovanadate (a mixture of orthovanadate and hydrogen peroxide) oxidizes the cysteine by forming cysteic acid (26), whereas alendronate forms sulfonic acid with the cysteine (27), and aromatic disulfides (28) and nitric oxide (29) form disulfides and inactivate the enzyme activity. Sodium orthovanadate acts as a competitive inhibitor for PTP1B, whereas peroxovanadate irreversibly inhibits the PTPase activity (26).

Several chemical modifications in the side chain of the analogs altered their effects on protein-tyrosine phosphorylation and PTPase inhibition. We found that an increase in the side chain length decreased the effects, suggesting the presence of the size limit in sulphydryl interaction of the side chain and the cysteine residue in the active site pocket. Addition of a methoxy group at the end of the side chain generated an analog, Cpd 38, as potent as Cpd 5, that has a hydroxy group at the end of the side chain instead. Because both hydroxy and methoxy groups are known to act as hydrogen bond acceptors, they may interact with hydrogen bonds present among the amino acid residues in the active site. This suggests that the interaction of inhibitors with non-cysteine residues is also important. Interestingly, a structure designed to bind to both active site and non-catalytic site of a PTPase has been demonstrated to be a very effective inhibitor (30).

EGFR was strongly tyrosine-phosphorylated by arylating K vitamin analogs. After ligand binding, EGFR molecules are dimerized, autophosphorylate their tyrosine residues, and are thereby activated for subsequent signal transduction (18). The status of EGFR phosphorylation is also controlled by PTPase-mediated dephosphorylation of the receptor. EGFR has been shown to associate with and be dephosphorylated by T cell PTPase (31), which was effectively inhibited by arylating analogs in this study. PTP1B is also known to associate with and interact with EGFR (32, 33). Thus, EGFR phosphorylation by arylating analogs might be due to inhibition of PTPases, which regulate the phosphorylation status of EGFR. The results of our EGFR dephosphorylation assay support this hypothesis. EGFR phosphorylation has also been shown to occur by inhibition of dephosphorylation by radiation, oxidants, and alkylating agents (34). Other examples of the association of protein-tyrosine kinases and PTPases have also been demonstrated, such as the interactions between insulin receptor and PTP1B (35), c-Src and PTP1C (36), and JAK tyrosine kinases and SHP-2 (37).

The two-dimensional gel analysis showed differences in the profiles of tyrosine-phosphorylated proteins between Cpd 5- and orthovanadate-treated cells. Cpd 5 induced tyrosine phosphorylation of EGFR, but not insulin receptor or HGF receptor (c-Met), whereas sodium orthovanadate induced phosphorylation of all of these receptor tyrosine kinases. Furthermore, PLC-γ was phosphorylated by treatment with sodium orthovanadate, but not with Cpd 5. These results suggest that Cpd 5 is more selective in phosphorylation of cellular proteins than sodium orthovanadate. Peroxovanadate has also been
shown to induce tyrosine phosphorylation of EGFR, insulin receptor, c-Met, and multiple signaling proteins in mouse liver and kidney in vivo (38). Cpd 5 and other thioether analogs may inhibit a selected class of PTPases. Because these K vitamin analogs have the common basic chemical structure 2-methyl-1,4-naphthoquinone, which is larger than orthovanadate, the interaction with the critical cysteine residue may not be possible for all PTPases, dependent on their active site structures. Recently, relatively modest chemical modifications in modular side chains have been shown to change the substrate specificity of other classes of tyrosine and dual specificity phosphatase inhibitors (39).

Cpd 5 induced tyrosine phosphorylation of ERK2, which is phosphorylated by MEK and has a pivotal role in the signaling pathways of receptor tyrosine kinases including EGFR (40). It is possible that Cpd 5-induced EGFR phosphorylation may activate the EGFR signaling pathways without ligand binding, as shown in the case of peroxovanadate (41). However, Cpd 5 did not affect the phosphorylation status of PLC-γ, which is known to be phosphorylated and thus activated following EGFR activation (23), suggesting that Cpd 5 may activate only a part of the EGFR signaling pathways. Cpd 5-mediated ERK2 phosphorylation could be independent of EGFR activation, because it has been demonstrated that kinase-negative EGFR can mediate ERK2 tyrosine phosphorylation (42, 43) and that ERK2 can be autophosphorylated (44). It is also possible that Cpd 5 inactivates dual specificity phosphatases that dephosphorylate ERK2, such as mitogen-activated protein kinase phosphatases (MKP)-1, MKP-2, MKP-3, and PAC1 (45, 46), thereby inducing tyrosine phosphorylation of ERK2. Peroxovanadate-mediated mitogen-activated protein kinase activation has been shown to be MEK-independent (47).

Arylating K vitamin analogs inhibited cell growth more strongly than non-arylating analogs. Among the arylating analogs, those that had stronger effects on protein-tyrosine phosphorylation and PTPase inhibition were more potent cell growth inhibitors. Furthermore, a hepatoma cell line (HepG2), which was relatively resistant to the growth inhibitory effect of Cpd 5, did not increase its protein-tyrosine phosphorylation levels upon Cpd 5 treatment. These data suggest that cell growth inhibition by the arylating analogs is related to increased protein-tyrosine phosphorylation and PTPase inhibition. However, it is important to note that non-arylating analogs can also inhibit cell growth, although at higher concentrations, indicating the presence of unidentified growth inhibitory mechanisms that are independent of increased protein-tyrosine phosphorylation.

Although protein-tyrosine phosphorylation has been generally supposed to mediate positive cellular events, such as cell signaling associated with growth stimulation, increased levels of protein-tyrosine phosphorylation have also been reported to be associated with cell growth inhibition and cell death in several cell types (48–51). It has been well established that many tumor cell lines stop cell growth in the presence of EGF at high concentrations (52, 53). It is also possible that PTPase inhibition itself might inhibit cell growth, because some PTPases have been demonstrated to act as positive regulators of cell growth (54). Interestingly, arylating K vitamin analogs induced sustained phosphorylation of EGFR, whereas EGF caused very rapid and transient EGFR phosphorylation. This different intensity and duration of phosphorylation of EGFR might be responsible for the growth inhibitory activities. In neuronal PC 12 cells, transient ERK phosphorylation induced by EGF is associated with cell proliferation, whereas sustained ERK phosphorylation induced by nerve growth factor is associated with growth inhibition and cell differentiation (55). We are currently investigating the mechanistic significance of transient or prolonged activation of tyrosine-phosphorylated proteins that seem to correlate with cell growth inhibition.

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