Critical Role of Extracellular Signal-regulated Kinase (ERK) Phosphorylation in Novel Vitamin K Analog-induced Cell Death

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In the present study, we show that 2-(2-hydroxyethylsulfaryl)-3-methyl-1,4-naphthoquinone, or CPD 5, is a potent growth inhibitor for pancreas cancer cell lines (ID50: 21.4±3.8, 31.8±2.7 and 55.2±4.5 µM for MiaPaCa, Panc-1 and BxPc3, respectively). It induced protein tyrosine phosphorylation of hepatocyte growth factor (HGF) receptor (c-Met) or epidermal growth factor receptor (EGFR), which increased progressively to a maximum level at 30 min in Panc-1 cells. The receptor phosphorylation by CPD 5 was indicated to be functional, since these receptors were found to bind with Grb2 or SOS1 protein. CPD 5 was also suggested to induce phosphorylation of external signal-regulated kinase (ERK). EGF induced cell proliferation through ERK phosphorylation, since U0126, which is an inhibitor of ERK phosphorylation, abrogated the increase of cyclin D1 by EGF. HGF increased the amount of p27 protein, suggesting that it is associated with cell differentiation. By contrast, U0126 reduced CPD 5-induced cell death. On two-dimensional electrophoresis, we found an extra type of phospho-ERK, and this was completely and selectively abolished by U0126. These results suggest that ERK phosphorylation, especially the extra spot on two-dimensional gel, is critically associated with CPD 5-mediated cell death.

Key words: Extracellular signal-regulated kinase (ERK) — Growth inhibition — Novel vitamin K analog — Phosphorylation — Pancreas cancer cells

In spite of a low incidence, pancreas cancer remains the fifth leading cause of death in cancer patients in America.1) Curative therapy by surgical removal of the tumor is rarely achieved because most tumors are not resectable at the time of diagnosis.2) Even if the tumor is removed, only about 14–33% of patients survive more than 5 years,3) since this type of tumor has poor profiles of sensitivity to commonly used chemotherapeutic agents. Studies are needed to examine the addition of adjuvant modalities or to develop new agents with greater anti-tumor activities.

Recently, we synthesized several K vitamin analogs, and found that 2-(2-hydroxyethylsulfaryl)-3-methyl-1,4-naphthoquinone (CPD 5) inhibited protein-tyrosine phosphatases (PTPases) through arylation of cellular thiols or thiol-dependent proteins by an addition-elimination mechanism.4, 5) We previously reported that CPD 5 mediated phosphorylation of epidermal growth factor receptor (EGFR) and acted as a potent growth inhibitor in Hep 3B hepatoma cell.6, 7) However, the relationship between growth factor receptor phosphorylation and growth-inhibitory action is unknown. This study was performed to find the basis of the CPD 5-induced growth-inhibitory effect.

In the present study, we show that CPD 5 induces phosphorylation of hepatocyte growth factor (HGF) receptor (c-Met) and EGFR simultaneously in pancreas cancer cells. We further found that CPD 5-induced phosphorylation of external signal-regulated kinase (ERK) plays a critical role in the growth-inhibitory action.

MATERIALS AND METHODS

Cell culture and cell growth inhibition assay CPD 5 was synthesized as described previously.5) The pancreas cancer cells were cultured in minimal essential medium (MEM) (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (FBS). For the growth inhibition assay, cells were plated at 2×10^4 cells/well, using 24-well plates (Corning, Inc. Science Products Div., Corning, NY). Twenty-four hours after plating, the medium was replaced with fresh medium containing CPD 5 at various concentrations. After treatment, the medium was aspirated and the plates were washed with phosphate buffered saline (PBS) and stored at −80°C. The fluorochrome Hoechst 33258 (20 µg/ml) in 200 µl of distilled water was added to each well and after incubation for 1 h at room temperature, the plates were frozen at −80°C. Cell number was counted by a DNA fluorometic assay.8) On the day of assay, 200 µl of distilled water was added to each well and after incubation for 1 h at room temperature, the plates were frozen at −80°C. The fluorochrome Hoechst 33258 (20 µg/ml) in 200 µl of TNE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 M NaCl) was added to each well and fluorescence was measured (excitation at 360 nm, emission at 460 nm).

Immunoblotting The cells were plated at 5×10^4 cells/dish in 6-well plates with MEM supplemented with 10% FBS. Twenty-four hours after plating, the cells were...
exposed to various agents at different concentrations. The cells were then washed with PBS and subsequently lysed in 75 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). For two-dimensional gels, almost confluent cells on a large dish were dissolved in 100 µl of standard urea solubilization buffer (9 M urea, 4% NP-40, 2% ampholytes, 2% 2-mercaptoethanol) with phosphatase inhibitor. Insoluble material was removed by microcentrifugation at 13 000 rpm for 15 min. Aliquots of samples were assayed for protein concentration, and cell lysates (40 µg of protein/lane) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels. Protein samples for two-dimensional gel electrophoresis (750 µg of protein/gel) were first applied to isoelectronic focusing gels (9.2 M urea, 5% ampholytes, 4% acrylamide/0.24% bisacrylamide, 2% NP-40, ammonium persulfate). The proteins were then transferred to a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA). The membrane was blocked with Tris-buffered saline containing Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) with 1% bovine serum albumin, then incubated with anti-phosphotyrosine monoclonal antibody (Ab-2, Oncogene Research Products, Calbiochem, Cambridge, MA) or anti-phospho-ERK monoclonal antibody (New England Biolabs, Beverly, MA), washed with Tris buffer and incubated with anti-mouse IgG coupled to horseradish peroxidase. Detection was performed with enhanced chemiluminescence reagents (NEN Life Science Products).

Immunoprecipitation Equal amounts of whole cell lysates (250 µg of protein) were incubated with 10 µg of polyclonal rabbit EGFR, c-Met or insulin receptor antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in lysis buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 ng/ml aprotinin and 1 mM orthovanadate) at 4°C for 3 h. Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) was added and samples were incubated overnight with mixing at 4°C. The samples were centrifuged at 2500 rpm for 5 min and washed with lysis buffer three times. The pellet was resuspended in electrophoresis sample buffer and heated at 95°C for 5 min.

RESULTS

CPD 5 inhibits cell growth and induces protein tyrosine phosphorylation in Panc-1 cells The effect of CPD 5 on the growth of three different pancreas cancer cell lines was examined. The ID_{50}s for CPD 5 were calculated as 21.4±3.8, 31.8±2.7 and 55.2±4.5 µM for MiaPaCa, Panc-1 and BxPc3 respectively (Fig. 1A). To see whether these cancer cell lines are stimulated by growth factors, we examined the influence of HGF or EGF under serum-free conditions. Fig. 1B shows that EGF clearly stimulates cell growth in a time-dependent manner, but HGF has no such activity in Panc-1. By contrast, HGF induces the growth of BxPc3 cells and EGF has no effect on growth of MiaPaCa.
and BxPc3 cell lines (data not shown). To examine whether CPD 5 is effective on protein tyrosine phosphorylation, Panc-1 cells were treated with 20 µM CPD 5, and cell lysates were tested by using a phosphorylation immunoblotting technique. In Panc-1 cells, phosphorylation of several proteins was not detectable at 2 min, but increased progressively to a maximum level at 30 min. After 60 min, the level of tyrosine phosphorylation was declining but was still distinguishable from that of untreated cells (Fig. 2A). The induction of protein tyrosine phosphorylation by CPD 5 was found to be dose-dependent (Fig. 2B).

**CPD 5 stimulates tyrosine phosphorylation of c-Met and EGFR in Panc-1 cells** To determine whether the protein phosphorylated by CPD 5 (Fig. 2) is 140 kDa c-Met or 170 kDa EGFR, cells were stimulated with CPD 5 for 30 min, and the cell lysates were immunoprecipitated.

![Fig. 2](image1.png)

**Fig. 2.** Effects of CPD 5 on protein tyrosine phosphorylation. Cells were exposed to 20 µM CPD 5 for the indicated time (A) or to several concentrations of CPD 5 for 30 min (B). The cells were lysed and cellular proteins (40 µg/lane) were resolved by SDS-PAGE. The fractioned proteins were transferred to a polyvinylidene difluoride membrane and tyrosine-phosphorylated proteins were visualized by using anti-phosphotyrosine antibody.

![Fig. 3](image2.png)

**Fig. 3.** CPD 5-mediated activation of c-Met and EGFR pathways. Panc-1 cells were plated on 6-well plates (5x10^4/well) and, after 24 h, treated with CPD 5 (20 µM), HGF (20 ng/ml) or EGF (10 ng/ml). The cells were lysed and 250 µg of lysate was subjected to immunoprecipitation as described under "Materials and Methods" for either c-Met (A) or EGFR (B). The c-Met or EGFR immunoprecipitates were probed with the Grb2 (C) or SOS (D) antibody.
with anti-c-Met or EGFR antibody. As shown in Fig. 3A, in HGF- or CPD 5-treated cells, anti-c-Met antibody immunoprecipitated similar amounts of protein and tyrosine phosphorylated c-Met. Further, phosphorylation of EGFR was detectable after stimulation with EGF or CPD 5 (Fig. 3B). These results suggest that CPD 5 mediated both c-Met and EGFR phosphorylation simultaneously. By contrast, in MiaPaCa cells, neither EGFR nor c-Met was phosphorylated by CPD 5. In BxPc3, CPD 5 induced phosphorylation of c-Met, but not EGFR (data not shown).

Activation of the signaling pathways by HGF or EGF requires the association of the receptor with cellular proteins. In fact, growth factor receptor binding protein 2 (Grb2) was noted to be co-immunoprecipitated with both c-Met and EGFR antibody (Fig. 3C). Since Grb2 connects receptor tyrosine kinases and SOS (son of sevenless ras-guanine nucleotide exchange factor), we also examined whether CPD 5-induced receptor activation was associated with SOS. At 30 min, SOS was not co-immunoprecipitated with c-Met or EGFR antibody, even though these growth factors mediated combination with Grb2. But at 60

Fig. 4. Comparison of signal transduction pathway in CPD 5- or growth factor-treated cells. Cells were exposed to 10 ng/ml EGF (open circles), 20 ng/ml HGF (open squares) or 20 µM CPD 5 (closed circles) for the indicated time. The cells were lysed and cellular proteins (40 µg/lane) were resolved by SDS-PAGE under reducing conditions. The fractioned proteins were transferred to a polyvinylidene difluoride membrane and phosphorylated proteins were visualized by using anti-phospho-ERK (A, B), -MEK (B), Elk-1 or c-Myc (C) antibody.
min, SOS was associated with CPD 5-induced phosphorylated receptors (Fig. 3D). Thus, these results suggest that CPD 5 activates the Ras signaling pathway through c-Met or EGFR phosphorylation.

Proteins of the EGFR or c-Met pathway are phosphorylated by CPD 5 Activation of the Ras signaling pathway is known to induce ERK phosphorylation. To estimate whether ERK activation is also associated with CPD 5-induced receptor tyrosine phosphorylation, we measured the amount by using phospho-ERK antibody. As shown in Fig. 4A, phospho-ERK appeared after HGF, EGF or CPD 5 treatment of Panc-1 cells. EGF-induced ERK phosphorylation appeared rapidly and declined after a transient peak, while HGF mediated a sustained induction of phosphorylated ERK. CPD 5-induced ERK phosphorylation showed the greatest peak and a more sustained level as compared with the other reagents. Thus, the induction pattern of phosphorylated ERK by CPD 5 was thought to be different from the signal of the growth factors. The CPD 5-induced phosphorylation of ERK and mitogen activated protein kinase (MAPK)/ERK kinase (MEK) was dose-dependent (Fig. 4B). Furthermore, phosphorylated Elk-1 and c-Myc, which are downstream of ERK, were also noted after 30 min (Fig. 4C).

Importance of ERK phosphorylation U0126, which is a potent ERK inhibitor, antagonized EGF- or CPD 5-induced ERK phosphorylation at 20 or 50 µM, respectively (Fig. 5A). Since EGF stimulated the growth of Panc-1 cells (Fig. 1B), EGF treatment may induce cell growth. U0126 (−), U0126 20 µM, U0126 50 µM, ∗P<0.05, ∗∗P<0.01 (C). Cells were cultured for the indicated time with 20 ng/ml HGF. The cellular proteins were resolved by SDS-PAGE and the fractioned proteins were visualized by using p27 antibody (D).

Fig. 5. Functional meaning of ERK phosphorylation. Panc-1 cells were plated on 6-well plates (5×10^4/well) and, after 24 h, treated with various concentrations of U0126 for 30 min prior to addition of 10 ng/ml EGF or 20 µM CPD 5. The cell lysates were subjected to phospho-ERK (A). Panc-1 cells were pretreated with 20 µM U0126 and were cultured for the indicated time with 10 ng/ml EGF. The cells were lysed and cellular proteins (40 µg/lane) were resolved by SDS-PAGE. The fractioned proteins were visualized by using cyclin D1 antibody (B). Panc-1 cells were treated with 20 µM CPD 5, with or without various concentrations of U0126. DNA content per well (means±SD) was determined by fluorescence measurement as described under “Materials and Methods.” U0126 (−), U0126 20 µM. U0126 50 µM, ∗P<0.05, ∗∗P<0.01 (C). Cells were cultured for the indicated time with 20 ng/ml HGF. The cellular proteins were resolved by SDS-PAGE and the fractioned proteins were visualized by using p27 antibody (D).

Fig. 6. ERK phosphorylation by CPD 5. Differences between growth factor- and CPD 5-induced ERK phosphorylation were examined on two-dimensional gels. Panc-1 cells were treated with 10 ng/ml EGF, 20 ng/ml HGF or 20 µM CPD 5. The same spot as in the case of growth factor is indicated by an arrow among the CPD 5-induced spots (A). Panc-1 cells were treated with 20 µM CPD 5 for the indicated time (B). Cell lysates in urea buffer were subjected to two-dimensional gel electrophoresis and blotted with phospho-ERK antibody as described under “Materials and Methods.”
cycle-dependent protein. In fact, EGF increased the level of cyclin D1 after 6 h in serum-starved cells, but the induction of cyclin D1 was completely antagonized by pretreatment of 20 µM U0126 (Fig. 5B). U0126 was also found to abrogate the CPD 5-induced growth-inhibitory effect (Fig. 5C). Thus, these results strongly suggest that ERK phosphorylation is critical for induction of cell proliferation by EGF and growth inhibition by CPD 5.

To address the effect of HGF on this cell line, another cell cycle-dependent protein was examined. As shown in Fig. 5D, p27 protein, a cyclin kinase inhibitor, appeared at 12 h after addition of HGF and increased progressively to a plateau level at 24 h. However, since U0126 itself mediated the increase of p27, the relationship between induction of p27 and phosphorylation of ERK could not be assessed. CPD 5 did not induce p27 protein (data not shown).

**Differences between growth factor and CPD 5 effects on ERK phosphorylation** As shown in Fig. 6A, phosphorylated ERK induced by HGF or EGF showed a single spot on two-dimensional gel, while additional spots of phosphorylated ERK induced by CPD 5 were seen in a higher pH zone. The main spot was noted in the highest pH area and the induction of this spot was almost completely abolished by 20 µM U0126. The time course study showed that this spot appeared at early time and increased gradually, while the lowest pH spot was hardly detectable at early time and diminished to the control level after a brief appearance (Fig. 6B). Over-expression of phosphorylated ERK, as shown in Fig. 3A, was indicated to originate from the high pH spot.

**DISCUSSION**

The pancreas cancer cell line, Panc-1 is one of the most useful human cells for testing the effects of chemotherapeutic agents, since it has low sensitivity to conventional chemotherapeutic drugs. In this study, we found that CPD 5 was a strong inducer of cell death in three different pancreas cancer cell lines. Since the purpose of this study was to clarify the relationship between growth factor receptor phosphorylation and the growth-inhibitory effect by CPD 5, we selected the Panc-1 cell line to examine the mechanism of CPD 5-induced growth inhibition.

Growth factor and/or growth factor receptor-induced signal transductions generate critical cell growth responses. For example, the c-Met system, which is encoded by the c-met proto-oncogene, has both positive and negative roles in tumor development. Namely, expression of c-Met is increased in tumor tissues and induces tumor formation or malignant progression, while HGF inhibits the growth of some cancer cell lines. Although EGF is known to stimulate the growth of many normal and cancer cell lines, the growth of some tumor cell lines is inhibited by nanomolar EGF. In the present study, we found that EGF stimulated Panc-1 cell growth and HGF had no effect on the proliferation. CPD 5 was found to induce both c-Met and EGFR phosphorylation simultaneously, and to inhibit cell growth.

As a first step of phosphorylated EGFR or c-Met-induced signal transduction, these receptors bind with common adaptor proteins, Grb2 and SOS. We showed that c-Met or EGFR activated by CPD 5 was co-immunoprecipitated with Grb2 and SOS antibody. This result indicates that CPD 5 activates the Ras signaling pathway through c-Met or EGFR phosphorylation in Panc-1 cells. In addition, we found (Fig. 4B) that CPD 5-induced signal transduction through receptor phosphorylation was activated with a lag of about 30 min.

The mitogen activated protein kinase (MAPK) family includes ERK, the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and P-38 kinases. The ERK pathway is thought to be activated by many stimuli, and phosphorylated ERK is important for several responses. For example, in neuronal PC 12 cells, transient ERK phosphorylation by EGF is associated with cell proliferation, while sustained ERK phosphorylation induced by nerve growth factor (NGF) is related to cell differentiation. As shown in Fig. 1B, EGF was observed to stimulate growth in Panc-1 cells. This result was supported by the finding that EGF-induced ERK phosphorylation is associated with an increase of cyclin D1 (Fig. 5B). By contrast, HGF mediated both phosphorylation of ERK and the increase of p27 protein, which binds to cyclin/cyclin dependent kinase complex and is involved in cell differentiation. Furthermore, since the activated c-Met pathway was reported to induce differentiation of several human pancreas cell lines, HGF may mediate differentiation of Panc-1 cells. Thus, we suggest that transient ERK phosphorylation by EGF is essential for cell proliferation, and sustained ERK phosphorylation by HGF may be associated with cell differentiation in this cell line. On the other hand, Xia et al. reported that the ERK pathway seemed to have an anti-apoptotic role, based on a study with NGF, which is a survival factor for PC 12, and several other reports described a protective effect of ERK phosphorylation against apoptosis. ERK phosphorylation has been indicated to have a positive effect on cell growth. By contrast, as shown in Fig. 5A, ERK phosphorylation induced by CPD 5 was quite marked and well sustained. Furthermore, ERK phosphorylation induced by CPD 5 was reduced by U0126, which also almost abrogated CPD 5-mediated growth inhibition (Fig. 6C). Thus, in the CPD 5-induced growth-inhibitory pathway, over-expression of ERK phosphorylation seems to play a critical role. Recently, Leppa et al. provided evidence that the ERK pathway stimulates c-jun synthesis. Since c-jun is implicated in apoptosis, activation of the ERK pathway...
may also correlate with apoptosis. Pumiglia and Decker reported that a sustained increase of ERK activity inhibited cell cycle-dependent kinase and caused growth arrest.\(^{30}\) Zhu et al. and Brink et al. showed a proapoptotic role of ERK phosphorylation in T cells, since inhibition of ERK phosphorylation antagonized apoptosis.\(^{31,32}\) We also found a growth-inhibitory effect of ERK phosphorylation in CPD 5-induced signal pathway.

Examination of phosphorylated ERK by two-dimensional electrophoresis (Fig. 6) showed high-pH spots, which might be hypo-phosphorylated ERK. As discussed above, since receptor phosphorylation by CPD 5 was only induced after 30 min, the Ras pathway should not be activated before that. However, ERK phosphorylation was already induced at 2 min after addition of CPD 5 (Fig. 5A). Namely, in the CPD 5-induced signaling pathway, ERK was suggested to be phosphorylated by both receptor-dependent and independent pathways (Fig. 3). In a time course study by two-dimensional electrophoresis, only the hypo-phosphorylated type of ERK was observed at early time. Since Elk-1 or c-Myc phosphorylation, which is downstream of ERK, was not seen at early time (Fig. 4C), this hypo-phosphorylated ERK appears not to cause activation downstream. As described above, U0126 abrogated CPD 5-induced growth inhibition and also abolished the hypo-phosphorylated ERK selectively. These results suggest that ERK phosphorylation induced by CPD 5 involves the hypo-phosphorylated ERK, and this type of ERK is closely related to the growth-inhibitory action. By contrast, hyper-phosphorylated ERK, which was the same as the product induced by EGF or HGF, emerged at 30 min and disappeared after a transient increase. Since the downstream proteins were also activated after 30 min, the hyper-phosphorylated ERK seems to transfer the signal downstream from the receptor. Thus, CPD 5 appears to be a candidate chemotherapeutic agent with a novel mechanism of action.

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