Antitumor and anticarcinogenic actions of Cpd 5: a new class of protein phosphatase inhibitor

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Dual specificity phosphatases (DSP) play an important role in control of the cell cycle and signal transduction. We have synthesized a new class of DSP inhibitors. Cpd 5 or [2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone] is one of the most potent of these. It inhibits DSPs of cells in culture and induces tyrosine phosphorylation of various DSP substrates, including the Cdc25 target Cdk5 and it potently inhibits cell growth. In this study, we have evaluated Cpd 5 in vivo for its antitumor and growth inhibitory activity on carcinogen-altered foci. Cpd 5 inhibited growth of the transplantable rat hepatoma cell line JM-1 in vitro, with concomitant phosphorylation of the mitogen-activated protein kinase ERK1/2 but not JNK1/2 or p38. ERK phosphorylation was associated with growth inhibition, as the ERK phosphorylation inhibitor PD098059 antagonized both ERK phosphorylation and growth inhibition. JM-1 cell lysates were found to contain ERK1/2-specific phosphatase(s) that could be inhibited by Cpd 5 and which are thought to be its major targets. Cpd 5 caused significant inhibition of both intrahepatic and subcutaneous (s.c.) growth of transplanted JM-1 cells in male Fischer F344 rats. The treatment was equally effective whether Cpd 5 was administered either as a single, acute dose or chronically as several lower doses. However, toxicity was much lower with chronic treatment. As in JM-1 cells in vitro, ERK1/2 was phosphorylated when rats in vivo were treated with Cpd 5 and tumor growth inhibition in vivo also was antagonized by treating rats with the ERK1/2 phosphorylation inhibitor PD098059. A single dose of Cpd 5 also inhibited the formation of glutathione S-transferase-pi enzyme-altered cells induced by the hepatocarcinogen N-nitrosodiethylamine. This is the first report of the in vivo activity and growth inhibitory mechanism of a novel class of K vitamin growth inhibitors that have potent tyrosine phosphatase activity.

Introduction

Reversible protein phosphorylation plays a major role in the control of cell-cycle and growth factor signaling pathways for mitogenesis and differentiation. Eukaryotic protein phosphatases have been classified into two basic types: S/T-specific (protein serine/threonine, PP) and Y-specific (protein tyrosine phosphatase, PTP). PTPs are a key group of signal transduction enzymes, which, together with protein tyrosine kinases, control the level of cellular protein phosphorylation. Protein tyrosine kinases phosphorylate cellular substrates on tyrosine residues, and much progress has been made over the last 20 years in elucidating their significance in signal transduction (1–3). However, it is only recently that the complexities of the PTPs have been appreciated. It is now recognized that selective dephosphorylation of phosphotyrosine residues plays a pivotal role in initiating, sustaining and terminating cellular signaling (4–6). Tyrosine-specific and dual-specific (both Tyr- and Ser/Thr-specific) phosphatases (DSP) are the two main classes of tyrosine phosphatases that are generally recognized. The PTPs are defined by the active site signature sequence motif H CX5R, where the five × (any amino acid) residues form a loop. The amide nitrogen of these five amino acids hydrogen bond to the phosphate of the substrate. R is a highly conserved arginine that also hydrogen bonds to the phosphorylated amino acid of the substrate (7). Both the catalytic and non-catalytic domains of the PTP confer substrate specificity. The non-catalytic domain may target the PTP to specific intracellular compartments where the substrate concentration is high (7,8). The catalytic domains themselves also have specificity for the phosphotyrosine residue and its flanking amino acids in the substrate (9,10). DSP (dual-specific protein phosphatases), which dephosphorylate tyrosine and threonine residues on the same substrate, are a subclass of PTP (11). The DSP subclass of PTP has emerged as a family of important regulators of cell-cycle control and mitogenic signal transduction (12–15). We have chemically synthesized a family of compounds based on the structure of vitamin K3 (16,17). They inhibit growth of various tumor cell lines in culture (18,19). One of the most potent is Cpd 5 or [2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone], which was found to induce ERK1/2 phosphorylation and activity, which in turn seemed to correlate with growth inhibition (18,20).

In experiments reported here, we have tested for the first time, the action of Cpd 5 on the growth in vivo of a transplantable rat hepatoma and on the formation of glutathione S-transferase-pi (GST-pi) positive liver cells, induced by N-nitrosodiethylamine (DEN) treatment.

Pre-neoplastic enzyme-altered foci and nodules represent important steps in chemical hepatocarcinogenesis (21–23). Single cells and mini-foci positive for the placental form of GST (GST-pi) develop very early in hepatocarcinogen-treated rat livers prior to the formation of nodules. These cells are detectable within days of a single administration of various initiating hepatocarcinogens, including DEN, but not with promoters and non-carcinogenic agents (24). This early, GST-pi positive population may be the precursor for pre-neoplastic foci and nodules (23). We examined the effect of Cpd 5 on the formation of the early GST-pi positive cells after a single DEN treatment.

Materials and methods

Materials
JM-1 rat hepatoma cells, which were established to grow in Fischer rats (25) were a gift (G. Michalopoulos, University of Pittsburgh). DEN was purchased...
from Aldrich Chemicals (St Louis, MO) and 2-week-old male Fischer rats were obtained from Hilltop labs (Scottsdale, PA). Cpd 5 was synthesized as described previously (17).

Cell culture and growth inhibition assay
JM-1 cells were cultured in minimum essential medium (MEM) (Life Technology, Gaithersburg, MD) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The medium contained 10% fetal bovine serum. Cells were plated at 2 × 10⁶ cells/well in 24-well dishes (Corning, Science Products Div., Corning, NY) for cell growth inhibition assays. After incubation in serum-free MEM for 24 h, the medium was replaced with a growth medium containing Cpd 5 for the indicated periods of time. The medium, with or without Cpd 5, was replaced every day with fresh medium. Removing the medium and immediately washing the cells with ice-cold phosphate-buffered saline (PBS) terminated the reaction. The cells were harvested and stored at –80°C. Cell number was measured by a DNA fluorometric assay with Hoechst 33258 (26).

In order to examine the effects of ERK1/2 inhibitor PD098059 (Calbiochem, La Jolla, CA), the cell cultures were incubated for 1 h with 10 μM of the inhibitors before adding Cpd 5.

Western blots and immunoprecipitation
Western blots and immunoprecipitation were done following our standard protocol (18).

Phospho-ERK2 dephosphorylation assay
Phospho-ERK1/2 was obtained from Cell Signaling Technologies (Beverly, MA). JM-1 cell lysate was immunoprecipitated with anti-ERK2 antibodies and cleared of endogenous ERK2 proteins by centrifugation. Phospho-ERK2 was incubated with ERK2-cleared cell lysate in phosphatase buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 10 mM diithiothreitol) for 30 min at 37°C in the presence or absence of Cpd 5. The phosphatase reaction was terminated by the addition of an equal volume of 2× sample buffer. The proteins were separated by 10% SDS–PAGE and transferred to a western blot that was probed with phospho-ERK1/2 and ERK2 antibodies.

Cell injection. JM-1 cells were grown in culture, harvested after trypsinization, washed twice with cold PBS and suspended at a concentration of 10⁷ cells/ml. 10⁶ cells were injected subcutaneously into the skin over the left and right shoulder or directly in five areas of the liver.

DEN injection. A stock solution of DEN at a concentration of 50 mg/ml was made in DMSO. Rats were injected intraperitoneally with a single injection at a dose of 200 mg/kg rat body wt.

Cpd 5 treatment. A stock solution of Cpd 5 was prepared at a concentration of 10 mg/ml in DMSO. It was injected either as an acute dose of 50 mg/kg rat body wt or as a series of five chronic doses (every other day) of 10 mg/kg body wt. Injection site was either directly into the JM-1 tumors on the shoulders, or subcutaneously at a site between the shoulders near the JM-1 injection, or intramuscularly into the thigh, at a distant site from the JM-1 growth site. PD098059 was injected subcutaneously on the shoulders of the rats 1 h before Cpd 5 treatment.

Tumor size. Animals were killed at the indicated times and the tumors were surgically removed. They were then weighed to determine size.

GST-pi staining. Immuno-histochemistry with GST-pi antibody was performed with an ABC kit (Vector Labs, Burlingame, CA) on 10% formalin-fixed liver sections of rats, which were harvested 3 weeks after an intraperitoneal injection of DEN.

In vivo half-life. Cpd 5 (50 mg/kg rat body wt) was injected intravenously and the concentration in blood at various times after injection was determined by its characteristic absorption maxima at 260 nm.

Statistical analysis. Statistical analysis of significance was determined by t-test.

Results

Growth inhibition of JM-1 hepatoma cells in vitro
The rat JM-1 cell line was established from a transplantable hepatoma grown in male Fischer rats by treatment with the chemical carcinogen DEN (25). We initially tested the effects of Cpd 5 in vitro on JM-1 cell proliferation. JM-1 cells were treated with increasing concentrations of Cpd 5 and the surviving cell number was counted after 3 days of treatment. Cpd 5 was found to inhibit their growth with an IC₅₀ = 20 μM (Figure 1A).

ERK activation and growth inhibition
We have seen previously that growth inhibition of some tumor cells in vitro by Cpd 5 was accompanied with dual phosphorylation and activation of ERK1/2 (20,27). This activation of ERK1/2 was strongly correlated with growth inhibition. We therefore looked for similar growth inhibitory mechanisms in the action of Cpd 5 on JM-1 cells. We found that dual phosphorylation of ERK1/2 was induced when JM-1 cells were treated with inhibitory doses of Cpd 5. This effect was specific for ERK1/2 phosphorylation. Two other MAPKs (JNK and p38) were not phosphorylated. The ERK1/2-kinase
(MEK1/2) inhibitor PD098059 antagonized ERK1/2 phosphorylation, as well as growth inhibition induced by Cpd 5 (Figure 1B). Thus, Cpd 5 induced ERK1/2 phosphorylation in JM-1 cells, which was important in mediating the growth inhibition.

### Phospho-ERK1/2 Phosphatase inhibition by Cpd 5

Cpd 5 has been shown to be a potent PTPase inhibitor (28,29). We therefore examined whether its effects on phospho-ERK induction could be due to inhibition of ERK phosphatases. JM-1 cell lysates were cleared of ERK2 by immunoprecipitation. The cleared lysates were then incubated with phospho-ERK2 proteins in a phosphatase reaction in the absence or presence of increasing concentrations of Cpd 5. Incubation with JM-1 cell lysates in the absence of Cpd 5 resulted in antagonism of the ERK phosphatase activity. These results show that JM1 cell lysates contain phospho-ERK2 phosphatase(s), which is antagonized by the effects of Cpd 5.

#### Cpd 5 effect on growth of transplantable tumors in rats

JM-1 hepatoma cells were shown previously to be able to grow and form transplantable tumors subcutaneously or in the liver parenchyma of male Fischer rats (25). The effects of Cpd 5 were tested in this transplantable hepatoma model. The kinetics of tumor growth was first determined after subcutaneous (s.c.) injection of 10⁶ JM-1 cells suspended in 1 ml of saline. The tumor volume increased linearly in the first 2 weeks (Figure 3A). Half-life of Cpd 5 in rat blood was determined to be ~45 min. A schematic outline of the experimental protocols is shown in Figure 3B and C. Rats were injected with 10⁶ JM-1 cells, either directly into the liver or subcutaneously on the two shoulders. Cpd 5 treatment was started a day after implantation of the injected JM-1 cells. The animals were treated with a single acute dose (50 mg/kg rat body wt) or chronically, as a series of five doses (10 mg/kg rat body wt, every other day). The route of Cpd 5 administration was i.p. in the case of intrahepatic tumors and s.c. (between the shoulders) or intramuscular (i.m.) (on the thighs) in the case of s.c. tumors. The experiments were terminated 2 weeks after the first injection of the Cpd 5.

In a second series of experiments, the transplanted tumors were first allowed to grow subcutaneously on the shoulders of the rats for 10 days and then challenged with Cpd 5 treatment, by intratumor, s.c. or i.m. injections. The dose of Cpd 5 used was either acute or chronic as in the first series of experiments.
Table I. JM-1 tumor growth inhibition in vivo by Cpd 5

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Location</th>
<th>Site</th>
<th>Cpd 5 treatment</th>
<th>Location</th>
<th>Site</th>
<th>% Inhibition</th>
<th>P value</th>
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<tr>
<td>A</td>
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<td>Shoulder</td>
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<td>Shoulder</td>
<td>s.c.</td>
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<tr>
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<td>Chronic</td>
<td>Shoulder</td>
<td>s.c.</td>
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<td>&lt;0.001</td>
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<tr>
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<td>Thigh</td>
<td>i.m.</td>
<td>50</td>
<td>&lt;0.002</td>
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<tr>
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<tr>
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<td>Peritoneum</td>
<td>i.p.</td>
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<tr>
<td>A</td>
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<td>i.p.</td>
<td>80</td>
<td>&lt;0.001</td>
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<tr>
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<td>Shoulder</td>
<td>i.t.</td>
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<td>&lt;0.001</td>
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<td>Thigh</td>
<td>i.m.</td>
<td>5</td>
<td>&gt;0.05</td>
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</table>

A: Cpd 5 injected the day after JM-1 cells; B: Cpd 5 injected after tumor growth for 10 days. I.t., intratumor. The location of the JM-1 tumors, site and mode of Cpd 5 treatment, percent inhibition of tumor mass compared with control rats and the P value were tabulated.

Fig. 4. Tumor growth and ERK1/2 phosphorylation in tumors. S.c. JM-1 tumors were grown on the shoulders of rats. PD098059 (1 ml at 375 µM) or vehicle control was injected under the skin near the tumors, 1 h before s.c. treatment with Cpd 5 (50 mg/kg) or vehicle control. ERK1/2 phosphorylation status of the tumors was examined 1 h and 2 weeks after Cpd 5 treatment (B), and the tumor mass (A), was determined 2 weeks after Cpd 5 treatment. Expression of ERK2 served as a control in (B).

Fig. 5. Toxicty of Cpd 5 administered either as acute or chronic doses. Rat body weights were monitored at the start and end of each experiment, in order to monitor general toxicity. After 2 weeks of the chronic dose regimen of 10 mg/kg rat body wt (five doses, given every other day), the body weight remained essentially the same, indicating no general toxicity. However, body weight was significantly lower after a single acute high Cpd 5 dose (50 mg/kg rat body wt) (Figure 5).

Effect of Cpd 5 on formation of DEN-induced GST-pi stained hepatocytes

GST-pi was found to be focally expressed in hepatocytes within 2 weeks after a single dose of the carcinogen DEN, as others have found (23). These cells are thought to be possible precursors to the pre-neoplastic foci and eventual liver tumors. Therefore, we studied the effects of Cpd 5 on the expression of GST-pi hepatocytes induced by DEN, as a possible marker of pre-neoplasia. Rats were injected intraperitoneally with a single dose of DEN at 200 mg/kg rat body wt. Cpd 5 or...
Few selective, potent and cell permeable inhibitors of PTP or DSP are currently available.

Cpd 5 was synthesized based on the structure of vitamin K3, and was hypothesized to arylate cellular cysteine (16). PTP and DSP contain a cysteine at their catalytic site. Hence, these phosphatases have been shown to be Cpd 5 targets. Cpd 5 was subsequently identified independently in a small molecule library as a potent inhibitor of the catalytic cysteine containing Cdc25 dual specificity phosphatase (20,30). It was also shown to be specific for the DSP, especially Cdc25. We have shown previously that Cpd 5 is a potent inhibitor of DNA synthesis in normal hepatocytes (29) and for growth of several cancer cell lines (17–19). The mechanisms of growth inhibitory action were shown to involve the inhibition of Cdc25, leading to cell-cycle arrest and the activation of signal transduction related protein kinase ERK1/2.

We found a strong correlation between ERK1/2 phosphorylation induced by Cpd 5 and growth inhibition. Therefore, similar mechanisms of growth inhibition through phosphorylation of ERK1/2 probably also work in Cpd 5 induced growth arrest of the JM-1 hepatoma cells in vitro. Cpd 5 effect was specific for ERK1/2 phosphorylation. Two other MAPKs, JNK and p38, were not phosphorylated by Cpd 5 action. This may be due to differential effects on the MAPK-specific phosphatases or activation of upstream kinases. It was noted that the basal levels of JNK and p38 in these cells were much lower that that of ERK2. Therefore, the failure to detect induction of phosphorylation of JNK and p38 by Cpd 5 might be due to this lower basal level of these kinases in the JM-1 cells. We also found that JM-1 cell lysates contain ERK1/2 phosphatase(s) that was inhibited by Cpd 5. Thus, ERK1/2 phosphorylation due to Cpd 5 action in JM-1 cells is probably due to inhibition of ERK1/2 phosphatase(s) in these cells.

In this study we have, for the first time, used this rather specific Cdc25 phosphatase inhibitor in animal tumor- and carcinogen-treatment models. Our results demonstrate the efficacy of Cpd 5 on JM-1 cells in an in vitro proliferation assay and in a rat tumor allograft model.

Cpd 5 inhibited both intrahepatic and s.c. growth of JM-1 tumors. S.c. tumor growth was suppressed even when Cpd 5 was administered intramuscularly at a site distant from the tumor. This probably is due to a systemic diffusion of Cpd 5 from the injection site to the tumor site. There was not much difference in the inhibitory effect between injection of Cpd 5 at a site near or far from the tumor, again suggesting a systemic diffusion.

Not only did Cpd 5 inhibit JM-1 tumor formation, but it also could reduce the size of a pre-formed tumor. However, the treatment was only effective on the pre-formed tumors when Cpd 5 was injected at a site near to the tumor. A distant i.m. injection did not have any effect on growth of pre-formed tumors. This probably suggests the requirement of a minimum inhibitory dose of Cpd 5 at the tumor site for tumors of a certain size, which could not be achieved by a distant i.m. injection. This minimum dose at the tumor site was probably sufficient to have an effect when the tumor cells were injected and closely followed in time by Cpd 5 treatment. However, it was not enough when the tumors were already formed and had grown beyond a certain size. Cpd 5 was also found to be more effective on pre-formed tumors when it was injected near the tumor, compared (80 versus 51%) with treatment right after JM-1 cell injection. This might be due to different diffusion rates after Cpd 5 injection. Cpd 5 might reach the preformed tumors more readily due to the extensive vasculature and increase the effective local concentration.
As seen in vitro, ERK1/2 was also phosphorylated transiently in the JM-1 tumors in rats that were treated with Cpd 5. ERK1/2 phosphorylation was detected in tumors within 1 h after Cpd 5 treatment. However, ERK1/2 phosphorylation returned to control levels after 2 weeks. This transient ERK1/2 phosphorylation in the tumors could be inhibited by pre-treatment of the rats with the ERK1/2 phosphorylation inhibitor PD98059. Inhibition of ERK1/2 phosphorylation also antagonized the growth inhibitory effect of Cpd 5 on tumor growth. This strongly suggests the involvement of ERK1/2 phosphorylation induced by Cpd 5 in tumor growth inhibition. This result is in contrast to a previous observation, where inhibition of ERK1/2 phosphorylation by the MEK inhibitor PD184352 resulted in tumor growth inhibition (31). However, we used a different PD, which was at a dose 50-fold less than used in the study referenced above. Tumor growth was not inhibited at our low PD dose. It could only antagonize the transient ERK1/2 phosphorylation in the tumors induced by Cpd 5 treatment.

The acute and chronic administration regimens of Cpd 5 did not have an appreciable difference in growth inhibitory effect of JM-1 tumors. However, the overall toxicity, as measured by decrease in body weight, was significant. Chronic dosing did not result in a reduction of body weight. Acute treatment, using higher doses of Cpd 5, on the other hand, resulted in an appreciable loss in rat body weight from the beginning of the experiment. This might indicate a maximum tolerated dose, which was not reached when Cpd 5 was administered chronically at the doses used in these experiments.

We also tested Cpd 5 effect on an in vivo carcinogen model. Rats had been shown to express GST-pi in their hepatocytes very early after the administration of a chemical hepatocarcinogen (23). These hepatocytes may include the precursors to neoplastic foci, which eventually develop into hepatomas. Cpd 5 was seen to significantly reduce the appearance of these GST-pi expressing cells. Hence, it is possible that it could block hepatoma formation in a complete tumorigenesis model.

In summary, these studies show that Cpd 5, a specific tyrosine phosphatase inhibitor, has demonstrated efficacy in an in vitro model of hepatoma cell growth and in an in vivo model of growth of the same hepatoma. Moreover, it can act as an antagonist of the in vivo growth of cells phenotypically altered by the in vivo action of a chemical carcinogen. These effects can be achieved with a non-toxic dose of Cpd 5. These experiments might encourage the further development of this and its related class of compounds as anticancer agents, or possibly in prevention of carcinogenesis.

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References


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