PM-20, a novel inhibitor of Cdc25A, induces extracellular signal–regulated kinase 1/2 phosphorylation and inhibits hepatocellular carcinoma growth in vitro and in vivo

Siddhartha Kar,1 Meifang Wang,1 Wei Yao,2 Christopher J. Michejda,2 and Brian I. Carr1

1Liver Cancer Center, Starzl Transplantation Institute, Pittsburgh, Pennsylvania and 2Structural Biophysics Laboratory, National Cancer Institute, Frederick, Maryland

Abstract
We have synthesized several new phenyl maleimide compounds, which are potent growth inhibitors of several human tumor cell lines. Among these, PM-20 was the most potent with an IC50 of 700 nmol/L for Hep3B human hepatoma cell growth. Two other derivatives, PM-26 and PM-38, did not inhibit Hep3B cell growth even at 100 μmol/L. Interestingly, under identical experimental conditions, PM-20 inhibited DNA synthesis of primary cultures of normal hepatocytes at a 10-fold higher concentration than that needed to inhibit the DNA synthesis of the Hep3B hepatoma cells. PM-20 affected two cellular signaling pathways in Hep3B cells: Cdc25 phosphatase and extracellular signal–regulated kinase (ERK) 1/2. It competitively inhibited the activity of Cdc25 (preferentially Cdc25A) by binding to the active site, likely through the catalytic cysteine, but did not inhibit PTP1B, CD45, or MKP-1 phosphatases. As a result of its action, tyrosine phosphorylation of the cellular Cdc25A substrates Cdk2 and Cdk4 was induced. It also induced strong and persistent phosphorylation of the Cdc25A substrate ERK1/2. Hep3B cell lysates were found to contain ERK2 phosphatase(s) activity, which was inhibited by the actions of PM-20. However, activity of exogenous dual-specificity ERK2 phosphatase MKP1 was not inhibited. Induction of ERK1/2 phosphorylation correlated with the potency of growth inhibition in tumor cell lines and inhibition of ERK1/2 phosphorylation by the mitogen-activated protein kinase (MAPK)/ERK kinase 1/2 inhibitor U0126 or overexpression of the cdc25A gene in Hep3B cells antagonized the growth inhibitory actions of PM-20. Growth of transplantable rat hepatoma cells in vivo was also inhibited by PM-20 action with a concomitant induction of pERK in the tumors. The mechanism(s) of growth inhibition of Hep3B hepatoma cells by the phenyl maleimide PM-20 involves prolonged ERK1/2 phosphorylation, likely resulting from inhibition of the ERK phosphatase Cdc25A. PM-20 thus represents a novel class of tumor growth inhibitor that inhibits mainly Cdc25A, is dependent on ERK activation, and has a considerable margin of selectivity for tumor cells compared with normal cells. [Mol Cancer Ther 2006;5(6):1511–9]

Introduction
Protein phosphorylation and dephosphorylation are essential mechanisms for intracellular control of protein function and signaling. Protein kinases are involved in phosphorylation whereas dephosphorylation is catalyzed by protein phosphatases (1). Two types of mammalian protein phosphatase have been identified: S/T specific (protein serine/threonine) and Y specific (protein tyrosine phosphatase). Dual-specificity phosphatases, which dephosphorylate both tyrosine and threonine/serine on the same protein, are a subclass of protein tyrosine phosphatase (2). Dual-specificity phosphatases share the protein tyrosine phosphatase active site sequence motif HC(X)sR, where H is a highly conserved histidine, C is the catalytic cysteine, the five X residues form a loop in which all the amide nitrogens hydrogen-bond to the phosphate of the substrate, and R is a highly conserved arginine that hydrogen-bonds to the phosphorylated amino acid of the substrate. Dual-specificity phosphatases and protein tyrosine phosphatases also have a similar mechanism of reaction although there is limited amino acid sequence identity beyond the active site region (3). The two important motifs for binding substrates in dual-specificity phosphatases are defined by the VH1-like phosphatases (pTEpY) and the Cdc25-like phosphatases (pTPY). Dual-specificity phosphatases display a marked preference for cyclin-dependent kinases (Cdk) and mitogen-activated protein kinases (MAPK) and they have been recognized as a family of important regulators of cell cycle control and mitogenic signal transduction (3–6).

The regulation of cell cycle is primarily due to the effect of the phosphorylation status of the Cdns (7). One of the major mechanisms of regulation is the phosphorylation and dephosphorylation of Thr14 and Tyr15 residues on Cdk1, Cdk2, and Cdk4. In mammalian cells Cdc25A, Cdc25B, and Cdc25C carry out this dephosphorylation reaction. Although Cdc25A mainly controls G1-S progression, and Cdc25B and Cdc25C activate the G2-M transition (8, 9), the role of Cdc25 phosphatases is more complex (10).
Consistent with their roles as positive regulators of cell proliferation, Cdc25A and Cdc25B are reported to act as oncogenes (11). Elevated mRNA and protein levels of Cdc25A and Cdc25B have been found in many human tumor types (12, 13). Thus, Cdc25s have emerged as important molecular targets for designing new anticancer agents.

We recently developed several novel thioalkyl K-vitamin analogues that inhibit the activity of several dual-specificity phosphatases and have specificity towards the Cdc25 family. They especially antagonize the action of extracellular signal–regulated kinase (ERK) phosphatases, resulting in prolonged ERK phosphorylation (14–19). One of these compounds, Cpd 5, was also found to bind to Cdc25 at its catalytic cysteine (20). Cpd 5 was also found to block hepatoma cell growth both in vitro and in vivo (16, 21–23). These compounds could thus be useful as pharmacologic probes for specific biochemical pathways and as possible anticancer drugs. However, the growth inhibitory activity of these compounds occurred at micromolar concentrations and the fact that they were quinones raised the possibility of toxicities induced by reduced oxygen species that are formed during redox cycling of many quinone drugs. We therefore used an alternate strategy to design more potent nonquinone antagonists of protein tyrosine phosphatases and of cell growth.

One approach to inactivating protein tyrosine phosphatases is to alkylate the catalytic site cysteine using thiol-specific agents. Maleimides are excellent reagents for thiol-selective modification (24). Maleimide reactions are specific for sulfhydryl groups in the pH range 6.5 to 7.5. At pH 7, the reaction of the maleimide itself with sulfhydryl proceeds at a rate 1,000 times greater than its reaction with amines. Structure-activity studies (to be reported separately) revealed that some maleimides substituted by thioethanol groups on the reactive double bond and by an aromatic residue on the nitrogen had interesting antitumor properties. We report here on one active maleimide derivative, PM-20, which inhibits growth of hepatoma cells in vitro and in vivo, likely through its action as a phosphatase antagonist. We also describe two inactive congeners, PM-26 and PM-38, which were used as negative controls.

Materials and Methods

Synthesis of PM-20 and Other Maleimides

A complete description of the syntheses of all the maleimide derivatives will be reported elsewhere. PM-20, PM-26, and PM-38 were synthesized by a two-step procedure described below, using PM-20 as the example. 4-Biphenylamine (1.1 equiv) was added dropwise to a stirred solution of 3,4-dibromomaleic acid in acetic acid in an ice-cooled bath. The mixture was heated to reflux overnight under argon. After cooling, the mixture was poured into ice water and extracted with ethylacetate. The combined organic layers were washed with saturated sodium bicarbonate and brine and dried over magnesium sulfate. Removal of solvent gave the crude product, which was recrystallized from methanol and purified by flash chromatography on silica gel. The compounds were characterized by 1H nuclear magnetic resonance and liquid chromatography-mass spectrometry. The N-(4-biphenyl)-3,4-bis-(2-hydroxy-ethylsulfanyl)maleimide was synthesized by treating a tetrahydrofuran solution of the relevant N-(4-biphenyl)-3,4-dibromomaleimide and imidazole (2.2 equiv) with 2-mercaptamethanol (2.2 equiv) under argon for 3 hours at room temperature. After quenching of the reactions with ammonium chloride and extraction, the crude products were purified by flash chromatography on silica gel and crystallized. The compounds were characterized by 1H nuclear magnetic resonance, liquid chromatography-mass spectrometry, and elemental analysis.

Cell Culture and Growth Inhibition Assay

Hep3B, MCF7, SKBR3, FemX, HR, PCI, and LS180 cells were cultured in MEM (Life Technologies, Gaithersburg, MD) in a humified atmosphere of 5% CO2 and 95% air at 37°C. The medium contained 10% fetal bovine serum. Cells were plated at 2 × 10⁴ per well in 24-well dishes (Science Products Division, Corning, Inc., Corning, NY) for cell growth inhibition assays. After cell attachment for a day, the medium was replaced with growth medium with or without the compounds. Cells were treated for 1 hour with the MAPK/ERK kinase (MEK) inhibitor U0126 before addition of FM-20. After 3 days of culture, the medium was removed and the cells were immediately washed with ice-cold PBS to terminate growth. Cells were then harvested and stored at −80°C until use. Cell number was measured by DNA fluorometric assay with Hoechst 33258 as previously described (25). To examine the effects of ERK1/2 inhibitor U0126 (Calbiochem, La Jolla, CA), we preincubated cells for 1 hour with the inhibitor before adding compound.

Cell Lysate Preparation, Western Blot, and Immunoprecipitation

Western blot and immunoprecipitation protocols were followed as described before (16, 22). Cells were plated at 5 × 10⁴ per well in six-well tissue culture plates. After 24 hours, the culture medium was replaced by control medium or medium containing the PM compounds at various concentrations and incubated for different times. They were then rinsed with PBS and lysed in 100 μL of radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin). The lysates were cleared by centrifugation at 12,000 rpm for 5 minutes in a microcentrifuge. Protein concentration of the lysates was determined by Bio-Rad assay (Bio-Rad, Hercules, CA).

Lysate proteins (40 μg/lane) were separated on a 10% gel by SDS-PAGE. The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). The membrane was incubated for 1 hour at room temperature in TBST buffer [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] containing...
lysates was measured by using the substrate 3-hydroxyanthranilic acid (Cell Signaling, Waltham, MA) and antibiotin and horseradish peroxidase–conjugated secondary antibody (Amersham, which was diluted in TBST-bovine serum albumin. The membrane was then washed four times with TBST. Enhanced chemiluminescence reagent (NEN Life Science, Boston, MA) was used for detection.

For immunoprecipitation, 200 μg of cell lysate were incubated with antibody and protein A-agarose (Sigma) overnight at 4°C. The immunoprecipitate was washed thrice with radioimmunoprecipitation assay buffer. The washed precipitate was suspended in SDS-sample buffer and electroblotted.

The antibodies used in these experiments (pY, ERK2, Cdk2, Cdk4, MKP1, and Cdc25A antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti–phospho-ERK1/Cdk2, Cdk4, MKP1, and Cdc25A antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti–phospho-ERK1/2 from Cell Signaling, Waltham, MA; and antibitin from Sigma) were commercially obtained.

**Protein Tyrosine Phosphatase Activity Assay**

Protein tyrosine phosphatase activity of liver protein lysates was measured by using the substrate 3-O-methyl fluorescein phosphate (Molecular Probes, Eugene, OR) as previously described (26). Cdc25A, Cdc25B, and Cdc25C were a gift from Dr. J. Rudolph (Duke University, Durham, NC); MKP1, PTP1B, and CD45 phosphatases were obtained commercially (Upstate, Lake Placid, NY). The PM analogues were solubilized in DMSO and all reactions including controls were done in 1% DMSO. The final incubation mixture (150 μL) was optimized for enzyme activity and composed of 30 mmol/L Tris (pH 8.5), 75 mmol/L NaCl, 1 mmol/L EDTA, 0.33% bovine serum albumin, and 1 mmol/L DTT. Reaction was initiated by adding enzyme. Fluorescence emission from the product was measured over a 10- to 60-minute time period at room temperature in a multiwell plate reader. The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentration. Half-maximal inhibition constant was calculated by curve fit by Cricket Graph III program.

**Phospho-ERK Dephosphorylation Assay**

Phospho-ERK1/2 protein was obtained from Cell Signaling Technologies (Beverly, MA). Hep3B 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 or the ERK2 phosphatase MKP1, either in the presence or absence of PM-20, in phosphatase buffer [50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 10 mmol/L DTT] for 30 minutes at 37°C in the presence or absence of Cpd 5. A phosphatase inhibitor cocktail (PPI, Sigma) was used as a control for the MKP1 assay. 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.

**Hepatocyte Preparation and DNA Synthesis**

Hepatocytes were isolated from rat livers and DNA synthesis in the hepatocytes was determined as previously described (27). To compare the growth inhibitory effects of PM-20, hepatocytes and Hep3B cells were both cultured under identical conditions. DNA synthesis was measured both in the hepatocytes and Hep3B cells by the incorporation of [3H]thymidine.

**Competition of PD 49 and PM-20 Binding to Cdc25Bcat**

One hundred-eighty nanograms of Cdc25Bcat were incubated in a 10-μL reaction volume for 18 hours at 4°C, with PD 49 at 1 μmol/L and PM-20 at a concentration of 0, 0.01, 0.1, and 1 μmol/L. PD 49 bound to Cdc25Bcat was detected on Western blots after probing with antibitin antibody.

**Cdc25A Gene Transfection**

The mammalian expression plasmids encoding the full-length wild-type Cdc25A (cdc25Awt) or a catalytically dead mutant Cdc25A (C430S, cdc25Amut) were generously provided by Dr. T. Roberts (Dana-Farber Cancer Institute, Boston, MA; ref. 28). Transfections were carried out by the LipofectAMINE method following the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Briefly, Hep3B cells were plated in 12-well plates and transfected with 0.8 μg/well plasmid DNA in OptiMEM transfection medium using the LipofectAMINE 2000 reagent. Five hours after transfection, the medium was replaced with complete growth medium and the cells were allowed to recover overnight before compound treatment for 24 hours. DNA synthesis was assessed by incorporation of 5 μCi of [3H]thymidine for 3 hours.

**In vivo Hepatoma Growth Assay**

JM-1 cells (gift from G. Michalopoulos, University of Pittsburgh, Pittsburgh, PA; ref. 29) were grown in culture. After trypsinization and harvest, the cells were washed twice with cold PBS and suspended at a concentration of 10⁶ cells/mL. Cells (10⁵) were injected into the liver via the mesenteric vein (two groups, with five rats per group). A stock solution of PM-20 was prepared at a concentration of 1 mg/mL in DMSO and was injected i.p., every other day at a dose of 1 mg/kg, for a total of five times. The control group had equal volume of DMSO. Animals were sacrificed after 2 weeks following cell transplantation and the tumors were surgically removed and the number of tumors per liver was determined. pERK induction was determined by Western blots in the tumor tissues from rats treated or untreated with PM-20, 4 hours after the last treatment.

**Statistical Analysis**

Statistical analysis of significance was determined by t test.

**Results**

**Growth Inhibition of Hep3B Cells**

PM-20, PM-26, and PM-38 were selected for this study from a library of 50 maleimide compounds. The PM
analogue was examined for growth inhibition of the well-characterized human hepatoma-derived Hep3B cell line. Hep3B cells in log-phase growth were treated with continuous exposure to various concentrations of the maleimides. Cell numbers in the culture dishes with or without maleimide treatment were measured after 3 days. IC50 (concentration for 50% reduction of cell number relative to untreated controls) of each maleimide was determined from the growth inhibition curves. IC50 of PM-20 was 700 nmol/L whereas IC50 of PM-26 and PM-38 was >100 μmol/L each (Fig. 1A).

We tested whether PM-20-mediated growth inhibition was specific for hepatoma cells. Various human tumor cell lines (MCF7 and SKBR3, mammary carcinoma; FemX, melanoma; HR, gastric carcinoma; PCI, squamous cell carcinoma from tongue; LS180, colon carcinoma; and Hep3B, hepatocellular carcinoma) were treated with either PM-20 or PM-26 and cell numbers were measured at 3 days after addition of the inhibitors to the culture medium. IC50 of PM-20 for these cell lines ranged from 700 nmol/L to 20 μmol/L. PM-26 did not inhibit growth of any of these cell lines up to a concentration of 20 μmol/L (Table 1).

Binding and Inactivation of Cdc25 Phosphatases in Cell-Free Systems and in Hep3B Cells

The structures of PM compounds were designed to be Cdc25 inhibitors. We therefore compared the inhibitory activity of PM-20 against the dual-specificity phosphatases Cdc25A, Cdc25B, Cdc25C, and MKP1 and the protein tyrosine phosphatases PTP1B and CD45. The IC50 of PM-20 was found to be 1, 10, 40, and 75 μmol/L for Cdc25A, Cdc25B, Cdc25C, and MKP1, respectively. In contrast, the IC50 was >100 μmol/L for PTP1B and CD45. PM-20 thus preferentially inhibited the Cdc25A phosphatase. A kinetic analysis of the inhibition of Cdc25Bcat (a COOH-terminal fragment containing the catalytic site) by PM-20 was consistent with competitive inhibition with a Ki of 0.7 μmol/L (Fig. 2A).

The dephosphorylation of phospho-tyrosines on Cdk2 and Cdk4 is an important biological role of Cdc25A (7, 9). Therefore, we examined whether PM-20 could inhibit Cdc25A phosphatase in intact Hep3B cells. Cells were treated with PM-20 and tyrosine phosphorylation of the Cdc25A substrates Cdk2 and Cdk4 was determined after immunoprecipitation with Cdk2 or Cdk4 antibodies and subsequent probing with phospho-tyrosine antibody on Western blots. PM-20 was found to inhibit the dephosphorylation of Cdk2 and Cdk4, resulting in elevated levels (Fig. 2B).

We previously found that PD 49, a biotin-tagged derivative of Cpd 5, interacted with the catalytic site of Cdc25Bcat (20). We explored whether PD 49 and PM-20 might bind to the same catalytic domain in Cdc25Bcat. PD 49 was incubated with Cdc25Bcat in the presence of increasing concentrations of PM-20. The PD 49-Cdc25Bcat complex was visualized on Western blot using antibiotin antibody. PM-20 was found to decrease the binding of PD 49 to Cdc25Bcat (Fig. 2C), suggesting a competitive binding to the catalytic region of Cdc25Bcat.

Hep3B Cells and Normal Hepatocytes Are Differentially Sensitive to PM-20

An anticancer compound will have potential clinical use if it has relatively low cytotoxicity against normal compared with tumor cells. Therefore, the inhibitory effects of PM-20 on DNA synthesis in mitogen-stimulated normal rat hepatocytes and Hep3B hepatoma cells were measured under identical experimental conditions. Rat hepatocytes or Hep3B hepatoma cells were plated in culture on fibronectin matrix (2 μg/dish). Three hours after plating, epidermal growth factor was added to the attached cells to stimulate DNA synthesis, which was measured by the incorporation of [3H]thymidine after 48 to 72 hours, in the presence or absence of PM-20. Under these similar culture conditions, the IC50 of PM-20 was found to be ~5 μmol/L for the rat hepatocytes but 0.5 μmol/L for Hep3B cells. Normal hepatocytes were thus ~10-fold more resistant to the growth inhibitory actions of PM-20 than Hep3B hepatoma cells (Fig. 3).

Inhibition of ERK1/2 Phosphatase(s) Activity in Cell Lysates by PM-20

We previously found that K-vitamin compounds were potent cellular phosphatase inhibitors (26, 27). To determine whether ERK1/2 phosphatase(s) might be inhibited by PM-20, Hep3B cell lysates were cleared of endogenous ERK2 protein by immunoprecipitation with ERK2 antibody. The cleared lysate proteins were incubated with exogenous pERK2 as target, in the presence or absence of 2 μmol/L PM-20. The phosphorylation status of ERK2 was determined on a Western blot after probing with pERK2 and ERK2 antibodies. Hep3B cell lysates were found to dephosphorylate exogenous pERK2 and this phosphatase activity was inhibited by PM-20 (Fig. 4A). In contrast, the
ERK2 phosphatase activity of the MAPK phosphatase MKP1 was not inhibited by PM-20 at similar concentration (Fig. 4B). The expression of MKP1 was also found to be very low in Hep3B cells (Fig. 5B). This suggested the presence of ERK2 phosphatase(s), other than MKP1, in Hep3B cell lysate proteins, which were inhibited by PM-20 action, leading to ERK phosphorylation. **ERK1/2 Phosphorylation and Cdc25A Inhibition Were Involved in Growth Inhibition**

We have previously found that growth inhibition of different cell types by the K-vitamin analogue Cpd 5 was associated with prolonged phosphorylation of ERK1/2 (22, 23). We therefore examined whether induction of ERK1/2 phosphorylation by PM-20 might also be involved in its mechanism of growth inhibition. pERK1/2 induction by PM-20 was assayed in four tumor cell lines (Hep3B, FemX, SKBR3, and LS180), which have different sensitivities to growth inhibition. pERK was found to be induced at lower concentrations in the more sensitive cell lines Hep3B and FemX (Fig. 5A) and at higher concentrations in the more resistant cell line (SKBR3 and LS180). The induction of pERK was also found to be persistent. It was detectable by 0.5 hour, remained high for 8 hours, and then returned to untreated levels by 24 hours (Fig. 5C). Hep3B cells were also pretreated with the MEK1/2 inhibitor U0126 to antagonize ERK1/2 phosphorylation induced by PM-20. We found that induction of ERK1/2 phosphorylation and growth inhibition by PM-20 were both antagonized by pretreatment of the cells with the MEK inhibitor (Fig. 5D). These two results suggested that the induction of ERK1/2 phosphorylation probably was involved in growth inhibition by PM-20.

We previously found that Cdc25A could act as a pERK phosphatase (30). Therefore, Cdc25A expression was determined in Hep3B cells by Western blotting. We found that Cdc25A was expressed more in Hep3B cells than other tumor cells (Fig. 5B). We also transfected plasmid vectors expressing Cdc25Awt (wild type), Cdc25Amut (catalytically inactive Cdc25A), or the vector control into Hep3B cells and examined the consequences for growth inhibition after addition of PM-20. Growth inhibition induced by PM-20 was observed in the cdc25Amut- and the vector-transfected cells. However, growth inhibition was antagonized in the cdc25Awt-transfected Hep3B cells. However, the cdc25awt-transfected Hep3B cells were growth inhibited at higher concentration of PM-20 (Fig. 5E). Cdc25A protein overexpression (5-fold) in the transfected cells was determined by Western blots (Fig. 5F).

| Table 1. IC50 of the PM-20 or PM-26 for growth inhibition of various human tumor cell lines |
|-----------------|-----------------|-----------------|-----------------|
| Organ | Cell line | IC50 (μmol/L) | PM-20 | PM-26 |
|-----------------|-----------------|-----------------|-----------------|
| Breast | MCF7 | 4 | >20 |
| Breast | SKBR3 | 6 | >20 |
| Skin | FemX | 2.5 | >20 |
| Stomach | HR | 6 | >20 |
| Tongue | PCI | 2.5 | >20 |
| Colon | LS180 | 20 | >20 |
| Liver | Hep3B | 0.7 | >20 |

NOTE: Tumor cell lines established from several human tumors (MCF7 and SKBR3, mammary carcinoma; FemX, melanoma; HR, gastric carcinoma; PCI, squamous cell carcinoma from tongue; LS180, colon carcinoma; and Hep3B, hepatocellular carcinoma) were cultured with PM-20 or PM-26 for 3 days. IC50 was determined from the growth inhibition curves. The maximum concentration of PM used in this experiment was 20 μmol/L.
PM-20 Inhibited Transplantable Hepatoma Growth 

PM-20 was injected i.p., every other day, into Fischer rats at a dose of 1, 10, or 20 mg/kg of body weight for a total of five injections. Four rats were treated in each group. All rats tolerated these doses without any sign of toxicity, which was monitored daily by measuring body weight and general reflexes for 3 weeks.

PM-20 was tested in vivo for activity against hepatoma cell growth in rat liver. A syngeneic transplantable rat hepatoma cell line, JM-1, was grown in tissue culture. The hepatoma cells were harvested, washed, and counted and then were directly injected under direct vision into rat livers through the mesenteric vein. The rats were treated i.p. with either PM-20 or its solvent (DMSO) as control (five rats in each group). Rats were sacrificed after 2 weeks and the number of tumor foci was counted in the PM-20-treated and untreated livers. PM-20 treatment was found to significantly reduce the number of liver tumors compared with the controls (Fig. 6A) showing that PM-20 was also an active tumor cell growth inhibitor in vivo. ERK induction was found to be induced in the tumor tissues of PM-20-treated but not in untreated rats (Fig. 6B).

Discussion

Expression of protein phosphatases is frequently induced in cancer (10, 31) and phosphatases are as important as the kinases for the maintenance of the cancer phenotype. Consequently, many phosphatases are potentially important targets for anticancer drugs. Unfortunately, it has been difficult to design selective phosphatase inhibitors because of considerable structural similarity of the active site regions in this group of proteins.

We previously examined a series of natural and synthetic K-vitamin analogues and found that several were cell growth inhibitors in vitro (IC50 in 5–100 μmol/L range) and in vivo (16, 17, 19, 21, 22–24). Cpd 5, the prototype analogue, was found to inhibit phospho-ERK phosphatase activity, as well as the Cdc25 family of protein tyrosine phosphatases, by binding to the catalytic cysteine of the protein tyrosine phosphatase enzyme (20). However, the limited potency of the quinone derivatives prompted us to examine a small synthetic library of N-arylmaleimide derivatives, which were designed to be protein tyrosine phosphatase inhibitors. One of these compounds, PM-20, was chosen for further study because it was a potent inhibitor of cell growth (IC50, 700 nmol/L in Hep3B cells). PM-20-induced growth inhibition was not specific only for Hep3B hepatoma cells. Other human tumor cell lines (FemX, MCF7, SKBR3, HR, PCI, and LS180) were also inhibited to a varying degree (2.5–20 μmol/L) although not as potently as Hep3B cells. PM-20 also showed 10-fold selectivity against Hep3B hepatoma cells compared with normal rat hepatocytes when both were cultured under identical conditions.

PM-20 retains the thioethanol side chain that was found to be important in the K-vitamin series. An important feature is that the maleimide derivatives are potent Michael acceptors and are expected to react covalently with protein tyrosine phosphatase active site cysteines. However, unlike the quinone derivatives, PM-20 is not able to undergo redox cycling, which is thought to limit the activity of the former because the reduced hydroquinone form is incapable of undergoing the Michael reaction (32).

The structure-activity data from the N-arylmaleimide series suggest that small variations in the structure of the...
proteins. We previously showed that tyrosine-phosphorylated Cdk4 without affecting the level of the unphosphorylated PM-20 up-regulated tyrosine-phosphorylated Cdk2 and Cdc25A substrates Cdk2 and Cdc4. Figure 2B shows that PM-20 would inhibit the dephosphorylation of the active site cysteine is relatively slow. The selective inhibition of the Cdc25 enzymes suggested that PM-20 would inhibit the dephosphorylation of the Cdc25A substrates Cdk2 and Cdk4. Figure 2B shows that PM-20 up-regulated tyrosine-phosphorylated Cdk2 and Cdk4 without affecting the level of the unphosphorylated proteins. We previously showed that tyrosine-phophorylated ERK1/2 is a substrate for Cdc25A (30). Consistent with that, we found that PM-20 induced persistent and strong ERK1/2 phosphorylation (Fig. 5C and D). We also found that ERK1/2 phosphorylation induced by PM-20 was probably responsible for the observed growth inhibition of Hep3B cells. Induction of ERK1/2 phosphorylation by PM-20 correlated with its potency of growth inhibition in several tumor cell lines (Fig. 5A). Moreover, when ERK1/2 phosphorylation was inhibited by the MEK1/2 inhibitor U0126 in PM-20-treated cells, the growth inhibitory effects of PM-20 were also almost completely antagonized (Fig. 5D). ERK1/2 is usually activated in response to mitogens, such as epidermal growth factor, in various cell types, as well as in response to differentiation factors, such as nerve growth factor (33). Although ERK is thought to play a key role in cell proliferation, it has been suggested that persistent ERK activation might also mediate cell cycle arrest and differentiation. For instance, in rat PC12 cells, both epidermal growth factor and nerve growth factor trigger ERK activation, but only nerve growth factor can induce PC12 cell differentiation associated with prolonged ERK phosphorylation (34, 35). In Hep3B cells, prolonged ERK activation has also been shown to be involved in growth inhibition by other protein tyrosine phosphatase antagonists (17, 21–23).
The expression of MKP1 was negligible and expression of Cdc25A was highest in the most sensitive Hep3B cells (Fig. 5B).

Normal liver epithelial cell lines are not readily available. Therefore, we have used cells derived from normal rat liver to study the antiproliferative effects of PM-20 and compared it with Hep3B hepatoma cells, which were cultured under identical condition. PM-20 seems to have a useful therapeutic index because it was significantly more inhibitory to Hep3B cells than to normal hepatocytes in similar culture conditions (Fig. 3). This finding was supported by an in vivo experiment involving an orthotopic model of hepatocellular carcinoma in Fischer 344 rats. JM-1 rat hepatoma was introduced into the liver via the mesenteric vein (21). Intraperitoneal treatment with 1 mg/kg of PM-20 in DMSO was initiated 2 days later and repeated five times every second day. The antitumor effect of PM-20 is apparent from Fig. 6A, which shows that the average tumor burden of untreated animals was 11 tumors per rat whereas the treated animals had an average of 2 tumors per rat. pERK was found to be induced in the tumor tissue 4 hours after the last PM-20 treatment, suggesting an in vivo molecular effect of PM-20 that was similar to in vitro effects.

PM-20 is thus a representative of a new class of protein tyrosine phosphatase inhibitors, which has in vitro activity against several tumor types and potent in vivo activity against a syngeneic hepatocellular carcinoma in the rat. It is a selective inhibitor of Cdc25A and induces hyperphosphorylation of ERK1/2. The data support the hypothesis that phosphorylation of ERK1/2 is responsible for tumor growth inhibition, likely as a result of Cdc25 inhibition.

To assess the role of Cdc25A further in up-regulation of ERK1/2 phosphorylation, plasmid vector expressing cdc25A was transfected into Hep3B cells and growth inhibition by PM-20 was measured in the transfected cells. PM-20-mediated growth inhibition of Hep3B cells was antagonized in the PM-20-transfected cells. However, transfectant cells expressing cdc25A transfected into the active site cysteine mutant, C430S) or vector-transfected cells were not resistant to PM-20, consistent with the obligatory role of the active site cysteine for binding to PM-20 (Fig. 5E and F).

The ability of PM-20 to inhibit ERK1/2 phosphatases was supported by our finding that cell lysates contained ERK phosphatase activity, which was inhibited by PM-20 action (Fig. 4A). We showed that the ERK-selective phosphatase MKP1 was effective in pERK2 dephosphorylation but this activity was not inhibited by PM-20 (Fig. 4B). This suggested that the levels of MKP1 are negligible in Hep3B cells, as confirmed by our experiment (Fig. 5B).

Table 1 shows the activity of PM-20 against several tumor cell lines from various organs. Whereas the hepatocellular carcinoma line Hep3B was the most sensitive line, there was a spectrum of activity in the other lines. Growth inhibitory activity correlated with the induction of pERK (Fig. 5A). The expression of MKP-1 was negligible and expression of Cdc25A was highest in the most sensitive Hep3B cells (Fig. 5B).

Figure 6. PM-20 inhibited growth of transplantable tumor cells in rat liver. JM-1 rat hepatoma cells were grown in tissue culture. $1 \times 10^6$ cells were injected into rat livers (two groups of five per group) through the mesenteric vein. Two days after cell transplantation, PM-20 was injected (group 1) i.p. at a dose of 1 mg/kg of body weight, which was administered every other day for a total of five injections. The control group had the DMSO solvent. Rats were sacrificed 2 wks after cell transplantation and the number of liver tumors was counted in the PM-20-treated and untreated rat (A). JM-1 tumor-bearing rats were treated with PM-20 (1 mg/kg) and the tumors were harvested after 1 h. Cell lysates prepared from the tumor tissue, 4 h after the last PM-20 treatment, were probed with pERK and ERK2 (control) antibodies (B). pERK expression in normal liver and JM-1 tumor tissues served as controls. SD and $P$ values were calculated from three measurements.
PM-20, a novel inhibitor of Cdc25A, induces extracellular signal–regulated kinase 1/2 phosphorylation and inhibits hepatocellular carcinoma growth in vitro and in vivo

Siddhartha Kar, Meifang Wang, Wei Yao, et al.

Mol Cancer Ther 2006;5:1511-1519.

Updated version  Access the most recent version of this article at: http://mct.aacrjournals.org/content/5/6/1511

Cited articles  This article cites 35 articles, 12 of which you can access for free at: http://mct.aacrjournals.org/content/5/6/1511.full#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/5/6/1511.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/5/6/1511. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.