

# Tautomycetin inhibits growth of colorectal cancer cells through p21<sup>Cip/WAF1</sup> induction via the extracellular signal-regulated kinase pathway

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## Abstract

Tautomycetin is an antifungal antibiotic retaining potent immunosuppressive function. We have identified the roles of tautomycetin on cellular proliferation and transformation of colorectal cancer cells. The proliferation and anchorage-independent growth of HCT-15, HT-29, and DLD-1 colorectal cancer cells were efficiently inhibited without induction of apoptosis by 150 nmol tautomycetin. These growth inhibitory effects were dependent on p21<sup>Cip/WAF1</sup> induction via the extracellular signal-regulated kinase pathway, and the tautomycetin effects were abolished in HCT-116 colon cells and eight other types of cells that did not induce p21<sup>Cip/WAF1</sup> by 150 nmol tautomycetin. The crucial role of p21<sup>Cip/WAF1</sup> in the extracellular signal-regulated kinase pathway-dependent antiproliferative responses by tautomycetin was confirmed by using p21<sup>Cip/WAF1</sup> gene-deleted HCT-116 cells. The growth inhibitory effect of tautomycetin was acquired by regulation of Raf-1 activity through inhibition of protein phosphatase type 1 and protein phosphatase type 2A with high preference toward protein phosphatase type 1. Tautomycetin could be a potential drug for colorectal cancer. [Mol Cancer Ther 2006;5(12):3222–31]

## Introduction

Tautomycetin, an antifungal antibiotic originally isolated from *Streptomyces griseochromogens*, has been identified as a

potent T cell-specific immunosuppressor (1, 2). Tautomycetin inhibits tyrosine phosphorylation of intracellular signaling molecules involved in various cellular responses such as T-cell receptor-proximal signaling (2). However, other functions of tautomycetin, such as growth control, have yet to be characterized.

The extracellular signal-regulated kinase (ERK) pathway is a major signaling pathway for cellular growth and transformation (3–5). On the other hand, a role for the ERK pathway in growth inhibition has also been reported (6–12). The ability of the ERK pathway to regulate these divergent effects on cellular growth is related to ERK signaling intensity, duration, and subcellular compartmentalization (13). ERK-mediated growth inhibition is associated with p21<sup>Cip/WAF1</sup> activation (11–14). Induction of p21<sup>Cip/WAF1</sup> occurs by both p53-dependent and ERK pathway-dependent mechanisms (15, 16). The latter occurs by hyperactivation of Raf-1 (10, 11). The p21<sup>Cip/WAF1</sup> protein is a cell cycle regulator that inhibits G<sub>1</sub> to S cell cycle progression (15, 16). Activation of cyclin D/cyclin-dependent kinase 4 and cyclin E/cyclin-dependent kinase 2 complexes is inhibited by p21<sup>Cip/WAF1</sup>, and induction of p21<sup>Cip/WAF1</sup> is an indicator for growth arrest in colorectal and other types of cells (6–12, 17).

Colorectal cancer is one of the most common human cancers in the Western world (18). Most of the clinically used drugs for treatment of colorectal cancer such as fluorouracil are systemic antiproliferative agents retaining cytotoxic effects (19). Currently, a limited number of effective anticancer drugs retaining specific curative potential for colorectal cancer are available. In this study, we identified tautomycetin as a drug specifically inhibiting growth of colorectal cancer cells without inducing any significant cellular toxicity and apoptosis at 150 nmol. The antiproliferative effect of tautomycetin was related with p21<sup>Cip/WAF1</sup> induction involving hyperactivation of ERKs, and the tautomycetin effect by 150 nmol tautomycetin was not observed in eight other cell types that abolished ERK activation and subsequent p21<sup>Cip/WAF1</sup> induction. With the use of chemical and genetic inhibitors of ERK pathway components, as well as with p21<sup>Cip/WAF1</sup> gene-deleted HCT-116 cells, we show that ERK pathway-dependent p21<sup>Cip/WAF1</sup> induction is required for tautomycetin-induced growth arrest and antiproliferative responses. We also provide evidence that tautomycetin regulates the ERK pathway by modulating Raf-1 activity through protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A) inactivation with preference toward PP1. Tautomycetin is a potential anticancer drug retaining specificity toward colorectal cancer.

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## Materials and Methods

### Materials

HCT-116  $p21^{Cip/WAF1}+/+$  human colon carcinoma cell line and the derivative line HCT-116  $p21^{Cip/WAF1}-/-$ , in which both  $p21^{Cip/WAF1}$  alleles are deleted by homologous recombination, were provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD; ref. 20). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). DMEM, McCoy's 5A, RPMI 1640, fetal bovine serum (FBS), antibiotics, and Lipofectamine plus reagents were purchased from Life Technologies, Inc. (Grand Island, NY). Phospho-ERK (p-ERK), phospho-mitogen-activated protein kinase/ERK kinase (p-MEK), phospho-c-jun NH<sub>2</sub>-terminal kinase (p-JNK), phospho-p38 (p-p38), phospho-GSK3 $\beta$ (Ser<sup>9</sup>) (p-GSK3 $\beta$ ),  $\beta$ -catenin, caspase-3, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody was obtained from Bio-Rad Laboratories (Hercules, CA). Anti-p21<sup>Cip/WAF1</sup> and phospho-Akt (p-Akt) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Annexin V-phycoerythrin and propidium iodide were purchased from BD Pharmingen (San Diego, CA). Anti-phospho-Raf-1(Ser<sup>338</sup>) (p-Raf-1), anti-PP2Ac, and anti-PP1c antibodies, as well as RAS activation assay kit and PP2A phosphatase assay kit, were purchased from Upstate Biotechnology (Lake Placid, NY).  $\alpha$ -Tubulin antibody was purchased from Oncogene Research Products (San Diego, CA). Protran nitrocellulose membrane was purchased from Schleicher & Schuell Co. (Dassel, Germany) and an enhanced chemiluminescence system was obtained from Amersham, Inc. (Buckinghamshire, United Kingdom). A luciferase assay kit was purchased from Promega Co. (Madison, WI). PD98059 was purchased from Calbiochem (La Jolla, CA). 4',6-Diamidino-2-phenylindole was purchased from Boehringer Mannheim (Mannheim, Germany). Goat anti-mouse Cy2-X-conjugated secondary antibody was purchased from Jackson ImmunoResearch laboratories, Inc. (West Grove, PA) and anti-BrdUrd monoclonal antibody was purchased from DAKO Co. (Carpinteria, CA). All other chemicals were purchased from Sigma (St. Louis, MO). The Elk-1-dependent PathDetect plasmids (pFR-Luc and pFA2-Elk-1) were purchased from Stratagene (La Jolla, CA). The  $p21^{Cip/WAF1}$ -promoter reporter construct p21P was provided by Dr. X.F. Wang (Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC; ref. 21) and pSVSport1-dn-Raf was obtained from J.H. Kim (Korea University, Seoul, Korea). pCMV-MEK-2a was obtained from G. Johnson (National Jewish Medical Research Center, Denver, CO). Purified tautomycetin was prepared as previously described (2).

### Cell Culture

The HCT-15, NIH 3T3, HEK293, L929, MCF-7, HeLa, Chang, and COS-7 cells were maintained in DMEM. The HT-29, HCT-116  $p21^{Cip/WAF1}+/+$ , and HCT-116  $p21^{Cip/WAF1}-/-$  cells were maintained in McCoy's 5A medium. DLD-1 and HepG2 cells were maintained in RPMI 1640.

Cell culture medium was supplemented with 10% (v/v) FBS, 100 units/mL penicillin, and 250 ng/mL streptomycin, and cultures were kept in 5% CO<sub>2</sub> at 37°C. To observe tautomycetin effects, cells were treated with tautomycetin at concentrations ranging from 0 to 500 nmol. The MEK inhibitor PD98059, at concentrations ranging from 0 to 60  $\mu$ mol, was added 1 h before tautomycetin treatment.

### Western Blot Analysis

Cells were collected by scrapping in 200  $\mu$ L of ice-cold PBS, centrifuged at 4,000  $\times$  g at 4°C for 5 min, and lysed directly in Laemmli SDS sample buffer. The samples were boiled at 100°C and the lysates were electrophoresed on an 8% to 12% SDS polyacrylamide gel. Western blot analysis was done using anti-p-ERK, anti-p-MEK, anti-p-Raf-1(Ser<sup>338</sup>), anti-p-Akt, anti-p21<sup>Cip/WAF1</sup>, anti-p-JNK, anti-p-p38, anti-p-GSK3 $\beta$ (Ser<sup>9</sup>), anti- $\beta$ -catenin, anti-caspase-3, or anti- $\alpha$ -tubulin antibodies as previously described (10).

### Transfection and Transient Reporter Assay

HCT-15 cells were plated onto six-well plates at 50% confluence. Twenty-four hours after plating, cell transfections were done using Lipofectamine plus transfection reagent according to the manufacturer's instructions. To normalize for variable transfection efficiencies, cells were cotransfected with 50 ng/ $\mu$ L pCMV  $\beta$ -gal reporter. Twenty-four hours after transfection, cells were incubated in 150 nmol tautomycetin for an additional 24 h. The cells were then rinsed twice in ice-cold PBS and resuspended in reporter lysis buffer for luciferase assay.

### Cell Counting, Flow Cytometry, and Apoptosis Assay

HCT-15 cells were seeded onto six-well plates at a density of  $1 \times 10^4$  per well. Tautomycetin was added at a concentration of 150 nmol. The cells were incubated in a CO<sub>2</sub> incubator for the indicated times at 37°C, and the attached cells were washed with PBS and harvested with Trypsin solution (0.05% trypsin, 0.5 mmol EDTA in PBS). A 1:1 mixture of 0.4% trypan blue dye and cell suspension was placed onto a Tiefe Depth Profondeur 0.0025-mm<sup>2</sup> cell counting plate (Superior Co., Lauda-Koenigshofen, Germany) and examined under a microscope to quantify cell numbers. For fluorescent activating cell sorting (FACS) analysis, cells were grown on coverslips at 30% confluence in medium supplemented with 10% FBS  $\pm$  150 nmol tautomycetin for 24 h. In some cases, 10 to 20  $\mu$ mol of PD98059 were added to HCT-15 cultures 1 h before tautomycetin treatment. The cells were labeled with 20  $\mu$ mol bromodeoxyuridine (BrdUrd) for 8 h before harvesting for immunocytochemical analysis. FACS analysis was done as previously described (22) using the ModFit LT 2.0 program (Verity Software House, Inc., ME) and the WinMOLDI 2.8 program created by Joseph Trotter of Scripps Research Institute (La Jolla, CA).

### Immunocytochemistry and BrdUrd Incorporation

Cells were grown on coverslips at 30% confluence in medium supplemented with 10% FBS  $\pm$  150 nmol tautomycetin for 24 h. In some cases, 10 to 20  $\mu$ mol of PD98059 were added to HCT-15 cultures for 1 h before tautomycetin treatment. The cells were labeled with 20  $\mu$ mol BrdUrd for 8 h before harvesting for immunocytochemical analysis.

The cells were fixed in methanol/formaldehyde (99:1), permeabilized with PBS containing 0.2% Triton X-100, and blocked in PBS containing 1% bovine serum albumin, 0.1% gelatin, and 5% goat serum. Cells were then incubated with p21<sup>Cip/WAF1</sup> antibody (1:100) for 3 h followed by incubation in rhodamine-conjugated goat anti-rabbit secondary antibody (1:200) for 1 h. Immunocytochemical analysis was done as previously described (22).

#### Foci Formation Assay

HCT-15, HT-29, DLD-1, HCT-116, COS-7, HEK293, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells were cultured for 24 h in six-well plates, and 0.5 µg/µL pcDNA3.0 was transfected into the cells using Lipofectamine plus reagent. After transfection, cells were maintained in the medium with 10% FBS for 2 weeks. G418 was added to the medium at a final concentration of 800 µg/mL. Medium containing G418 was exchanged twice a week. After 2 weeks, resistant colonies were stained with crystal violet.

#### RAS-GTP Loading Assay

The capacity of Ras-GTP to bind to the RAS-binding domain of Raf-1 was used as an indicator of Ras activation status (23). Ras-GTP loading assay was done as described in previous assay (22).

#### Purification of PP1 $\gamma$ and PP2Ac

The 1-kb cDNA fragment of the catalytic subunit of PP1 $\gamma$  (PP1 $\gamma$ ) was obtained by reverse transcription-PCR (RT-PCR) against human cDNA library from HEK293 cells by using 5'-AAAAGGATCCGCGGATTTAGATAAACT-CAACATC-3' and 5'-AACTCGAGTTTCTTTGCTTGCTTTGTGATTCA-3' primers. The PCR product was cleaved with *Bam*H1 and *Xho*I restriction enzymes and cloned into identical sites of pGEX-4T3 bacterial expression vector (Amersham Bioscience, Piscataway, NJ) and named as pPP1 $\gamma$ . The pPP1 $\gamma$  was transformed into *Escherichia coli* BL21 (DE3) pLysE. The vector for bacterial production of glutathione *S*-transferase (GST)-fused PP2Ac (24) was kindly provided by Dr. Kenneth J. Soprano (Temple University School of Medicine, Philadelphia, PA). GST-PP1 $\gamma$  and GST-PP2Ac proteins were overexpressed by 16-h induction with 1 mmol isopropyl  $\beta$ -D-1-thiogalactopyranoside at 22°C, and purified with glutathione-Sepharose beads.

#### PP1 and PP2A Phosphatase Assays

PP1 and PP2A activities were measured using a malachite green-based phosphatase assay system (PP2A Immunoprecipitation Phosphatase Assay Kit, Upstate Biotechnology). For *in vitro* PP1 and PP2A phosphatase assays, HCT-15 cells were grown at a density of  $1 \times 10^5$  per well, and cells were lysed in buffer devoid of phosphatase inhibitors [20 mmol imidazole-HCl, 2 mmol EDTA, 2 mmol EGTA (pH 7.0) with 10 µg/mL each of aprotinin, leupeptin, pepstatin, 1 mmol benzamidine, and 1 mmol phenylmethylsulfonyl fluoride]. Whole-cell lysate (500 µg) was incubated with protein A/G agarose slurry in the presence of 4 µg of anti-PP2Ac or anti-PP1c antibody (Upstate Biotechnology) at 4°C with constant rocking for 8 h. After being washed with 700 µL TBS (thrice) and 500 µL optimized Ser/Thr buffer (final wash), agarose-bound

immune complexes were resuspended in 50 µL of Ser/Thr buffer [50 mmol Tris-HCl (pH 7.0), 100 µmol CaCl<sub>2</sub>]. These immunoprecipitated PP1c and PP2Ac and purified GST-PP1 $\gamma$  and GST-PP2Ac were then incubated with tautomycetin (0–150 nmol) for 30 min at 30°C. The phosphopeptide substrate for PP2Ac and PP1c (amino acid sequence, K-R-pT-I-R-R) was added and samples were incubated at 30°C in a shaking incubator for 10 min. Twenty-five microliters of supernatant were transferred to a 96-well plate and released phosphate was measured by adding 100 µL of malachite green phosphate detection solution. Color was allowed to develop for 15 min before reading the plate at 650 nm. For *in vivo* PP1 and PP2A phosphatase assays, HCT-15 cells were grown with or without 150 nmol tautomycetin for 24 h and the extracts were processed as described above for the measurement of PP1 and PP2A activity.

## Results

### Tautomycetin Inhibits Proliferation and G<sub>1</sub>-to-S Cell Cycle Progression in HCT-15 Cells

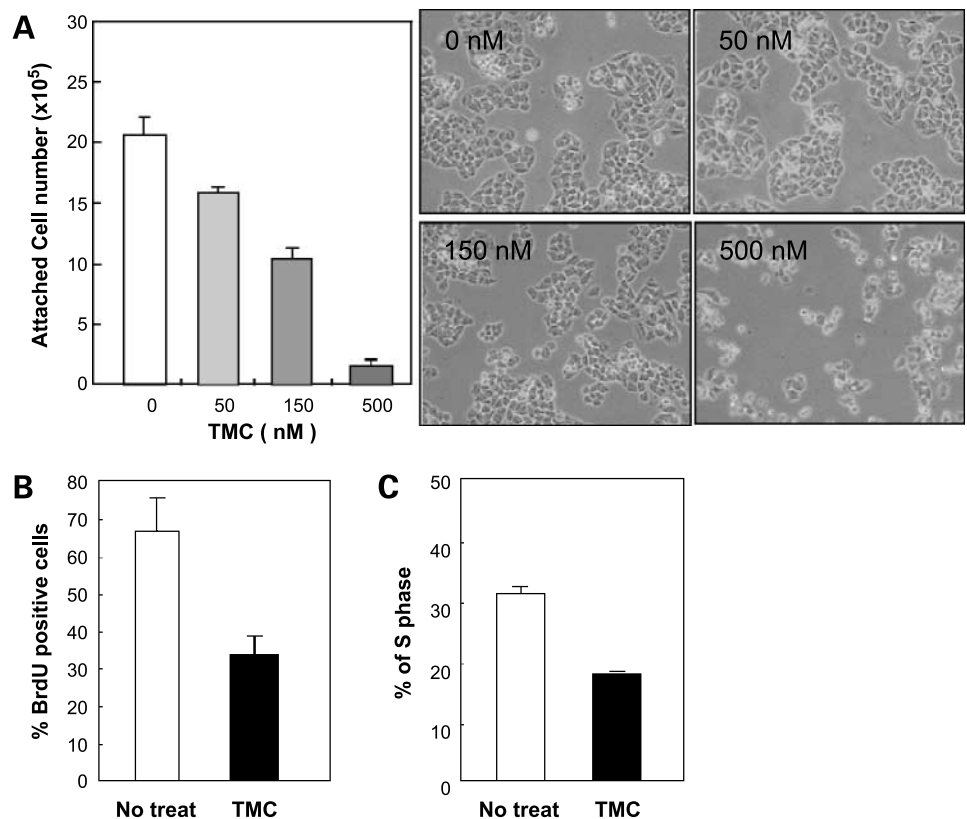
HCT-15 cells were cultured in increasing concentrations of tautomycetin (0–500 nmol) for 96 h. As shown in Fig. 1A, the number of attached cells was decreased in a stepwise fashion. The cells were not significantly detached from the plates when grown in medium containing 150 nmol tautomycetin. In contrast, significant numbers of cells were detached and floated to the surface when cultured in 500 nmol tautomycetin (Fig. 1A, right). Based on these results, we used 150 nmol tautomycetin throughout the remainder of the study.

Next, the effect of tautomycetin on cellular proliferation was examined using BrdUrd incorporation as an index of proliferation. The percentage of cells incorporating BrdUrd was 33% in cultures treated with 150 nmol tautomycetin and 67% in untreated cultures (Fig. 1B). The effect of tautomycetin on cell cycle progression was also examined. FACS analysis revealed that the percentage of cells in S phase was reduced from 31.9% to 17.3% when cultures were treated with 150 nmol tautomycetin (Fig. 1C).

### Tautomycetin Induces p21<sup>Cip/WAF1</sup> via the Raf-1 → MEK → ERK Cascade in HCT-15 Cells

To characterize the involvement of the ERK pathway in tautomycetin-mediated growth regulation, phosphorylation of ERK, MEK, and Raf-1 was examined by Western blot analysis. As shown in Fig. 2A, the phosphorylation of ERKs, as well as that of the upstream kinases, Raf-1 and MEK, was simultaneously increased in cultures treated with 150 nmol tautomycetin for up to 24 h. In contrast, no increase in levels of  $\beta$ -catenin and phosphorylation of Akt, JNK, p38, and GSK3 $\beta$ (Ser<sup>9</sup>), an alternative proliferative pathway component, was observed under these conditions (Fig. 2A). In addition, there were no significant changes of amounts of the fully processed fragments of caspase-3 (p17) after tautomycetin treatment. Because induction of the cell cycle regulator p21<sup>Cip/WAF1</sup> is a well-established marker of ERK-dependent growth arrest, we measured

**Figure 1.** Effect of tautomycetin on growth, proliferation, and cell cycle progression of HCT-15 cells. **A**, HCT-15 cells were treated with varying concentrations of tautomycetin (TMC; 0, 50, 150, and 500 nmol) and the number of attached cells was counted 96 h later. Cell morphologies were analyzed by phase-contrast microscopy and photographed (magnification,  $\times 200$ ). **B**, BrdUrd (BrdU) incorporation was measured after 24 h treatment with 150 nmol tautomycetin. Quantitative measurements of the percentage of BrdUrd positive cells are presented. **C**, HCT-15 cells were grown and synchronized with double thymidine blocking. Tautomycetin was added 24 h before the cells were harvested for FACS analysis. The relative percentage of cells in S-phase is presented. Columns, mean of three independent experiments; bars, SD.



p21<sup>Cip/WAF1</sup> levels in cells treated with tautomycetin. As shown in Fig. 2A, p21<sup>Cip/WAF1</sup> protein levels were increased in cells treated with tautomycetin. Moreover, p21<sup>Cip/WAF1</sup> induction coincided with activation of the ERK pathway components (Fig. 2A).

To determine if tautomycetin-induced p21<sup>Cip/WAF1</sup> induction is dependent on activation of the ERK pathway, cultures were incubated with the MEK-specific inhibitor PD98059 before tautomycetin treatment. PD98059 blocked tautomycetin-induced ERK activation and abolished p21<sup>Cip/WAF1</sup> induction in a dose-dependent manner (Fig. 2B). At 60  $\mu$ mol PD98059, a significant level of p21<sup>Cip/WAF1</sup> protein was still detectable, whereas phosphorylated ERK was not (Fig. 2B, bottom). The partial reduction of p21<sup>Cip/WAF1</sup> by PD98059 may indicate that some p21<sup>Cip/WAF1</sup> was induced independently of ERK.

To determine whether p21<sup>Cip/WAF1</sup> induction is due to transcriptional activation, we measured tautomycetin-induced p21<sup>Cip/WAF1</sup> promoter activation in the presence and absence of ERK pathway inhibition. First, Elk-1 reporter activity was measured to confirm ERK pathway activity. Elk-1 reporter activity was increased by tautomycetin treatment, and this increase was blocked by PD98059. Elk-1 reporter activity was even more significantly blocked by cotransfection of dominant negative Raf-1 (dn-Raf-1) or catalytically inactive MEK (MEK-2a; Fig. 2C, bottom). These results indicate that tautomycetin-induced ERK pathway activation is transmitted into nucleus. Tautomycetin

treatment also resulted in an increase in p21<sup>Cip/WAF1</sup> promoter activity, and this increase was also blocked by PD98059 and by cotransfection of the vector for dn-Raf-1 or MEK-2a (Fig. 2D).

#### ERK-Dependent Nuclear Accumulation of p21<sup>Cip/WAF1</sup> Mediates Tautomycetin-Induced Growth Arrest

BrdUrd incorporation was monitored in individual cells in which p21<sup>Cip/WAF1</sup> had been induced by tautomycetin treatment. HCT-15 cells actively incorporated BrdUrd when grown in 10% FBS medium (Fig. 3A). Tautomycetin treatment increased the number of cells positive for p21<sup>Cip/WAF1</sup> and the number of cells that had p21<sup>Cip/WAF1</sup> enriched in their nuclei (Fig. 3A). Importantly, BrdUrd incorporation was reduced from 63% to 25% by tautomycetin treatment and was not observed in cells that displayed accumulated nuclear p21<sup>Cip/WAF1</sup>.

The tautomycetin-induced reduction of BrdUrd incorporation was significantly rescued by PD98059 pretreatment (25% versus 52%). BrdUrd-positive cells were reduced from 63% to 53% by treatment with PD98059 alone (Fig. 3B). A similar effect of PD98059 was observed on tautomycetin-induced inhibition of G<sub>1</sub> to S phase progression. The percentage of cells in S phase was increased from 22% to 33% by PD98059 pretreatment (Fig. 3C). Treatment with PD98059 alone reduced G<sub>1</sub> to S phase progression, as compared with untreated cells. On the other hand, 150 nmol tautomycetin did not induce apoptosis of HCT-15 cells as shown in Annexin V and

propidium iodide costaining followed by FACS analyses (Fig. 3D).

### ERK-Inducible p21<sup>Cip/WAF1</sup> Activation Is Essential for Tautomycetin-Induced Antiproliferation of Colorectal Cancer Cell

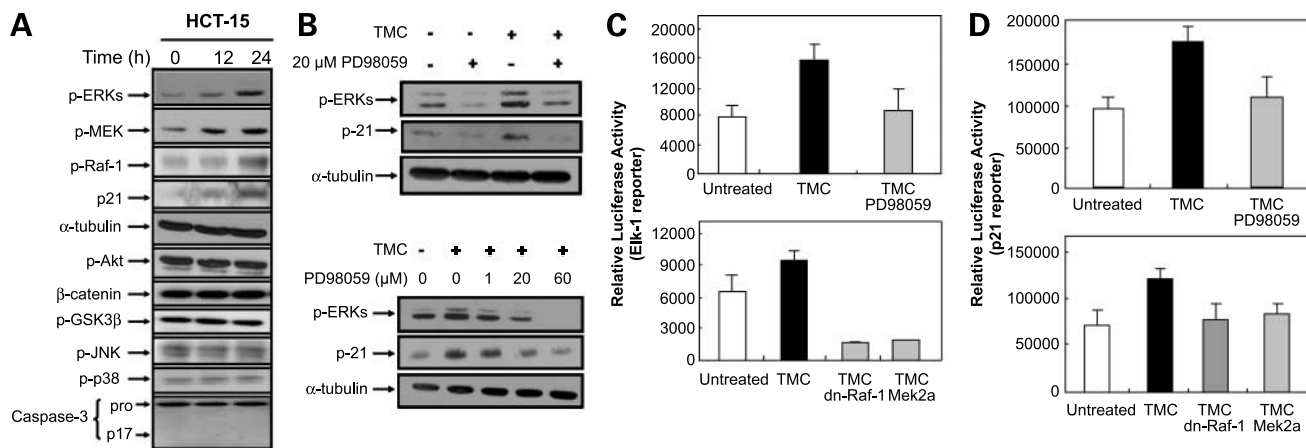
To determine if our observations of tautomycetin effects on HCT-15 colorectal cancer cells could be extended to other colorectal cancer cell lines, proliferation was measured in HT-29, DLD-1, and HCT-116 cells exposed to 150 nmol tautomycetin. The percentage of BrdUrd-positive cells in HT-29 and DLD-1 cultures was reduced by tautomycetin treatment, but this was not the case in HCT-116 cultures (Fig. 4A). As observed in HCT-15 cells, levels of p21<sup>Cip/WAF1</sup> protein were significantly increased by 150 nmol tautomycetin exposure in HT-29 and DLD-1 cells (Fig. 4B). However, basal p21<sup>Cip/WAF1</sup> levels were aberrantly high in HCT-116 cells and p21<sup>Cip/WAF1</sup> levels did not increase in response to tautomycetin treatment in these cells (Fig. 4B).

To understand the requirements for p21<sup>Cip/WAF1</sup> in tautomycetin-induced antiproliferative responses in colorectal cancer cells, we examined the effect of tautomycetin on proliferation of p21<sup>Cip/WAF1</sup> -/- HCT-116 cells and p21<sup>Cip/WAF1</sup> +/+ HCT-116 cells. Tautomycetin did not induce any changes in the amount of nuclear p21<sup>Cip/WAF1</sup> in p21<sup>Cip/WAF1</sup> -/- and p21<sup>Cip/WAF1</sup> +/+ cells (Fig. 4C). Moreover, the percentage of BrdUrd-positive cells was not significantly altered in p21<sup>Cip/WAF1</sup> -/- or in p21<sup>Cip/WAF1</sup> +/+ cultures exposed to tautomycetin (Fig. 4D). Interestingly, the basal level of BrdUrd-positive cells was 2-fold higher in HCT-116 p21<sup>Cip/WAF1</sup> -/- cells compared that in p21<sup>Cip/WAF1</sup> +/+ cells. The percentage of BrdUrd-

positive cells was not decreased by 150 nmol tautomycetin treatment in noncolorectal cancer cells including COS-7 monkey kidney cells, HEK293 human embryonic kidney cells, Chang human liver cells, HepG2 human liver cancer cells, HeLa human cervical cancer cells, MCF-7 breast cancer cells, and L929/NIH 3T3 mouse fibroblast cells (Fig. 5A). Moreover, treatment of these cells with 150 nmol tautomycetin did not induce ERK activation or p21<sup>Cip/WAF1</sup> induction (Fig. 5B). Others have shown that ERK activity is decreased in COS-7 cells in response to 5  $\mu$ mol tautomycetin (25). We have also observed a decrease in ERK activity in COS-7 cells treated with tautomycetin at concentrations >150 nmol. Under these conditions and at even greater tautomycetin concentrations (5  $\mu$ mol), p21<sup>Cip/WAF1</sup> levels did not increase (Fig. 5C). On the other hand, ERK activity and p21<sup>Cip/WAF1</sup> levels were increased in a stepwise fashion in HCT-15 cells treated with 0 to 5  $\mu$ mol tautomycetin (Fig. 5C).

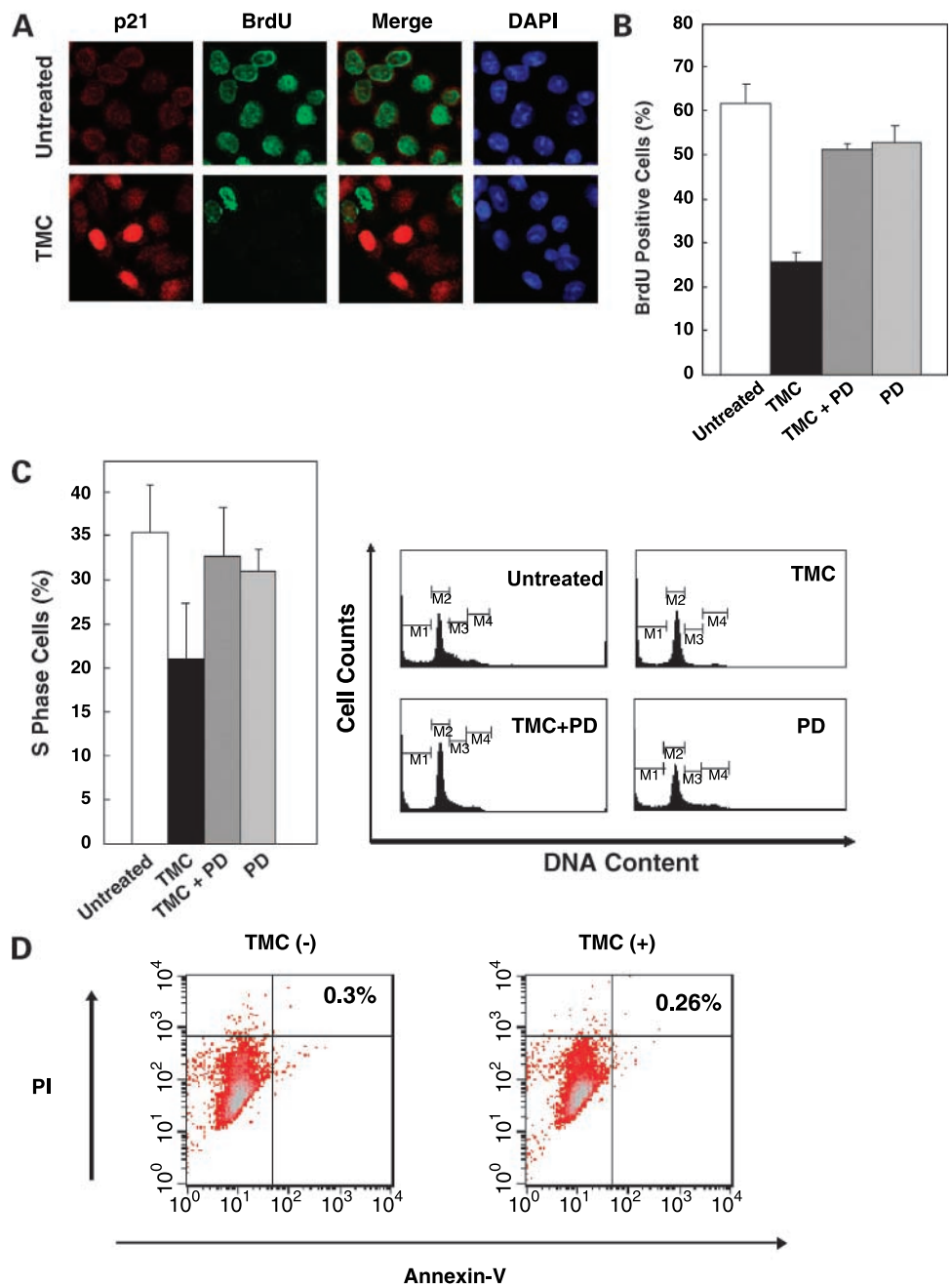
### ERK-Inducible p21<sup>Cip/WAF1</sup> Activation Is Associated with Inhibition of Anchorage-Independent Growth by Tautomycetin

To understand the role of tautomycetin in colorectal cellular anchorage-independent growth, the effects of 150 nmol tautomycetin on foci formation in colorectal cancer cells and other types of cells were examined. HCT-15, HT-29, and DLD-1 cancer cells exhibited a highly transforming phenotype. Therefore, numerous transforming foci were formed in the absence of tautomycetin treatment (Fig. 5D). The numbers of foci in HCT-15, HT-29, and DLD-1 cells were significantly reduced by tautomycetin treatment. The HCT-116 cells, which retained high p21<sup>Cip/WAF1</sup> levels, formed fewer foci than did the



**Figure 2.** Effect of ERK pathway inhibition on p21<sup>Cip/WAF1</sup> induction by tautomycetin. **A**, HCT-15 cells were harvested at 0, 12, and 24 h after 150 nmol tautomycetin exposure. Extracts were analyzed for p21<sup>Cip/WAF1</sup>,  $\alpha$ -tubulin,  $\beta$ -catenin, caspase-3, and the phosphorylated forms of ERK, MEK, Raf-1, JNK, p38, GSK3 $\beta$ (Ser<sup>3</sup>), and Akt by Western blot analyses. **B**, HCT-15 cells were treated with 150 nmol tautomycetin  $\pm$  PD98059 and harvested 24 h later. Extracts were analyzed for p21<sup>Cip/WAF1</sup>, phosphorylated ERK, and  $\alpha$ -tubulin. **C**, *top*, HCT-15 cells were cotransfected with the reporter plasmid pFR-Luc and transactivator pFA2-Elk-1 (9) and treated with 150 nmol tautomycetin for 24 h. Cultures were pretreated with 20  $\mu$ mol PD98059 where indicated. *Bottom*, HCT-15 cells were transfected with pFR-Luc and pFA2-Elk-1 plasmids, along with MEK-2a or dn-Raf-1 expression vectors, as described in Materials and Methods. Cultures were pretreated with 20  $\mu$ mol PD98059 where indicated. **D**, *top*, HCT-15 cells were transfected with 0.5  $\mu$ g/ $\mu$ L of the p21<sup>Cip/WAF1</sup>-promoter reporter p21P (21). Cultures were left untreated or treated with 150 nmol tautomycetin for 24 h in the presence or absence of 20  $\mu$ mol PD98059 for 1 h. *Bottom*, HCT-15 cells were transfected with an empty vector or with p21P, together with an expression vector encoding kinase-inactive MEK-2a or dn-Raf-1. In all transfection experiments, cells were cotransfected with plasmid containing the gene for  $\beta$ -galactosidase. Luciferase activity was then normalized to  $\beta$ -galactosidase to control for transfection efficiency. *Columns*, mean of three independent experiments; *bars*, SD.

**Figure 3.** Effect of PD98059 on tautomycetin-induced inhibition of proliferation and G<sub>1</sub>-to-S cell cycle progression in HCT-15 cells. **A**, HCT-15 cells were incubated for 24 h in the presence or absence of 150 nmol tautomycetin. BrdUrd (green) and p21<sup>Cip/WAF1</sup> labeling (red) was then done as described in Materials and Methods. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (blue). **B**, the effects of tautomycetin ± 10 μmol PD98059 on BrdUrd incorporation and p21<sup>Cip/WAF1</sup> inductions were examined. Quantitative measurements of the percentage of BrdUrd-positive cells are shown. **C**, HCT-15 cells were synchronized with double thymidine blocking, treated with 150 nmol tautomycetin for 24 h in the presence or absence of 10 μmol PD98059, and harvested for FACS analysis. The percentages of cells in S phase were estimated in each case. *Right*, representative results. *M1* to *M4*, cell cycle phases. *M1*, debris and apoptosis; *M2*, G<sub>0</sub>-G<sub>1</sub> phase; *M3*, S-phase; *M4*, G<sub>2</sub>-M phase. *Columns*, mean of three independent experiments; *bars*, SD. **D**, effect of tautomycetin on apoptosis of HCT-15 cells. HCT-15 cells were cultured for 24 h in the absence or presence of 150 nmol tautomycetin and harvested for FACS analysis (36). *Top*, percentages of apoptotic cells including both early-stage (Annexin V+, propidium iodide<sup>-</sup>) and late-stage (Annexin V+, propidium iodide+) apoptotic cells.

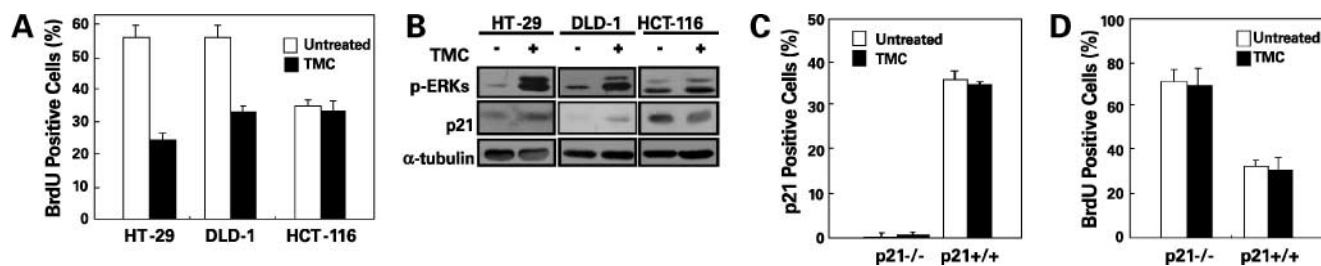


HCT-15 and DLD-1 cells under normal conditions. Tautomycetin treatment did not significantly alter the numbers of foci in HCT-116. In the same way, the number of transforming foci was not altered in COS-7, HEK293, L929, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells (Fig. 5E).

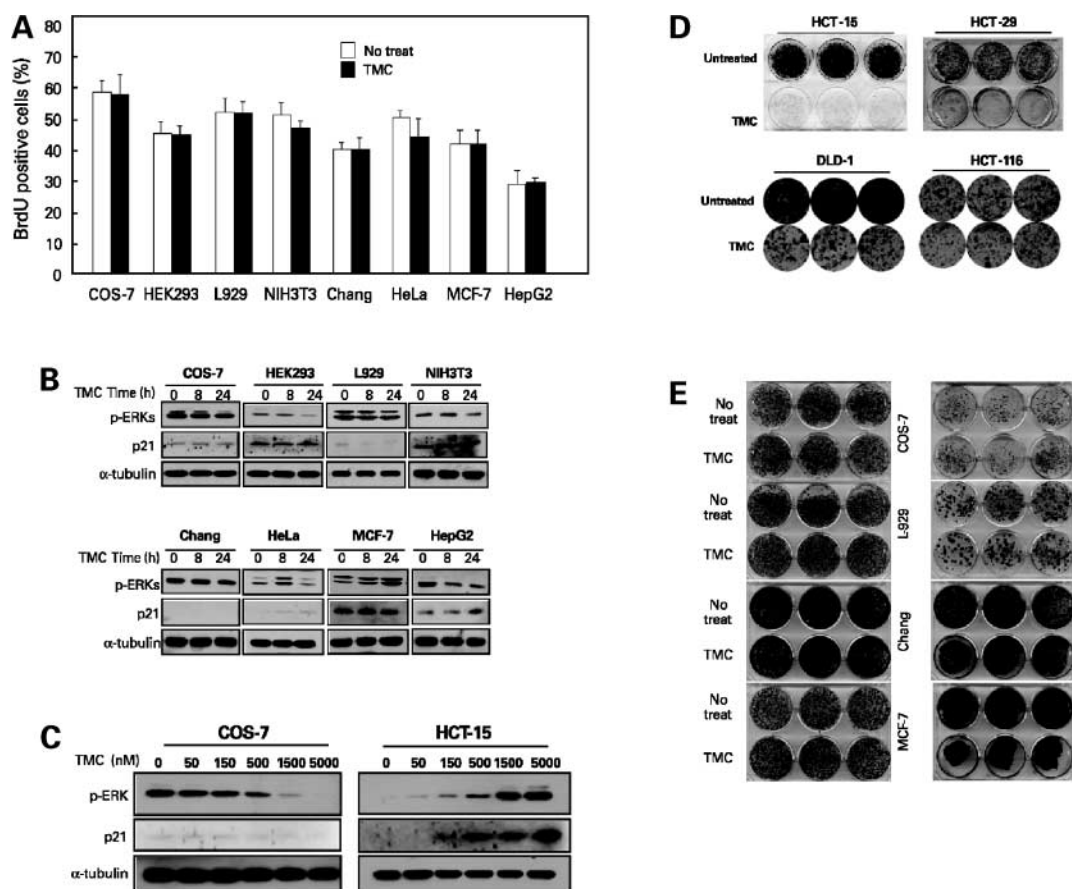
#### Tautomycetin Activates the ERK Pathway by Suppressing the Activity of PP1 and PP2A, Possible Regulators of Raf-1, with High Preference toward PP1

To identify the site of action for tautomycetin in the Raf-1→MEK→ERK cascade, we examined the effect of

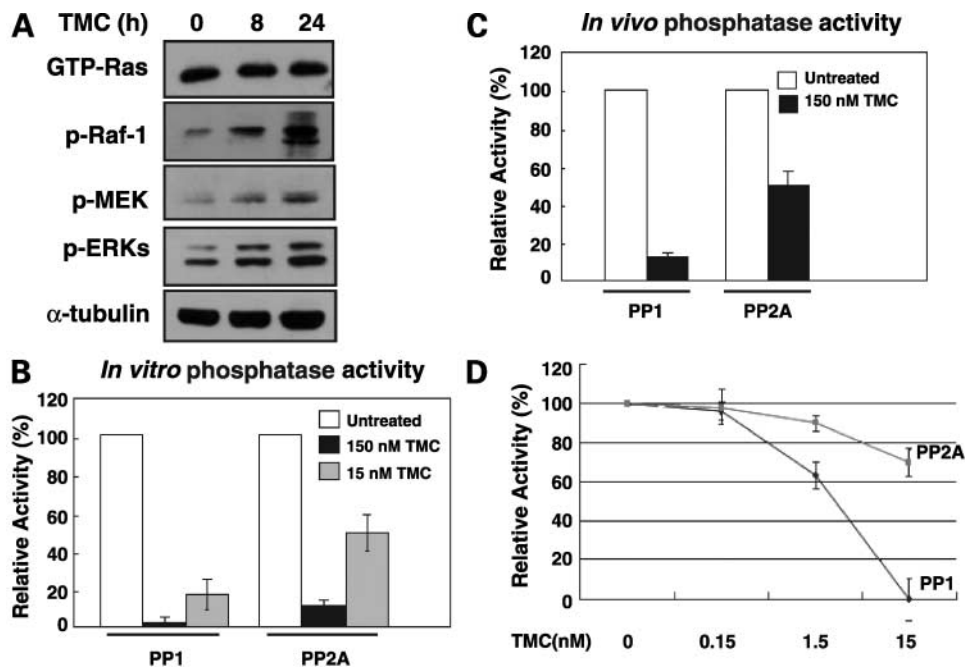
tautomycetin on the levels of GTP-bound Ras (Ras-GTP) by using GTP loading analysis (23). The level of Ras-GTP was not altered in response to tautomycetin, although the activities of Raf-1, MEK, and ERK were concomitantly increased, suggesting that Raf-1 is the point of tautomycetin action (Fig. 6A). Tautomycetin has been shown to inhibit Raf-1 activation by specifically inhibiting PP1 (25). Therefore, we measured *in vitro* phosphatase activities of PP1 and PP2A in HCT-15 cell extracts treated for 30 min with 150 nmol tautomycetin (Fig. 6B). Tautomycetin (150 nmol) decreased PP1 and PP2A activities by ~97% and 90%,



**Figure 4.** Effect of tautomycetin on BrdUrd incorporation and ERK and p21<sup>Cip/WAF1</sup> activation in HT-29, DLD-1, and HCT-116 colorectal cancer cells. **A**, cells were treated with 150 nmol tautomycetin and immunocytochemical staining for p21<sup>Cip/WAF1</sup> and BrdUrd incorporation, as well as nuclear counterstaining, was done. Quantitative measurements of the percentage of BrdUrd-positive cells are shown. **B**, lysates obtained from cells treated with 150 nmol tautomycetin for 24 h were analyzed for p-ERK, p21<sup>Cip/WAF1</sup>, and  $\alpha$ -tubulin by Western blot analyses. **C** and **D**, p21<sup>Cip/WAF1</sup><sup>-/-</sup> and p21<sup>Cip/WAF1</sup><sup>+/+</sup> HCT-116 colorectal cancer cells were treated with 150 nmol tautomycetin, and immunocytochemical staining for p21<sup>Cip/WAF1</sup> and BrdUrd incorporation was done. Quantitative measurements of the percentage of BrdUrd-positive cells and p21<sup>Cip/WAF1</sup>-positive cells are shown. Columns, mean of three independent experiments; bars, SD.



**Figure 5.** Effect of tautomycetin on BrdUrd incorporation, ERK and p21<sup>Cip/WAF1</sup> activation, and transformation of various types of cells. **A**, COS-7, HEK293, L929, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells were grown and treated with 150 nmol tautomycetin and immunocytochemical staining for BrdUrd incorporation, as well as nuclear counterstaining (4',6-diamidino-2-phenylindole), was done. Cells containing nuclear BrdUrd incorporation were scored as BrdUrd-positive cells. Quantitative measurements of the percentage of p21<sup>Cip/WAF1</sup> and BrdUrd positive cells are shown. Columns, mean of three independent experiments; bars, SD. **B**, COS-7, HEK293, L929, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells were treated with 150 nmol/L tautomycetin and harvested 0, 8, and 24 h later. Phosphorylated ERK, p21<sup>Cip/WAF1</sup>, and  $\alpha$ -tubulin levels were examined by Western blot analyses of total lysates. **C**, COS-7 and HCT-15 cells were treated with 0, 50, 150, 500, 1,500, or 5,000 nmol tautomycetin for 24 h. Phosphorylated ERK, p21<sup>Cip/WAF1</sup>, and  $\alpha$ -tubulin levels were examined by Western blot analysis of total lysates. **D**, HCT-15, HT-29, DLD-1, and HCT-116 cells were transfected with a control vector expressing the neo gene. Cells were selected with 800  $\mu$ g/mL G418 in the presence or absence of 150 nmol tautomycetin for 2 wk. Then cells were stained with crystal violet. **E**, COS-7, HEK293, L929, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells were transfected with a control vector expressing the neo gene. Cells were selected with 800  $\mu$ g/mL G418 and grown in the presence or absence of 150 nmol tautomycetin for 2 wk. Then cells were stained with crystal violet.



**Figure 6.** Effect of tautomycetin on Ras activation and *in vitro* and *in vivo* PP1 and PP2A phosphatase activities in HCT-15 cells. **A**, HCT-15 cells were treated with 150 nmol tautomycetin and the level of GTP loading of Ras (GTP-loading) was measured as described in Materials and Methods. Western blot analyses were done to detect p-ERK, p-Raf-1, p-MEK, and  $\alpha$ -tubulin. The Ras-GTP levels were also detected by Western blot analysis with anti-pan-RAS antibody. **B**, to measure *in vitro* PP1 and PP2A activity, HCT-15 cell extracts were incubated for 30 min with or without 150 or 15 nmol tautomycetin, and phosphatase activities were measured as described in Materials and Methods. **C**, to measure *in vivo* PP1 and PP2A activity, HCT-15 cells were treated with 150 nmol tautomycetin for 0 or 24 h and cell lysates were assayed for phosphatase activities of PP1 and PP2A as described in Materials and Methods. *Columns*, mean of three independent experiments; *bars*, SD. **D**, GST-PP1 $\gamma$  or GST-PP2Ac proteins were incubated for 30 min with or without 0.15, 1.5, or 15 nmol tautomycetin, and phosphatase activities were measured as described in Materials and Methods.

respectively (versus activities in untreated extract). The differences in the phosphatase activities of PP1 and PP2A were more significant when 15 nmol tautomycetin was used, 82% and 50%, respectively.

*In vivo* activities of PP1 and PP2A in extracts prepared from 24-h tautomycetin (150 nmol)-treated cells were also reduced by 85% and 50%, respectively (Fig. 6C). The most significant difference in the phosphatase activities between PP1 and PP2A was observed by assaying the enzyme activities with purified recombinant proteins (GST-PP1 $\gamma$  and GST-PP2Ac; Fig. 6D). Here, GST-PP1 $\gamma$  phosphatase activity was totally abolished although 72% of PP2Ac phosphatase activity remained by treatment with 15 nmol tautomycetin. Tautomycetin retains high specificity toward PP1.

## Discussion

Here we show that 150 nmol tautomycetin specifically inhibits proliferation of colorectal cancer cells without significantly affecting the proliferation of eight other types of cells. The effect of tautomycetin in antiproliferation is acquired by induction of the cell cycle regulator p21<sup>Cip/WAF1</sup> via the ERK pathway. Accordingly, we observed that cells exhibiting reduced or no BrdUrd incorporation exhibited an induction of p21<sup>Cip/WAF1</sup> in

response to tautomycetin treatment. HCT-116 cells, which had relatively high levels of p21<sup>Cip/WAF1</sup>, were seldom observed to be BrdUrd positive and did not change proliferation or anchorage-independent growth by tautomycetin. Likewise, tautomycetin did not change the proliferation of HCT-116 cells, regardless of the mutational status of p21<sup>Cip/WAF1</sup>, and, therefore, available p21<sup>Cip/WAF1</sup> -/- HCT-116 cells might not be an ideal cell line to confirm the role of p21<sup>Cip/WAF1</sup> in tautomycetin-induced antiproliferation. The role of p21<sup>Cip/WAF1</sup> in antiproliferation, however, was still indicated by significant up-regulation of the proliferation of p21<sup>Cip/WAF1</sup> -/- HCT-116 cells.

Tautomycetin treatment led to p21<sup>Cip/WAF1</sup> induction and growth inhibition through activation of the ERK pathway, rather than the inhibition of this pathway, as confirmed with the use of PD98059, dn-Raf-1, and catalytically inactive MEK-2a (6–9). The observation that p21<sup>Cip/WAF1</sup> induction occurred in HT-29, DLD-1, and HCT-15 cells, which all harbor p53 mutations, provides support for the hypothesis that tautomycetin-induced increases in p21<sup>Cip/WAF1</sup> protein levels occur via the ERK pathway, not a pathway dependent on p53 (26–28). Moreover, the ability of PD98059 to restore BrdUrd incorporation and G<sub>1</sub> to S cell cycle progression in the presence of tautomycetin underscores the antiproliferative role of the ERK



pathway. The finding that treatment with PD98059 alone diminished BrdUrd incorporation and G<sub>1</sub>-to-S phase progression shows that the ERK pathway exerts both positive and negative regulatory effects on cell growth.

It has previously been shown that the ERK pathway is inhibited in COS-7 cells treated with 5 μmol/L tautomycetin (25), and our results confirm this finding (Fig. 5C). In contrast, tautomycetin increased ERK activity in HCT-15 cells, showing that tautomycetin regulates ERK activity in a cell type-specific manner. In addition, we did not observe any significant effect of 150 nmol tautomycetin on ERK activation and antiproliferation activities in other types of cells: COS-7, HEK293, L929, NIH 3T3, Chang, HeLa, MCF-7, and HepG2 cells (Fig. 5A and B). These results indicate that activation of the ERK pathway by low concentrations (i.e., 150 nmol) of tautomycetin specifically inhibits growth of colorectal cancer cells. Tautomycetin could be a potential anti-cancer drug retaining high specificity toward colorectal cancer.

Although tautomycetin treatment led to activation of the Raf-1→MEK→ERK cascade, the level of GTP-bound Ras (Ras-GTP) was not altered, indicating that tautomycetin may mediate its effects through regulation of Raf-1 activity. PP1 has been shown to interact with Raf-1 and positively regulate its activity *in vivo* (25). Moreover, tautomycetin has been shown to specifically inhibit PP1 activity (25). In the current study, we have confirmed this finding although we have also found that PP1 negatively regulates the ERK pathway in colorectal cancer cells. Tautomycetin also inhibited PP2A activity, although to a lesser extent. PP2A has also been shown to interact with and activate Raf-1 (29). Interestingly, there is evidence that PP2A can positively and negatively regulate the ERK pathway (30, 31). This mode of ERK regulation is not unprecedented as okadaic acid, another PP2A and PP1 inhibitor (PP2A is the favored target in this case), is known to trigger ERK pathway activation (32–35). Based on these results, it is likely that ERK pathway activation by tautomycetin is achieved through inhibition of PP1 and PP2A, with a preference toward PP1. It is possible that the different regulatory roles of PP1 and PP2A may account for the ability of tautomycetin to elicit opposing effects on the ERK pathway in colorectal cancer cells and COS-7 cells, as has been described here. Additional further studies will be necessary to clarify the role of PP1 and PP2A in the cell type-specific regulation of the Raf-1→MEK→ERK pathway by tautomycetin.

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