

# Perillyl alcohol causes G1 arrest through p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction

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**Abstract.** The monoterpene perillyl alcohol (POH) is a naturally occurring compound derived from citrus fruits, mint and herbs. It exhibited chemotherapeutic potential against various malignant tumors in preclinical models and is currently being tested in clinical trials in patients with refractory advanced cancers. POH inhibits cellular proliferation at the G1 phase of the cell cycle *in vitro*. However, the molecular mechanisms responsible for this effect have not been sufficiently elucidated. Here we showed that 1.0 mM POH upregulates p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup>, resulting in hypophosphorylation of the retinoblastoma (RB) protein and subsequent G1 arrest in human immortalized keratinocyte HaCaT cells. The induction of p15<sup>INK4b</sup> was mediated through its promoter, but that of p21<sup>WAF1/Cip1</sup> was not. The small interfering RNA (siRNA) of either p15<sup>INK4b</sup> or p21<sup>WAF1/Cip1</sup> significantly attenuated the increase in the G1 cell population caused by POH. The induction of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> and subsequent G1 arrest by POH was also observed in other cancer cell lines. These results suggest that the induction of p15<sup>INK4b</sup> as well as p21<sup>WAF1/Cip1</sup> is associated with the antiproliferative effect of POH.

## Introduction

Regulation of the cell cycle is important in cellular proliferation, and therefore the loss of cell cycle control is involved in carcinogenesis (1). Cyclins and cyclin-dependent kinases (CDKs), in association with each other, play pivotal roles in

promoting the transition of cells from the G1 phase to the S phase of the cell cycle by phosphorylating the tumor-suppressor retinoblastoma (RB) protein (2,3). Cyclin-CDK complex activation is negatively regulated by CDK inhibitors (CKIs). The first family of CKIs, referred to as the CIP/KIP family, consists of p21<sup>WAF1/Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>. Each member inhibits a broader spectrum of cyclin/CDK complexes including cyclin E/A-CDK2 and cyclin D-CDK4/6 (4,5). The second family of CKIs is called the INK4 family, which comprises p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>. These molecules are specific inhibitors of cyclin D-CDK4/6 complexes (5,6). Many studies have shown that RB is directly, or indirectly, inactivated in most human cancers. Abnormalities leading to malignancies frequently relate to loss or dysfunction of tumor-suppressor molecules including members of these two CKI families, which activate the RB pathway (5,7).

Perillyl alcohol (POH) is a naturally occurring monoterpene found in the essential oils of cherry, lemongrass, gingergrass, cranberry, perilla, mint, lavender, sage, wild bergamot, caraway and celery seeds (8,9). It has been shown that POH exerts antitumor activity against malignant tumor cells *in vitro* and *in vivo*. POH inhibits the growth of various types of malignant tumor cells *in vitro* through blockade of proliferation, angiogenesis and migration, and induction of differentiation and apoptosis (10-14). Regarding the antiproliferative activity of POH, this monoterpene is reported to cause cell cycle arrest at the G1 phase through downregulation of cyclin D1 and upregulation of p21<sup>WAF1/Cip1</sup> in murine mammary transformed cells or through upregulation of both p21<sup>WAF1/Cip1</sup> and p27<sup>Kip1</sup> in human pancreatic adenocarcinoma cells (15,16). Moreover, POH significantly inhibits the growth of mammary and liver tumors in rodent models (12,17). Based on these preclinical data, clinical studies using POH have commenced in patients with advanced malignancies. However, two phase II studies in patients with refractory metastatic breast cancer and metastatic androgen-independent prostate cancer reported that no objective responses were observed (18,19). On the other hand, a recent clinical study showed that intranasal administration of POH increased the overall survival of patients with recurrent glioblastoma (20).

In this study, we demonstrated that POH caused G1 arrest in malignant tumor cells through p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction leading to the dephosphorylation of the RB protein.

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*Abbreviations:* POH, perillyl alcohol; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; RB, retinoblastoma; siRNA, small interfering RNA; RT-PCR, reverse transcription-PCR

*Key words:* perillyl alcohol, G1 arrest, retinoblastoma protein, p15<sup>INK4b</sup>, p21<sup>WAF1/Cip1</sup>

We suggest that not only p21<sup>WAF1/Cip1</sup> but also p15<sup>INK4b</sup> could be important molecular targets that mediate the antitumor effects of POH.

## Materials and methods

**Cell culture and reagents.** Human immortalized keratinocyte HaCaT cells were a kind gift from Dr N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany. Human colon cancer cell lines HT-29 and SW620 were obtained as cell lines of NCI-60 from the NCI Developmental Therapeutics Program (NCI DTP). These cells were maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. POH was purchased from Wako (320-52902; Osaka, Japan), dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentrations in each volume of culture medium used.

**Growth inhibition assay.** Cells were plated at 5x10<sup>4</sup> cells in 12-well plates. One day after inoculation of cells, various concentrations of POH were added to the culture medium. From the first to the second day after plating, the numbers of viable cells were counted using a trypan blue dye exclusion test.

**Cell cycle analysis.** For flow cytometry, 5x10<sup>4</sup> cells were plated in 12-well plates. One day later, unsynchronized cells were exposed to 1.0 mM POH for 24 h. The cells were then treated with Triton X-100 and RNase A, and their nuclei were stained with propidium iodide before DNA content was measured using a BD FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). At least 10,000 cells were counted and the ModFit LD V2.0 software package (BD Biosciences) was used to analyze the data.

**Protein isolation and western blot analysis.** Cells were lysed in SDS buffer [50 mM Tris-HCl (pH 7.5), 1% SDS]. The protein extract was then boiled for 5 min and loaded onto a 12% (for p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> detection), a 10% (for α-tubulin detection) or a 5% (for RB detection) polyacrylamide gel, subjected to electrophoresis and transferred to a nitrocellulose membrane. The following primary antibodies were used: anti-p15<sup>INK4b</sup> (sc-612; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) anti-p21<sup>WAF1/Cip1</sup> (sc-397; Santa Cruz Biotechnology, Inc.), anti-p27<sup>Kip1</sup> (sc-528; Santa Cruz Biotechnology, Inc.), anti-pRB (554136; BD Pharmingen) and anti-α-tubulin (CP06; Calbiochem, San Diego, CA). The signal was then developed with Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan) or Immobilon Western (EMD Millipore, MA).

**RNA isolation and real-time reverse transcription (RT)-PCR.** Real-time RT-PCR analysis was performed as previously described (21). The GeneAmp5700 (Applied Biosystems, CA) was used to quantify the expression level of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> mRNAs and normalized to β2MG mRNA. Real-time RT-PCR primer probes for p15<sup>INK4b</sup> (Hs00394703), p21<sup>WAF1/Cip1</sup> (Hs00355782) and β2MG (Hs99999907) were purchased from Applied Biosystems.

**Transfection and luciferase assay.** The p15<sup>INK4b</sup>-luciferase fusion plasmid was described previously (22). HaCaT cells were seeded at 1.6x10<sup>5</sup> cells/well in 6-well plates. One day later, cells were transfected with the plasmid or pGVB2 (a vacant control; 2.5 µg) using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h, transfected cells were treated with POH at various concentrations for 12 h and then harvested. Luciferase assays were then performed using luciferase assay reagents (Promega, Madison, WI) and a luminometer.

**Small interfering RNA (siRNA).** The p15<sup>INK4b</sup> (CDKN2B HSS141533) and the negative control (Negative Universal Control High #3) siRNAs were purchased from Invitrogen. The p21<sup>WAF1/Cip1</sup> siRNA (s415) was purchased from Ambion (Carlsbad, CA). One day before transfection, HaCaT cells were seeded at 9x10<sup>4</sup> cells/well in 6-well plates without antibiotics. The p15<sup>INK4b</sup>, p21<sup>WAF1/Cip1</sup> or a negative control siRNA (20 nM) was transfected into cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after the transfection, cells were treated with 1.0 mM POH for 24 h and then harvested.

**Statistical analysis.** Statistical evaluation of the data was performed using the Student's t-test for simple comparison between treatments and controls. p<0.05 was considered to indicate a statistically significant difference.

## Results

**Cell growth inhibition and G1 arrest by POH in HaCaT cells.** We first investigated the antiproliferative effects of POH in human immortalized keratinocyte HaCaT cells. The growth of HaCaT cells was measured in the presence or absence of various concentrations of POH (Fig. 1A). POH inhibited the growth of HaCaT cells in a dose-dependent manner. Notably, 1.0 mM POH had a cytostatic effect. To examine the effects of POH on cell cycle progression, the DNA content of cell nuclei was measured by flow cytometry. POH increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase in a dose-dependent manner (Fig. 1B). These data demonstrate that POH arrests the HaCaT cell cycle at the G1 phase.

**p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction and hypophosphorylation of the RB protein by POH in HaCaT cells.** We aimed to elucidate whether cell cycle-associated molecules are influenced by treatment with POH in HaCaT cells. We discovered that POH increased p15<sup>INK4b</sup> protein expression in a dose-dependent manner (Fig. 2). Additionally, POH increased p21<sup>WAF1/Cip1</sup>, which is consistent with previous studies (15,16). Of note, POH had no effect on the protein expression levels of p27<sup>Kip1</sup>. Both p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> are members of the CKI families and subsequently dephosphorylate the RB protein leading to G1 cell cycle arrest. We, therefore, examined whether POH alters the phosphorylation status of RB. A hyperphosphorylated form of the RB protein was converted into a hypophosphorylated form by POH treatment in a dose-dependent manner (Fig. 2). Taken together, these results indicate that POH elevates p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> protein levels, and subsequently

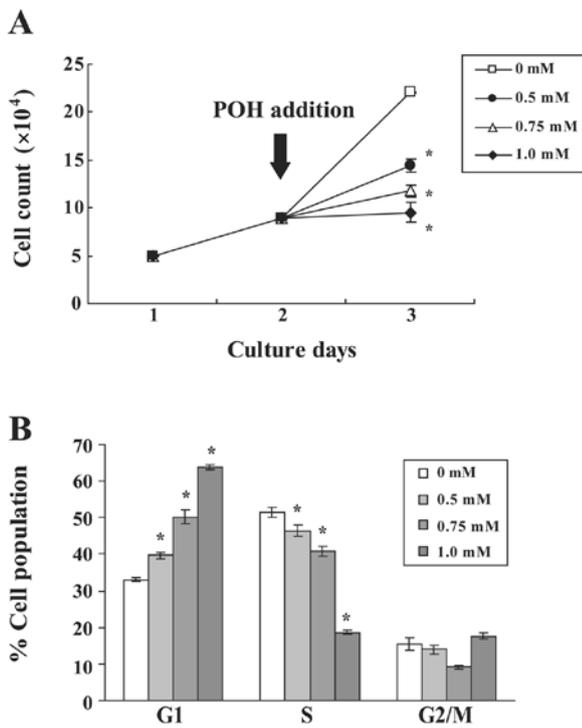


Figure 1. Effects of POH on cell growth in HaCaT cells. (A) One day after the inoculation of HaCaT cells, POH at 0.5 (black circle), 0.75 (white triangle) and 1.0 mM (black diamond) was added. After 24 h, cell growth was compared with a DMSO-treated control (white square). The number of viable cells was counted using a trypan blue dye exclusion test. Data represent means  $\pm$  SD of triplicate experiments; \* $p < 0.05$ , significantly different compared with DMSO-treated control. (B) Unsynchronized HaCaT cells were incubated for 24 h in the presence of either DMSO or POH at various concentrations as indicated, and cell cycle analysis was performed by measuring the DNA content of cells using flow cytometry. The percentage of cells at G1, S and G2/M phases are indicated. Data represent means  $\pm$  SD of triplicate experiments; \* $p < 0.05$ , significantly different compared with DMSO-treated control.

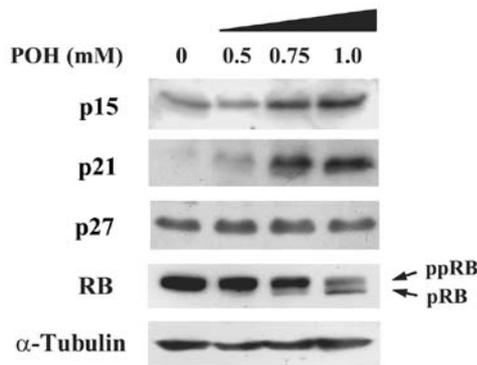


Figure 2. Effects of POH on the expression of cell cycle-related molecules in HaCaT cells. HaCaT cells were exposed for 24 h to either DMSO (0 mM) or to POH at various concentrations as indicated. The expression levels of CKIs, p15<sup>INK4b</sup> (p15), p21<sup>WAF1/Cip1</sup> (p21) and p27<sup>Kip1</sup> (p27), and RB were analyzed using western blotting. Hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms of RB are indicated.  $\alpha$ -tubulin was used as the loading control.

converts a hyperphosphorylated form of the RB protein into a hypophosphorylated form in HaCaT cells.

**Mechanisms of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction by POH in HaCaT cells.** We next investigated whether POH affects

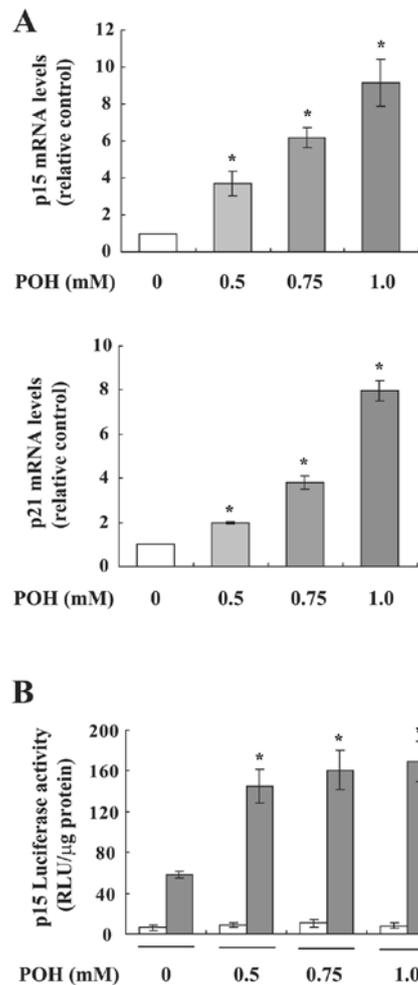


Figure 3. Mechanisms of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction by POH in HaCaT cells. (A) HaCaT cells were exposed to DMSO (0 mM) or POH at the indicated concentrations for 18 h, then the expression of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> mRNAs was examined using real-time RT-PCR. Data represent means  $\pm$  SD of triplicate experiments; \* $p < 0.05$ , significantly different compared with DMSO-treated control. (B) HaCaT cells transiently transfected with a vacant control (PGVB2) or the p15<sup>INK4b</sup>-luciferase fusion plasmid (p15) were treated with or without POH at the indicated concentrations for 12 h, and luciferase activity was then measured. Data represent means of triplicate experiments  $\pm$  SD; \* $p < 0.05$ , significantly different compared with DMSO-treated control.

p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> mRNA expression in HaCaT cells using real-time RT-PCR. Both mRNAs were significantly increased by POH in a dose-dependent manner (Fig. 3A). Since these mRNAs are induced by POH, we analyzed the effect of POH on the promoter activity using p15<sup>INK4b</sup> or p21<sup>WAF1/Cip1</sup> promoter-luciferase fusion plasmids in a transient assay. POH upregulated the promoter activity of p15<sup>INK4b</sup> (Fig. 3 B), however, POH did not elevate that of p21<sup>WAF1/Cip1</sup> (data not shown). These results suggest that p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> are differentially regulated by POH.

*p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> are important targets of POH-induced G1 arrest.* The present results raise the possibility that upregulation of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> proteins by POH contributes to its induction of G1 arrest. If these molecules are key targets of POH-induced G1 arrest, p15<sup>INK4b</sup> or p21<sup>WAF1/Cip1</sup>-depleted cells should be insensitive to the effect of POH. Transfection of HaCaT cells with p15<sup>INK4b</sup> or p21<sup>WAF1/Cip1</sup> siRNA impaired

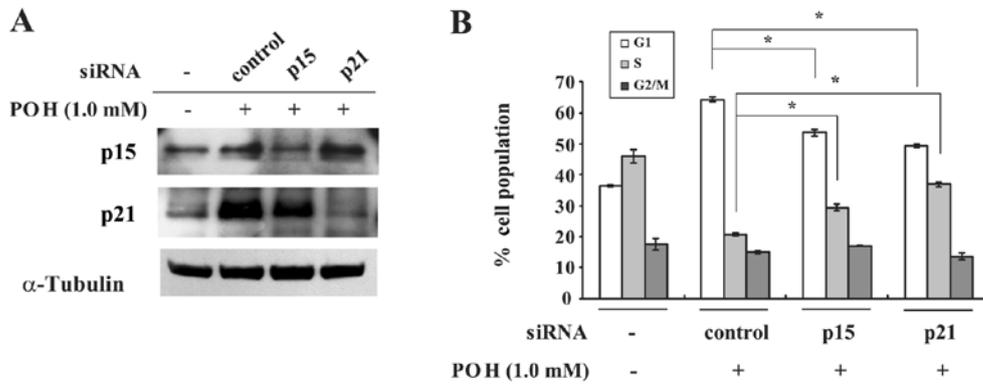


Figure 4. p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> induction is associated with POH-mediated G1 arrest. HaCaT cells were transfected with siRNAs as indicated, then treated with or without 1.0 mM POH for 24 h. (A) The expression levels of p15<sup>INK4b</sup> (p15), p21<sup>WAF1/Cip1</sup> (p21) and  $\alpha$ -tubulin (a loading control) proteins was assessed using western blotting. (B) Cell cycle analysis was performed by measuring the DNA content of cells using flow cytometry. The percentage of cells in the G1, S and G2/M phases are indicated. Data represent means  $\pm$  SD of triplicate experiments. \*p<0.05.

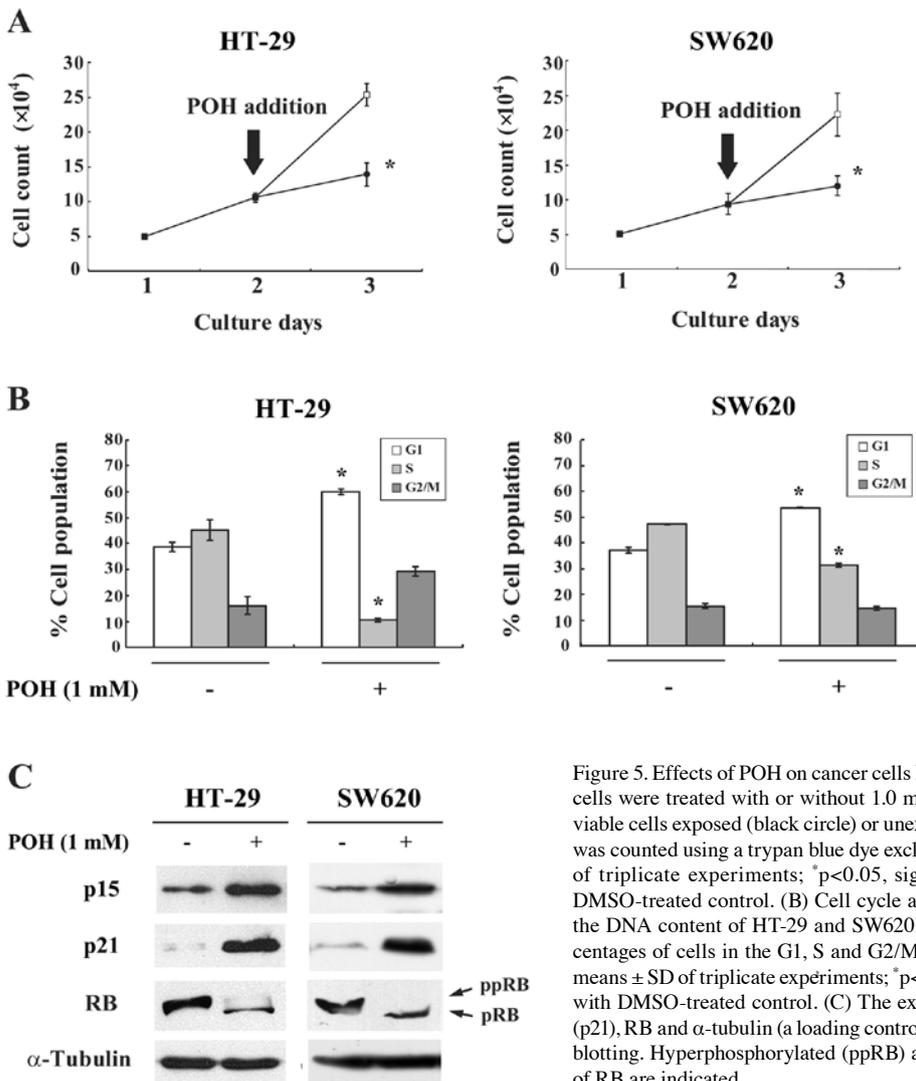


Figure 5. Effects of POH on cancer cells HT-29 and SW620. HT-29 and SW620 cells were treated with or without 1.0 mM POH for 24 h. (A) The number of viable cells exposed (black circle) or unexposed (white square) to 1.0 mM POH was counted using a trypan blue dye exclusion test. Data represent means  $\pm$  SD of triplicate experiments; \*p<0.05, significantly different compared with DMSO-treated control. (B) Cell cycle analysis was performed by measuring the DNA content of HT-29 and SW620 cells using flow cytometry. The percentages of cells in the G1, S and G2/M phases are indicated. Data represent means  $\pm$  SD of triplicate experiments; \*p<0.05, significantly different compared with DMSO-treated control. (C) The expression of p15<sup>INK4b</sup> (p15), p21<sup>WAF1/Cip1</sup> (p21), RB and  $\alpha$ -tubulin (a loading control) proteins was assessed using western blotting. Hyperphosphorylated (ppRB) and hypophosphorylated (pRB) forms of RB are indicated.

the induction of these proteins by POH (Fig. 4A). Additionally, these siRNAs significantly restored POH-altered percentages of the G1 and S cell populations when compared with the control siRNA (Fig. 4B). These results imply that both p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> play pivotal roles in POH-induced G1 arrest.

POH causes G1 arrest through induction of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> in other cancer cell lines. To investigate whether the effects of POH on G1 arrest could be observed more generally, other cancer cell lines, HT-29 and SW620, were similarly assayed. POH inhibited the proliferation and caused G1 arrest

in these cells (Fig. 5A and B). Moreover, POH increased p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> protein expression and hypophosphorylated the RB protein in both cell lines (Fig. 5C). Taken together, these results suggest that POH has antitumor activity against various malignant tumor cells through induction of p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> and subsequent G1 arrest.

## Discussion

Numerous studies have shown that dysfunction of the RB pathway is the most frequent event in human malignant tumors (6,7). Therefore, we focused our studies on agents that reactivate RB function through induction of the two CKI families. As a result, we previously demonstrated that p15<sup>INK4b</sup> is upregulated by a histone deacetylase inhibitor trichostatin A, a naturally occurring compound indole-3-carbinol, an epidermal growth factor receptor inhibitor gefitinib (ZD1839) and a novel MEK inhibitor JTP-70902 (22-25). Additionally, we found that p21<sup>WAF1/Cip1</sup> is increased by trichostatin A, a dietary flavonoid apigenin and a plant alkaloid cryptolepine (26-28).

p16<sup>INK4a</sup> and p15<sup>INK4b</sup> are encoded within the *INK4a/ARF/INK4b* locus on chromosome 9p21. Deletion of this locus is the most frequent cytogenetic abnormality of the RB pathway in human hematopoietic malignancies (6). On the other hand, in many malignant solid tumors, p16<sup>INK4a</sup> is inactivated through not only gene deletions, but also point mutations or transcriptional silencing by methylation of the promoter. In contrast to p16<sup>INK4a</sup>, however, alteration of the p15<sup>INK4b</sup> gene is a rare event in solid tumors (6,29). Moreover, among the INK4 family, p15<sup>INK4b</sup> has a function similar to that of p16<sup>INK4a</sup>. These studies suggest that p15<sup>INK4b</sup> may act as a replacement for p16<sup>INK4a</sup> when p16<sup>INK4a</sup> is inactivated. Krimpenfort *et al* indicated that p15<sup>INK4b</sup> can fulfill a critical backup function for p16<sup>INK4a</sup> in human tumors with p16<sup>INK4a</sup> deficiency (30). In the present study, we showed that depletion of p15<sup>INK4b</sup> protein using siRNA suppressed the G1-arresting activity of POH. These findings suggest that induction of p15<sup>INK4b</sup> by POH could be, at least partially, involved in its antiproliferative activity. Taken together, the ability of POH to induce p15<sup>INK4b</sup> might be useful for inhibiting the growth of solid tumors where the p16<sup>INK4a</sup>-RB pathway is inactivated.

p21<sup>WAF1/Cip1</sup> is known to be a major effector of the tumor suppressor p53. Therefore, p21<sup>WAF1/Cip1</sup> is regarded as a tumor-suppressor gene (31). On the other hand, it has been shown that p21<sup>WAF1/Cip1</sup> plays oncogenic roles in certain cellular circumstances through its ability to suppress apoptosis and promote the assembly of cyclin D with CDK4 and CDK6 (32-34). Thus, these data indicate that p21<sup>WAF1/Cip1</sup> induction confers a growth advantage in tumor development in certain type of cancers, while it has the opposite effect in others. We revealed that POH upregulated p21<sup>WAF1/Cip1</sup> as well as p15<sup>INK4b</sup> proteins and subsequently caused G1 arrest in three malignant tumor cell lines. Additionally, we showed that depletion of p21<sup>WAF1/Cip1</sup> protein using siRNA rendered HaCaT cells insensitive to POH-induced G1 arrest. These data suggest that the induction of p21<sup>WAF1/Cip1</sup> by POH could be at least partially involved in its antiproliferative activity.

POH is readily metabolized to perillidic acid (PA) and dihydroperillidic acid (DHPA) in animals, whereas in humans PA is the major circulating metabolite (17,35). Haag *et al* (17)

reported that in a rat mammary cancer model administration of a 2.5% POH diet for 3 weeks caused complete regression in 22 out of 27 (81%) primary tumors, while the plasma levels of POH metabolites were approximately 800  $\mu$ M in rats given a 2% POH diet for 10 weeks. Based on the data from preclinical models, POH has been tested in phase I and II clinical trials in patients with refractory solid malignancies. The mean peak PA plasma levels ranged between 415 and 630  $\mu$ M and minimal toxicities were observed in patients when doses of POH at 1600 or 2100 mg/m<sup>2</sup> were administered orally (36-38). Recently, an encouraging clinical study carried out by da Fonseca *et al* (20), showed that intranasal administration of 440 mg POH increased the overall survival of patients with recurrent GBM when compared with untreated controls.

We revealed that POH upregulated p15<sup>INK4b</sup> as well as p21<sup>WAF1/Cip1</sup> protein and subsequently caused G1 arrest in three malignant tumor cell lines. Additionally, we showed that depletion of the p15<sup>INK4b</sup> or p21<sup>WAF1/Cip1</sup> protein rendered HaCaT cells resistant to POH-induced G1 arrest. These results indicate that induction of both p15<sup>INK4b</sup> and p21<sup>WAF1/Cip1</sup> is at least partially associated with sensitivity to the antiproliferative effect of POH. POH driven activation of RB function through induction of CKIs may contribute to new strategies which have been termed 'gene-regulating chemotherapy' for the treatment of malignancies. (39,40). In short, POH is promising as a molecular-targeted anticancer drug against a variety of malignant tumors.

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