

Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression

Sylvie Bardon^{a,*}, Valérie Foussard^b, Sophie Fournel^b, Agnès Loubat^c

^aLaboratoire de Nutrition et Sécurité Alimentaire, UR909, INRA, 78352 Jouy-en-Josas cedex, France

^bLaboratoire d'Hépatogastro-Entérologie et Nutrition, UFR de Médecine, 28, Avenue de Valombrose, 06107 Nice cedex, France

^cLaboratoire d'Immunologie Cellulaire et Moléculaire, INSERM Unité 364, UFR de Médecine, 28, Avenue de Valombrose, 06107 Nice cedex, France

Received 12 September 2001; received in revised form 5 October 2001; accepted 11 January 2002

Abstract

The monoterpene perillyl alcohol (POH) is a naturally occurring anti-cancer compound which is effective against a variety of rodent organ-specific tumor models. To establish the molecular mechanisms of POH and its major metabolite perillic acid (PA) as anti-proliferative agents, their effects on cell proliferation, cell cycle and cell cycle regulatory proteins were studied in HCT 116 human colon cancer cells. POH, and to a lesser extent, PA, exerted a dose-dependent inhibitory effect on cell growth correlated with a G1 arrest. Analysis of G1 cell cycle regulators expression revealed that monoterpenes increased expression of cdk inhibitor p21^{Waf1/Cip1} and cyclin E, and decreased expression of cyclin D1, cyclin-dependent kinase (cdk) 4 and cdk2. Our results suggest that monoterpenes induce growth arrest of colon cancer cells through the up-regulation of p21^{Waf1/Cip1} and the down-expression of cyclin D1 and its partner cdk4. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Colon cancer; HCT 116 cells; Monoterpene; Perillyl alcohol; Cyclin; p21^{Waf1/Cip1}

1. Introduction

Perillyl alcohol (POH) is a naturally occurring monoterpene found in the essential oils of numerous species of plants including mints, cherries, and celery seeds. This monocyclic monoterpene has shown chemopreventive and therapeutic activity in rodent mammary [1], skin [2], lung [3], liver [4], pancreatic [5], and colon tumor models [6]. Phase I clinical studies have shown that POH is well tolerated in cancer patients at doses which may have clinical activity [7–9]. Several patients with refractory colon

cancer had stable disease for at least 2 months [9] and evidence of anti-tumour activity has been seen in a patient with metastatic colorectal cancer who had an ongoing near-complete response of >2 years duration [8]. Pharmacokinetic studies have indicated that POH is subject to rapid absorption from the gastrointestinal tract followed by efficient metabolism to perillic acid (PA), the predominant circulating metabolite, and dihydroperillic acid detected in lesser amounts [1,7–9]. Several cellular and molecular activities of POH have been reported. Monoterpenes can inhibit the posttranslational isoprenylation of small G proteins such as growth-controlling *ras* oncoproteins [10]. However, it has been reported that monoterpenes are unlikely to inhibit cell growth by inhibiting *ras* function [11–13]. The chemopreventive activity of POH is

* Corresponding author. Tel.: +33-1-4459-4781; fax: +33-1-4459-4773.

E-mail address: bardon@unice.fr (S. Bardon).

associated with a marked increase in tumor cell loss by apoptosis in mammary, liver and colon tumors [4,6,14], and activation of the transforming growth factor β signaling pathway has been shown in mammary carcinomas treated with POH [14]. Yet, the mechanisms by which POH inhibit tumor growth have not been firmly established.

We focused on the effects of POH and its major metabolite PA on colon cancer cells, since colon cancer is one of the most common cause of cancer deaths in Western countries. It has been reported that dietary administration of POH results in an inhibition of the incidence of adenocarcinomas and an increase in apoptosis in azoxymethane-induced rat colon tumors [6]. In vitro studies have indicated that the growth of HT29 and SW480 colon carcinoma cell lines is inhibited by POH [15,16].

In order to improve our understanding of the mechanisms of action of POH, we analysed the effects of POH and PA on HCT 116 colonic carcinoma cells. We showed that growth inhibition induced by these monoterpenes is associated with cell cycle changes, and modulation of the expression of cell cycle-related proteins p21^{Waf1/Cip1}, cyclin D1, cyclin E, and cyclin-dependent kinases cdk4 and cdk2.

2. Materials and methods

2.1. Cell culture and reagents

The HCT 116 human colon carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was maintained in Dulbecco's modified Eagle medium (DMEM)/F12 medium 1:1 supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μ g/ml streptomycin, at 37°C in a 5% CO₂ incubator. All cell culture reagents were from GibcoBRL (Life Technologies, Cergy-Pontoise, France). POH and PA were from Sigma–Aldrich Chemical (St. Quentin Fallavier, France) and were stored as a 1000-fold concentration in ethanol at 4°C. 3,5-Diamino-benzoic acid dihydrochloride (DABA) was from Sigma–Aldrich.

2.2. DABA assay

The effects of monoterpenes on cell proliferation was evaluated by measuring in situ the amount of

cellular DNA, using the DABA fluorometric assay [17]. Gillery et al. [18] described this technique as an efficient tool for evaluating the number of cells. They showed that the intensity of fluorescence measured after the DABA assay increases linearly with the increasing number of cells. HCT 116 cells were seeded in 24-well plates at 1.5×10^4 cells/well. One day after plating, monoterpenes were added to cells and the medium was changed every 2 days. At the end of the treatment, cells were rinsed with phosphate-buffered saline (PBS) and fixed with methanol. Then, 100 μ l of DABA solution (300 mg/ml double distilled water) were added in each well. The reaction was carried out at 60°C for 1 h and stabilised by adding 1.5 ml of 1 N HCl. The fluorescence intensity of the DABA–DNA complexes was determined using a fluorescence spectrophotometer (Hitachi F-2000, excitation wavelength 405 nm, emission wavelength 495 nm). Standards containing 1–20 μ g of calf thymus DNA were assayed at the same time as cell culture samples.

2.3. Cell cycle analysis

HCT 116 cells were treated with or without monoterpenes for 48 h in 10% FCS-supplemented medium while in the exponential growth phase. Cells were harvested by trypsinization, washed with PBS, resuspended in a citrate buffer and stained with a propidium iodide solution according to Vindelov et al. [19]. Flow cytometric analysis of stained cells was performed on a FACScan flow cytometer (Becton Dickinson).

2.4. Western blot analysis

Subconfluent cells on 80 cm² plates were incubated for 1 h at 4°C in 500 μ l lysis buffer consisting of 10 mM Tris–HCl, 20 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P40, 0, 50 mM NaF, 0.1 mM Na₃VO₄, and the protease inhibitors pepstatin A (1 μ g/ml), anti-pain (2 μ g/ml), leupeptin (10 μ g/ml) and phenylmethylsulfonyl fluoride (0.1 mM) (Sigma–Aldrich). Cell debris was removed by centrifugation at 10,000 \times g for 10 min at 4°C, and the supernatants stored at –80°C. Total-cell protein extracts were normalised for concentration by the Bradford assay (Bio-Rad, Ivry sur Seine, France) and 40 μ g of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were

then blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech) using a trans-blot apparatus (Bio-Rad). The membranes were blocked in 5% non-fat dry milk in TBS–Tween (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, and were incubated with primary antibodies in 5% non-fat milk in TBS–Tween. Rabbit polyclonal antibodies directed against cyclin D1, cdk4 and cdk2 as well as mouse monoclonal antibody directed against cyclin E were obtained from Santa Cruz Biotechnology (Tebu SA, Le Perray en Yvelines, France), and mouse monoclonal antibody directed against p21^{Waf1/Cip1} was from BD Transduction Laboratories (Franklin Lakes, NJ). The blots were washed for 1 h in TBS–Tween with three changes and then incubated with anti-rabbit IgG (Sigma–Aldrich) diluted 1/10,000 in 5% milk/TBS–Tween or with anti-mouse IgG (Sigma–Aldrich) diluted 1/5000, for 1 h at room temperature. The membranes were then washed in TBS–Tween for 1 h. Immunodetection was performed with a chemiluminescence system (ECL, Amersham Pharmacia Biotech) and was followed by autoradiography.

2.5. RT-PCR

Cells were seeded in 20 cm² dishes at 400,000 cells per dish. One day later, cells were incubated either with ethanol or with POH and PA for 18 h. Total RNA was isolated from cells by direct lysis, using the specifications provided by the manufacturer (Talent, Rome, Italy) and quantified spectrophotometrically. After quantification, reverse transcription (RT) reaction was performed with 5 µg of each sample and oligo dT primer, using the first-strand cDNA synthesis kit (Sigma–Aldrich). PCR reactions were carried out in a final volume of 100 µl consisting of 20 mM Tris–HCl, pH 9.0, 0.2 mM MgSO₄, 200 µM amounts of each deoxyribonucleoside triphosphate, 5 µl of the RT first-strand cDNA product, 20 µM of each forward and reverse primer, and 5 units of Taq DNA polymerase. The reactions were heated to 94°C for 45 s and then immediately cycled through a 1.5 min annealing step at 72°C. Aliquots were taken off and analysed after 20–30 cycles in order to ensure that they remained in the exponential amplification phase. The p21^{Waf1/Cip1} primer was sense 5′-GGCGGCAGACCAGCATGA-

CAGATT-3′, and anti-sense 5′-CAGCGGACAAGT-GGGGAGGAGGAA-3′. The cyclin E primer was sense 5′-GACCGGTATATGGCGACACAAGAA-3′, and anti-sense 5′-GTCGCACCACTGATACCT-GAAAC-3′ (Genset Oligos, Paris, France). PCR amplified products were fractionated on a 2% agarose gel containing ethidium bromide (0.1 µg/ml) and visualised by UV transillumination.

2.6. Statistical analysis

Statistical analysis was performed using StatView Statistical Analysis software (SAS Institute, San Francisco, CA). Differences between group mean values were determined by one-way analysis of variance (ANOVA) followed by a two-tailed Student's *t*-test for unpaired samples, assuming equal variances.

3. Results

3.1. Anti-proliferative effect of PA and POH

Proliferation of exponentially growing HCT 116 cells was dose-dependently inhibited by both PA

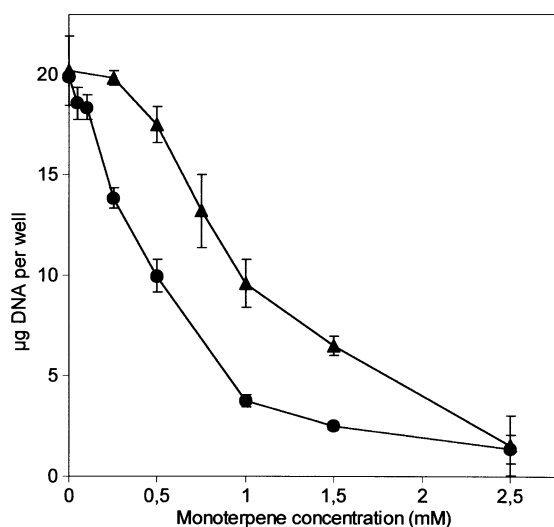
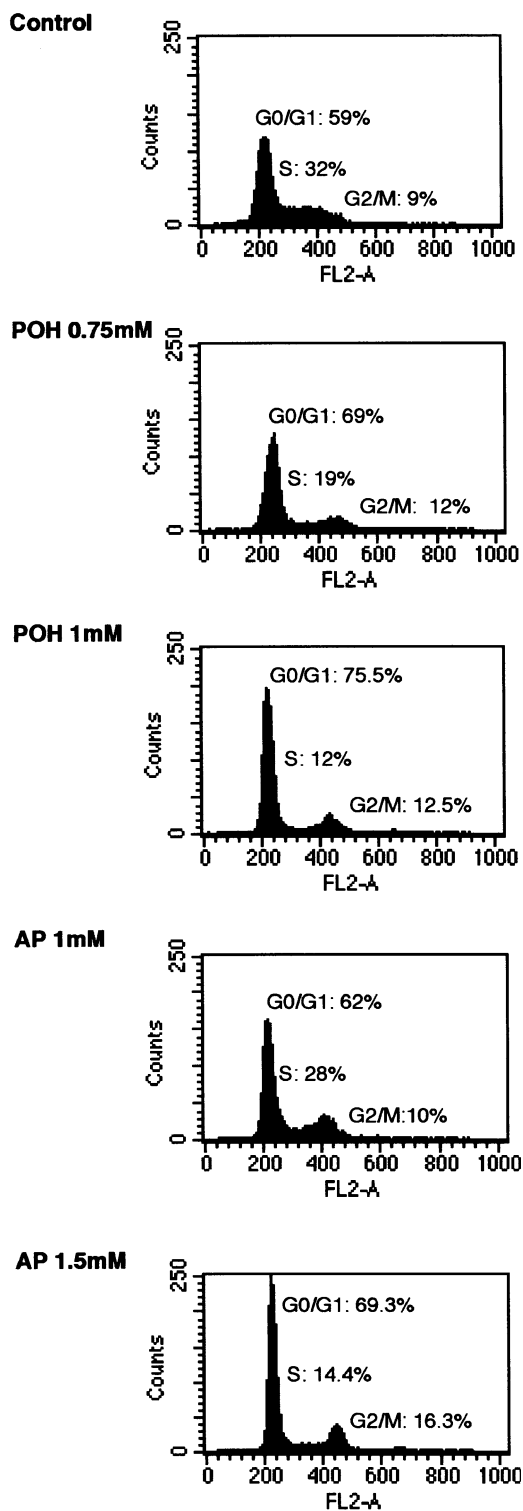


Fig. 1. Inhibitory effect of monocyclic monoterpenes on HCT 116 cell growth. Cells were exposed to increasing concentrations of PA (triangles) or POH (circles) for 6 days. At the end of the treatment, cells were fixed and the amount of DNA was evaluated using the DABA fluorometric assay. Data are the means \pm SD of three determinations.



and POH as shown in Fig. 1. POH was found to be the most potent inhibitor of cell proliferation with a significant inhibition ($P < 0.01$) at a concentration of 0.25 mM. PA was less effective with a significant reduction of growth rate at a concentration of 0.75 mM. POH produced a 50% inhibition of cell proliferation at 0.5 mM whereas 1 mM PA was necessary to attain the same reduction of growth rate. Both monoterpenes had a cytotoxic effect at 2.5 mM. To further understand the nature of the growth arrest, colon cancer cells were cultured for 48 h in the presence of various concentrations of PA or POH and subjected to cell cycle analysis (Fig. 2). Both monoterpenes induced an accumulation of HCT 116 cells in the G1 phase. This increase was associated with a marked decrease in the percentage of S phase cells. Twelve percent and 14% of the HCT 116 cells, respectively, treated with 1 mM POH and 1.5 mM PA were in S phase compared with 32% of control cells. The proportion of cells in the G2M phase was slightly increased.

3.2. Effects of monoterpenes on the expression of G1–S cell cycle regulators

We examined the effects of monoterpenes on the expression of cell cycle-regulatory proteins operative in the G1–S phase transition including p21^{Waf1/Cip1}, cyclin D1, cyclin E, cdk4 and cdk2. Immunoblot analysis revealed that POH (0.25–1 mM) and, to a lesser extent, PA treatment (0.75–2 mM) resulted in a dose-dependent induction of p21^{Waf1/Cip1} (Fig. 3). A 3.5-fold increase was obtained for a 1 mM POH concentration as measured after densitometric analysis of the blots. Cyclin D1 expression was found to be markedly decreased in a dose-dependent manner after treatment with monoterpenes. At 1 mM POH, cyclin D1 protein level was reduced by 89%. In contrast, a dose-dependent increase of cyclin E was observed. A 10-fold up-regulation of cyclin E protein was found when cells were incubated with 1 mM POH. The expression of cyclin-dependent kinases cdk4 and cdk2 was dose-dependently decreased after treatment

Fig. 2. Effect of monocyclic monoterpenes on cell cycle progression. Cells were exposed for 48 h to PA or POH at indicated concentrations. At the end of the treatment, cells were harvested and processed for flow-cytometric DNA analysis.

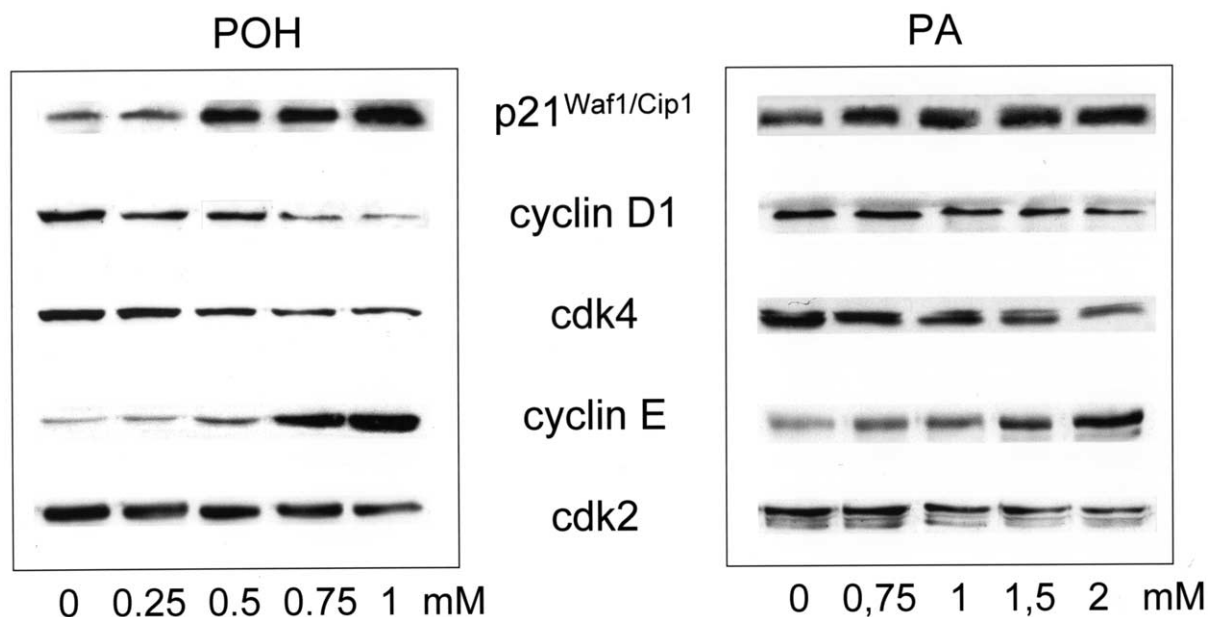


Fig. 3. Western blot analysis of G1–S cell cycle regulatory protein expression in HCT 116 cells incubated for 24 h with POH or PA. Cells were treated with POH or PA at indicated concentrations. Control cells received ethanol alone. Equal volumes of whole cell extracts containing 30 μg of proteins were resolved by SDS-PAGE, transferred to PVDF membranes and probed with antibodies to p21^{Waf1/Cip1}, cyclin D1, cdk4, cyclin E, and cdk2 as indicated. Proteins were detected by enhanced chemiluminescence.

with increasing concentrations of monoterpenes. The most effective monoterpene was POH in all cases and the maximal effect was observed after the addition of 1 mM POH. The time-course study showed that changes in the expression of cell cycle-related proteins was observed within 6 h of treatment by 1 mM POH (Fig. 4). The maximal effect was observed after 24 h of treatment. Analysis of the expression of p21^{Waf1/Cip1} and cyclin E genes by RT-PCR revealed that both mRNA were up-regulated after treatment with POH (Fig. 5).

4. Discussion

The present study was undertaken with the objective of determining cellular and molecular effects of POH and its major metabolite PA in colon cancer cells, with regard to cell proliferation. In our experiments, we used HCT 116 human colon carcinoma cells which have been reported to express cell cycle components including p21^{Waf1/Cip1}, cyclin D1, cdk4, cyclin E and cdk2 [20,21]. In addition, HCT 116

cell line has been shown to be highly responsive to cell cycle anti-cancer agents [21]. Our data showed that monoterpene treatment of HCT 116 cells resulted in a significant dose-dependent cell growth inhibition with a greater effect for POH. Similar effect of POH and PA has been previously reported on HT29 colon cancer cells [15], and on human breast cancer cell lines [22]. The concentrations of monoterpenes required to inhibit cell proliferation were similar to the serum levels of terpene metabolites in patients treated with POH [8,9].

To elucidate the effect of monoterpenes on the growth of colon cancer cells, we assessed the effect of POH and PA on cell cycle progression. Both compounds induced an increase in the G0/G1 fraction of the cell cycle and a concomitant decrease in cell population in the S phase, suggesting a G1/S arrest. Many studies have shown an association between cell cycle and cancer, and in recent years, inhibition of the cell cycle has been considered as a target for the management of cancer. Passage through the cell cycle is governed by the function of a family of protein kinase complexes. Each complex is composed

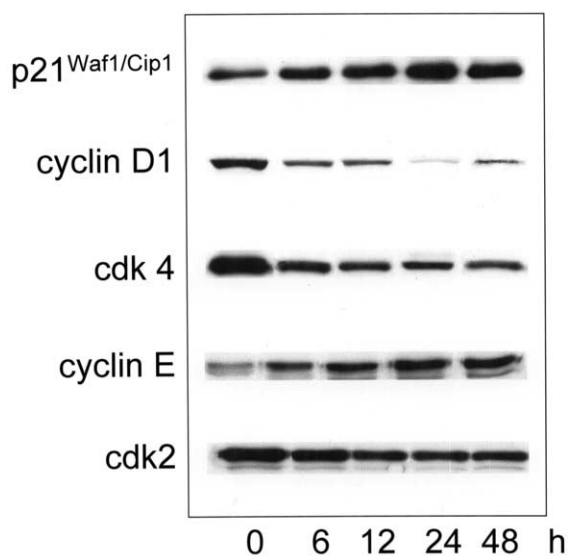


Fig. 4. Kinetic analysis of the effect of POH on G1–S cell cycle regulatory protein expression. HCT 116 cells were exposed to ethanol alone or with 1 mM POH, and proteins were extracted at the indicated time after incubation. Equal volumes of whole cell extracts containing 30 μ g of proteins were resolved by SDS-PAGE, transferred to PVDF membranes and probed with antibodies to p21^{Waf1/Cip1}, cyclin D1, cdk4, cyclin E, and cdk2 as indicated. Proteins were detected by enhanced chemiluminescence.

minimally of a catalytic subunit, the cdk, and its essential activating partner, the cyclin [23,24]. During the progression of the cell cycle, the cdk–cyclin complexes are inhibited via binding to cdk-inhibitors (ckis) such as the Cip/Kip and INK4 families of proteins. The INK4 family (p16, p15, p18 and p19) can inhibit Cdk4 and Cdk6 activity, while the Cip/Kip family members (p21^{Waf1/Cip1}, p27^{Kip1} and p57^{Kip2}) act as broad specific inhibitors of cyclin D, E, and A complex [25]. G1-phase progression is mediated by the combined activity of the cyclin D1/Cdk4,6 and cyclin E/Cdk2 complexes [23]. Cyclin D1-associated kinase activity increases in mid-G1, while cyclin E/Cdk2 activity increases in late G1 and peaks in early S phase. Any defect in this machinery causes an altered cell cycle regulation. Because our studies have demonstrated that monoterpene treatment resulted in a G1/S arrest of the cell cycle, we examined the effects of monoterpenes on cell cycle-regulators operative in the G1 phase of the cell cycle.

We showed a significant dose- and time-dependent up-regulation of the cki p21^{Waf1/Cip1} which is known to

play a critical role in regulating entry of cells at the G1–S phase transition [26]. In addition, monoterpene treatment of HCT 116 cells resulted in a marked dose-dependent decrease in the expression of cyclin D1 and its partner cdk4. The down-modulation of both regulatory molecules was observed within 6 h of exposure to POH and was maximal at 24 h. Inhibition of cyclin D1 and cdk4 expression has previously been reported in colon cancer cells in response to several anti-tumour agents including NA22598 [27], FR901228 [28], and resveratrol [29] which all induced cell cycle arrest. As a positive regulator of cdk4 and cdk6, cyclin D1 has been implicated in controlling the G1 phase of the cell cycle and is frequently over-expressed in human colon adenocarcinomas [30,31]. A recent study has shown that elevated cyclin D1 and cdk4 correlated with enhanced dysplasia in human colon [32]. On the other hand, up-regulation of p21^{Waf1/Cip1} has been shown to exert a G1 cell cycle arrest in a variety of cell types and particularly in colon cancer cells in response to stimuli such as butyrate, a product of colonic fermentation [33]. Decreased expression of p21^{Waf1/Cip1} occurs frequently in cancers including colorectal cancers [34]. A recent study showed that subcellular localisation of cyclin D1 protein in colorectal tumour is associated with p21^{Waf1/Cip1} expression and correlates with patient survival, suggesting the importance of these proteins in colorectal tumorigenesis [35]. In contrast, mono-

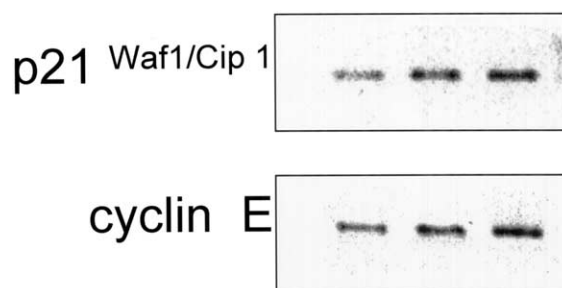


Fig. 5. PCR amplifications. HCT 116 cells were incubated with or without POH and PA at the indicated concentrations for 18 h. Total RNA was then purified and first strand cDNA performed. After PCR amplification from 5 μ g cDNA with primers specific for p21^{Waf1/Cip1} and cyclin E, PCR products were analysed on agarose gel electrophoresis and visualised by UV transillumination. The results of the amplification with p21^{Waf1/Cip1} were obtained after 27 cycles of amplification, and the results of the amplification with cyclin E were obtained after 29 cycles of amplification.

terpene treatment of colon cancer cells resulted in a dose-dependent increase in cyclin E whereas its partner cdk2 was inhibited. Thus, there was an apparent contradictory down-regulation of cyclin D1 and up-regulation of cyclin E. Previous studies have shown that other agents, which are histone deacetylase inhibitors, may also cause G1 arrest by increasing cdk inhibitor p21^{Waf1/Cip1}, decreasing cyclin D1 expression, and increasing cyclin E expression [28,36]. Induction of cyclin E, despite cyclin D down regulation, could move cells into S phase. However, we reported that p21^{Waf1/Cip1} was also induced at the time of cyclin E induction, and it has been shown that p21^{Waf1/Cip1} can inhibit cyclin E-driven initiation of S phase [37]. Therefore, our results suggest that increased p21^{Waf1/Cip1} counterbalanced increased cyclin E. Additional studies will be needed to elucidate the mechanism of action through which monoterpenes up-regulate p21^{Waf1/Cip1} expression.

It has been mentioned in a recent paper that molecular and cellular effects of monoterpenes were specific to carcinoma tissues and were not found in normal mammary gland or in intestinal tissues chronically treated with POH [14]. Similarly, the POH-mediated induction of TGF β receptors and apoptosis in a liver tumor model were absent in its tissue of origin [38]. Additionally, the apoptotic effects of POH in malignant pancreatic cells were more pronounced than in non-malignant pancreatic cells [39]. These tumor-specific events induced by monoterpenes suggest the interest of these compounds as potential chemotherapeutic agents.

In conclusion, treatment of HCT 116 colon carcinoma cells with POH and its metabolite PA, caused a down-regulation of cyclin D1 and its partner cdk4 and an induction of the cdk inhibitor p21^{Waf1/Cip1}, resulting in growth arrest in the G1 phase of the cell cycle. Our data suggest that the anti-proliferative effect of monoterpenes is linked to their ability to regulate the expression of cell cycle-related proteins.

References

- [1] J.D. Haag, M.N. Gould, Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene, *Cancer Chemother. Pharmacol.* 34 (1994) 477–483.
- [2] M. Barthelman, W. Chen, H.L. Gensler, C. Huang, Z. Dong, G.T. Bowden, Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation, *Cancer Res.* 58 (1998) 711–716.
- [3] L.E. Lantry, Z. Zhang, F. Gao, K.A. Crist, Y. Wang, G.J. Kelloff, R.A. Lubet, M. You, Chemopreventive effect of perillyl alcohol on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced tumorigenesis in (C3H/HeJ X A/J) F1 mouse lung, *J. Cell Biochem.* 27 (Suppl.) (1997) 20–25.
- [4] J.J. Mills, R.S. Chari, I.J. Boyer, M.N. Gould, R.L. Jirtle, Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol, *Cancer Res.* 55 (1995) 979–983.
- [5] M.J. Stark, Y.D. Burke, J.H. McKinzie, A.S. Ayoubi, P.L. Crowell, Chemotherapy of pancreatic cancer with the monoterpene perillyl alcohol, *Cancer Lett.* 96 (1995) 15–21.
- [6] B.S. Reddy, C.X. Wang, H. Samaha, R. Lubet, V.E. Steele, G.J. Kelloff, C.V. Rao, Chemoprevention of colon carcinogenesis by dietary perillyl alcohol, *Cancer Res.* 57 (1997) 420–425.
- [7] G.H. Ripple, M.N. Gould, J.A. Stewart, K.D. Tutsch, R.Z. Arzooonian, D. Alberti, C. Feierabend, M. Pomplun, G. Wilding, H.H. Bailey, Phase I clinical trial of perillyl alcohol administered daily, *Clin. Cancer Res.* 4 (1998) 1159–1164.
- [8] G.H. Ripple, M.N. Gould, R.Z. Arzooonian, D. Alberti, C. Feierabend, K. Simon, K. Binger, K.D. Tutsch, M. Pomplun, A. Wahamaki, R. Marnocha, G. Wilding, H.H. Bailey, Phase I clinical and pharmacokinetic study of perillyl alcohol administered four times a day, *Clin. Cancer Res.* 6 (2000) 390–396.
- [9] G.R. Hudes, C.E. Szarka, A. Adams, S. Ranganathan, R.A. McCauley, L.M. Weiner, C.J. Langer, S. Litwin, G. Yeslow, T. Halber, M. Qian, J.M. Gallo, Phase I pharmacokinetic trial of perillyl alcohol (NSC 641066) in patients with refractory solid malignancies, *Clin. Cancer Res.* 6 (2000) 3071–3080.
- [10] P.L. Crowell, R.R. Chang, Z. Ren, C.E. Elson, M.N. Gould, Selective inhibition of isoprenylation of 21–26-Kda proteins by the anticarcinogen *d*-limonene and its metabolites, *J. Biol. Chem.* 266 (1991) 17679–17685.
- [11] R.J. Ruch, K. Sigler, Growth inhibition of rat liver epithelial tumor cells by monoterpenes does not involve Ras plasma membrane association, *Carcinogenesis* 15 (1994) 787–789.
- [12] J. Karlson, A.K. Borg-Karlson, R. Unelius, M.C. Shoshan, N. Wilking, U. Ringborg, S. Linder, Inhibition of tumor cell growth by monoterpenes in vitro: evidence of a Ras-independent mechanism of action, *Anticancer Drugs* 7 (1996) 422–429.
- [13] G.R. Hudes, C.E. Szarka, A. Adams, S. Ranganathan, R.A. McCauley, L.M. Weiner, C.J. Langer, S. Litwin, G. Yeslow, T. Halber, M. Qian, J.M. Gallo, Phase I pharmacokinetic trial of perillyl alcohol (NSC 641066) in patients with refractory solid malignancies, *Clin. Cancer Res.* 6 (2000) 3071–3080.
- [14] E.A. Ariazi, Y. Satomi, M.J. Ellis, J.D. Haag, W. Shi, C.A. Sattler, M.N. Gould, Activation of the transforming growth factor beta signaling pathway and induction of cytotostasis and apoptosis in mammary carcinomas treated with the anticancer agent perillyl alcohol, *Cancer Res.* 59 (1999) 1917–1928.
- [15] P.L. Crowell, Z. Ren, S. Lin, E. Vedejs, M.N. Gould, Structure–activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation, *Biochem. Pharmacol.* 47 (1994) 1405–1415.
- [16] S.R. Cerda, J. Wilkinson, S. Thorgeirsdottir, S.A. Broitman,

- R-(+)-perillyl alcohol-induced cell changes, altered actin cytoskeleton and decreased *ras* and p34^{cdc2} expression in colonic adenocarcinoma SW480 cells, *J. Nutr. Biochem.* 10 (1999) 19–30.
- [17] J.M. Kissane, E. Robins, The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system, *J. Biol. Chem.* 233 (1958) 184–188.
- [18] P. Gillery, A. Bonnet, J.P. Borel, A fluorometric deoxyribonucleic acid assay for tridimensional lattice cultures of fibroblasts, *Anal. Biochem.* 210 (1993) 374–377.
- [19] L.L. Vindelov, I.J. Christensen, N.I. Nissen, A detergent-trypan method for the preparation of nuclei for flow cytometric DNA analysis, *Cytometry* 3 (1983) 323–327.
- [20] X. Qiu, H.J. Forman, A.H. Schonthal, E. Cadenas, Induction of p21 mediated by reactive oxygen species formed during the metabolism of aziridinylbenzoquinones by HCT116 cells, *J. Biol. Chem.* 271 (1996) 31915–31921.
- [21] K. Lu, C. Shih, B.A. Teicher, Expression of pRB, cyclin/cyclin-dependent kinases and E2F1/DP-1 in human tumor lines in cell culture and in xenograft tissues and response to cell cycle agents, *Cancer Chemother. Pharmacol.* 46 (2000) 293–304.
- [22] S. Bardon, K. Picard, P. Martel, Monoterpenes inhibit cell growth, cell cycle progression and cyclin D1 gene expression in human breast cancer cell lines, *Nutr. Cancer* 32 (1998) 1–7.
- [23] C.J. Sherr, G1 phase progression: cycling on cue, *Cell* 79 (1994) 551–555.
- [24] T. Jacks, R.A. Weinberg, Cell-cycle control and its watchman, *Nature* 381 (1996) 643–644.
- [25] C.J. Sherr, J.M. Roberts, CDK inhibitors: positive and negative regulators of G1-phase progression, *Genes Dev.* 13 (1999) 1501–1512.
- [26] S.I. Reed, E. Bailly, V. Dulic, L. Hengst, D. Resnitzky, J. Slingerland, G1 control in mammalian cells, *J. Cell Sci.* 18 (Suppl.) (1994) 69–73.
- [27] M. Kawada, A. Kuwahara, T. Nishikiori, S. Mizuno, Y. Uehara, NA22598, a novel antitumor compound, reduces cyclin D1 levels, arrests cell cycle at G1 phase and inhibits anchorage-independent growth of human tumor cells, *Exp. Cell Res.* 249 (1999) 240–247.
- [28] V. Sandor, A. Senderowicz, S. Mertins, D. Sackett, E. Sausville, M.V. Blagosklonny, S.E. Bates, P21-dependent G1 arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228, *Br. J. Cancer* 83 (2000) 817–825.
- [29] F. Wolter, B. Akoglu, A. Clausnitzer, J. Stein, Downregulation of the cyclin D1/cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines, *J. Nutr.* 131 (2001) 2197–2203.
- [30] Q.S. Wang, A. Papanikolaou, C.L. Sabourin, D.W. Rosenberg, Altered expression of cyclin D1 and cyclin-dependent kinase 4 in azoxymethane-induced mouse colon tumorigenesis, *Carcinogenesis* 19 (1998) 2001–2006.
- [31] T. Zhang, L.B. Nanney, C. Luongo, L. Lamps, K.J. Heppner, R.N. DuBois, R.D. Beauchamp, Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients, *Cancer Res.* 57 (1997) 169–175.
- [32] J. Bartkova, M. Thullberg, P. Slezak, E. Jaramillo, C. Rubio, L.H. Thomassen, J. Bartek, Aberrant expression of G1-phase cell cycle regulators in flat and exophytic adenomas of the human colon, *Gastroenterology* 120 (2001) 1680–1688.
- [33] S. Siavoshian, J.P. Segain, M. Kornprobst, C. Bonnet, C. Cherbut, J.P. Galmiche, H.M. Blottiere, Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression, *Gut* 46 (2000) 507–514.
- [34] K.M. Ropponen, J.K. Kellokoski, P.K. Lipponen, T. Pietilainen, M.J. Eskelinen, E.M. Alhava, V.M. Kosma, p21/WAF1 expression in human colorectal factor carcinoma: association with p53, transcription factor AP-2 and prognosis, *Br. J. Cancer* 81 (1999) 133–140.
- [35] T.A. Holland, J. Elder, J.M. McCloud, C. Hall, M. Deakin, A.A. Fryer, J.B. Elder, P.R. Hoban, Subcellular localisation of cyclin D1 protein in colorectal tumours is associated with p21(WAF1/CIP1) expression and correlates with patient survival, *Int. J. Cancer* 95 (2001) 302–306.
- [36] Y.B. Kim, K.H. Lee, K. Sugita, M. Yoshida, S. Horinouchi, Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase, *Oncogene* 18 (1999) 2461–2470.
- [37] Z.A. Stewart, S.D. Leach, J.A. Pietenpol, p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption, *Mol. Cell Biol.* 19 (1999) 205–215.
- [38] J.J. Mills, R.S. Chari, I.J. Boyer, M.N. Gould, R.L. Jirtle, Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol, *Cancer Res.* 55 (1995) 979–983.
- [39] K.R. Stayrook, J.H. McKinzie, Y.D. Burke, Y.A. Burke, P.L. Crowell, Induction of the apoptosis-promoting protein Bak by perillyl alcohol in pancreatic ductal adenocarcinoma relative to untransformed ductal epithelial cells, *Carcinogenesis* 18 (1997) 1655–1658.