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Perillyl alcohol selectively induces G0/G1 arrest and apoptosis in Bcr/Abl-transformed myeloid cell lines

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The Bcr/Abl tyrosine kinase that is expressed from the Philadelphia chromosome protects leukemia cells from apoptosis caused by removal of growth factors or by cytotoxic agents and ionizing irradiation. This resistance to apoptosis is associated with a Bcr/Abl-mediated G2/M delay. Therefore, inhibiting Bcr/Abl signaling pathways should block the ability of the Bcr/Abl kinase to protect cells from apoptosis. The monoterpenes, limonene and perillyl alcohol (POH) are new anticancer agents that selectively induce apoptosis in neoplastic cells of a variety of rodent carcinoma models. Since the potential antitumor activities of monoterpenes overlap with signaling pathways affected by the Bcr/Abl kinase, POH and limonene were tested for antileukemia activity. POH, but not limonene selectively induced G0/G1 arrest followed by apoptosis in Bcr/Abl-transformed, but not nontransformed FDC.P1 and 32D myeloid cell lines. In contrast to their greater sensitivity to POH, Bcr/Abl-transformed cells were more resistant than nontransformed cells to several chemotherapy agents and ionizing irradiation. Since in Bcr/Abl-transformed cells, POH induces apoptosis associated with G0/G1 arrest, POH must activate an apoptotic pathway that is not protected by the Bcr/Abl-induced G2/M delay. Monoterpenes may represent novel agents for treating Ph$^+$ leukemias.

Keywords: Bcr/Abl; leukemia; monoterpenes; perillyl alcohol; cell cycle; apoptosis

Introduction

Patients with chronic myelogenous leukemia (CML), as well as subgroups of patients with acute lymphoblastic and acute myeloblastic leukemias (ALL and AML, respectively) carry the t(9;22), or Philadelphia chromosome (Ph) in their leukemic stem cells.[1–4] While significant progress has been made in treating many types of leukemias, those that are Ph$^+$ respond poorly to conventional therapies.[5–7] Although with standard chemotherapy, 80% of children with Ph$^+$ ALL achieve remission, only 20% remain event-free after 4 years.[6] Generally, it is even more difficult to treat adult patients with Ph$^+$ ALL; none have attained long-term disease-free survival after treatment with conventional chemotherapy.[5] The experience with Ph$^+$ CML is similar to that of Ph$^+$ ALL in that conventional treatment offers little chance of cure.[7] Bone marrow transplant or, in the case of CML, treatment with alpha interferon can sometimes cure Ph$^+$ leukemia; however, a significant proportion of patients who initially respond to these therapies quickly relapses with an even more aggressive malignancy.[7] More effective treatment strategies are needed in order to improve the survival of patients with Ph$^+$ leukemia.

The Ph translocation fuses the c-Abl gene on chromosome 9 to the c-Bcr gene on chromosome 22. This results in expression of either a p210 or a p185 chimeric Bcr/Abl protein depending on how much of the c-Bcr gene is joined to c-Abl.[1] Both Bcr/Abl fusion proteins express constitutive tyrosine kinase activity that interacts with several cell signaling pathways.[8–10] Thus, either dominant negative suppression of c-Myc or c-H-Ras,[11,12] or inhibition of PI-3 kinase, NF-κB or Bcl-2[13–16] can prevent Bcr/Abl transformation of hematopoietic and fibroblast cell lines. By interfering with these cell regulatory mechanisms, Bcr/Abl enables Ph$^+$ leukemia cells to survive regimens that induce apoptosis in other leukemias that lack the Ph chromosome. Thus, in the absence of specific growth factors normal hematopoietic cells undergo cell cycle arrest and apoptosis.[17–19] In contrast, cells that express the Bcr/Abl tyrosine kinase are able to survive and proliferate without required growth factors.[20–23] Bcr/Abl-transformed cells also resist apoptosis induced by a number of cytotoxic agents that have distinct intracellular targets.[17–19,24–28] Together, these observations suggest that signaling pathways downstream of Bcr/Abl represent potential targets for the development of new chemotherapy agents for treating Ph$^+$ leukemias that are resistant to apoptosis induced by conventional therapies.

Monocyclic monoterpenes represent a new class of anticancer compounds that are synthesized in the mevalonate pathway of plants but not mammals.[29] Limonene, the simplest monoterpene (Figure 1) is able to both prevent and treat rat mammary tumors, and to treat advanced stomach, lung and liver cancers in rats.[30–35] The monohydroxylated derivative of limonene, perillyl alcohol (POH) (Figure 1) is 5–10 times more effective than limonene at treating advanced carcinomas.[36] In tumor-bearing animals fed monoterpenes, tumor regression is associated with an increase in apoptotic cells and a decrease in cell proliferation within the tumor parenchyma. However,

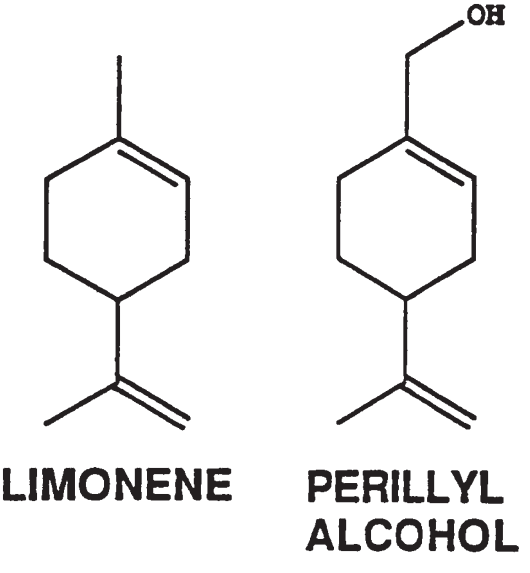

Figure 1 Chemical structure of limonene and perillyl alcohol (POH).

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these antitumor effects are not seen in adjacent normal tissues.[34,37] Importantly, therapeutic doses of either limonene or POH do not cause significant toxicity in rodents, dogs or humans.[33,38,39]

Although the specific mechanism(s) responsible for the anticancer activity of monoterpenes are not known, the compounds affect several cell regulatory activities, some of which may be related to their anticancer activities. For instance, in mammalian cells, monoterpenes block intermediate activities of the mevalonate metabolic pathway[40–45] that in turn inhibit production of ubiquinone, cholesterol and precursors of protein farnesylation and geranylgeranylation.[41] *In vitro*, monoterpenes are also weak, but specific inhibitors of farnesyl and geranylgeranyl transferases.[44] Finally, monoterpene-induced regression of rat carcinomas is associated with significant increases in tumor expression of the mannose 6-phosphate/IGFII and TGF-β1 receptors as well as increased inter- and intracellular levels of TGF-β1.[34,46] While it is unclear which, if any, of these cellular regulatory mechanisms that are affected by monoterpenes are responsible for tumor regression, the potential antitumor activities of monoterpenes may overlap with some of the biological activities that are affected by the Bcr/Abl tyrosine kinase. For this reason, monoterpenes are logical compounds to test for antileukemia activity in Bcr/Abl-induced malignancies. In this report, we document the cellular effects of monoterpenes on Bcr/Abl transformed and nontransformed cells.

Materials and methods

Virus and cell lines

Helper-free P210 Bcr/Abl virus was produced from COS-7 cells that were transiently transfected with a pSRαMSVtkneo plasmid engineered to carry Bcr/Abl cDNA between two murine sarcoma virus long terminal repeats.[47] Virus that only expressed tkneo and not Bcr/Abl was used for control. Culture supernatant collected from the transfected COS cells was used to infect the IL-3-dependent murine myeloid cell lines, FDC.P1[48] and 32D.[49] Two-to-three days after infection, multiple transformed clonal sublines were isolated from the infected cells by G418 selection and limiting dilution. Cell lines infected with the Bcr/Abl virus were shown to express a high level of kinase-active Bcr/Abl protein. Bcr/Abl transformed FDC.P1 and 32D cells also efficiently formed agar colonies in the absence of IL-3 and gave rise to hematopoietic malignancy in syngeneic mice. All cells were maintained in RPMI 1640 media containing 10% fetal bovine serum, plus 10% WEHI-3 conditioned medium (WEHI CM) as a source of IL-3. Whether grown in the presence or absence of factor-conditioned media, transformed FDC.P1 cell lines demonstrated doubling times of about 16 h that was only slightly faster than their nontransformed parental cells or cells infected with the tkneo virus (18 h). In contrast, transformed 32D cells grew slightly faster than their nontransformed counterparts (16 *vs* 24 h doubling times, respectively). Unless otherwise indicated, cells were treated with monoterpenes in the presence of 10% WEHI CM.

Reagents

Stock solutions (100 μm) of POH and limonene (Aldrich, Milwaukee, WI, USA) were made by diluting the monoterpene in fetal bovine serum at 37°C for 30 min with frequent mixing. The stock solutions were then diluted to the desired concentration in cell growth media at 37°C for 30–60 min with mixing. Melphalan, cytosine arabinoside, and hydroxyurea were purchased from Sigma (St Louis, MO, USA). Stock (0.15 m) solutions of melphalan were prepared by dissolving the drug in absolute ethanol plus 10 μ/ml of 12 n HCl. The stock solution was further diluted with growth medium to the desired concentration. Stock solutions of cytosine arabinoside and hydroxyurea were prepared in water and RPMI, respectively, then diluted in growth medium.

Analysis of cell growth and apoptosis

The effect of monoterpenes and other drugs on cell growth and viability were measured either by visually counting the number of cells able to exclude trypan blue, or by the MTT assay using a 'Cell Growth Determination Kit, MTT Based' purchased from Sigma. The MTT assay is based on the cleavage of the yellow dye, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT), to purple formazen crystals by mitochondrial dehydrogenase activity that is only found in living cells. To analyze the cell cycle distribution and quantitate apoptotic cells with subdiploid DNA content, cells were fixed in 90% ethanol, permeabilized with 0.1% Triton-X-100, treated with 5 μg/ml RNase and stained with 50 μg/ml propidium iodide (PI). Nuclear DNA content was measured by flow cytometry using a FACScan Plus flow cytometer. In some experiments apoptosis was confirmed by DNA laddering or with the TUNEL assay in which terminal deoxynucleotidyl transferase is used to add fluorescein-conjugated dUTP to 3'-DNA ends generated by the DNA fragmentation that accompanies apoptosis. The TUNEL reactions were carried out using a kit purchased from Boehringer Mannheim (Indianapolis, IN, USA) and fluorescein-labeled cells were quantitated by flow cytometry.

Cell cycle kinetics

For analysis of cell cycle kinetics, cells (0.4×10^6 per ml) were pulsed for 10–20 min with 30 μm BrUdR at 37°C. Cells were then washed and replated in media containing 100 μm thymidine, either in the presence or absence of POH. At the indicated times after the BrUdR pulse, $1–2 \times 10^6$ cells were collected and fixed with 70% ethanol. After the cells were collected from each time point, they were centrifuged and resuspended in 1 ml of pepsin (0.4 mg/ml in 0.1 m HCl) for 30 min. Three ml of PBS were added and the cells were pelleted and resuspended in 1 ml of 2 m HCl for 30 min at 37°C, then washed in 3 ml of 0.1 m Na tetra borate buffer pH 8.3 to neutralize the acid. The neutralized cell pellet was washed in PBS-Tween (0.5%) containing 1 mg/ml BSA, then incubated with 100 μl PBS containing 10 μl of FITC-conjugated anti-BrUdR antibody (Becton Dickinson, Mountain View, CA, USA) for 30 minutes at room temperature. After the cells were washed, they were labeled with PI and analyzed by flow cytometry.

Irradiation of cells

In order to measure the radiation response of Bcr/Abl-transformed and nontransformed cells, cells in 5 ml growth media

were subjected to 5 Gy radiation at a rate of 5.79 Gy/min from a ^{137}Cs source. After irradiation, cells were washed once and plated at 0.5×10^6/ml for 20 h at which time their viability was assessed by trypan blue exclusion and their cell cycle status determined by flow cytometry after labeling with PI.

Results

POH but not limonene selectively inhibits growth of Bcr/Abl-transformed cells

In order to test the antileukemia effects of monoterpenes on Bcr/Abl-transformed cells, two different factor-dependent cell lines were infected either with a helper-free Bcr/Abl retrovirus or a control virus as described in the Materials and methods section. This allowed us to compare the effects of the monoterpenes on Bcr/Abl-transformed cells and their nontransformed counterparts. The ability of monoterpenes to inhibit growth of the different cell lines was then evaluated either by counting trypan-excluding viable cells or by the MTT assay. There was a close correlation in the results obtained using both assays. Figure 2 shows representative MTT data, which demonstrates that Bcr/Abl-transformed 32D cells are more sensitive than their nontransformed counterparts to growth inhibition by POH. Whereas up to 800 μm POH had no apparent effect on nontransformed 32D cells, two different Bcr/Abl-transformed 32D cell lines were equally inhibited with an IC_{50} of 300–400 μm POH. The factor-independent Bcr/Abl-transformed 32D cells were equally sensitive to POH when treated in the presence or absence of WEHI CM (data not shown). Finally, treatment with up to 800 μm limonene had no apparent effect on either nontransformed or Bcr/Abl-transformed 32D cell lines (data not shown). Results identical to these were obtained in three different experiments using a total of three different Bcr/Abl-transformed 32D cell lines.

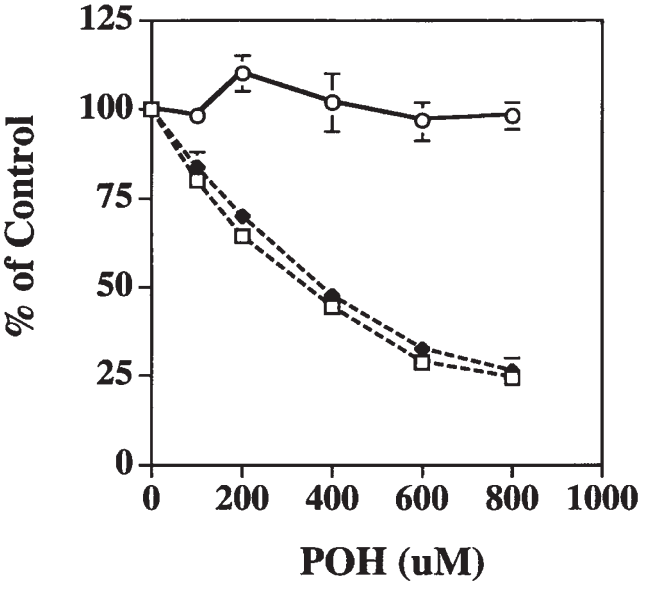

Figure 2 POH specifically inhibits proliferation of Bcr/Abl-transformed 32D myeloid cells. 1×10^4 transformed or nontransformed cells were plated in quadruplicate wells of 96-well plates in the presence of increasing concentrations of POH. Following treatment for 20 h, cell viability was quantitated by the MTT assay using a kit from Sigma. At each drug concentration, the results are expressed as percent MTT activity relative to nontreated control cells. Data are shown for two independently transformed lines (dashed lines) and for one control line (solid line). Similar results were obtained for three independent Bcr/Abl-transformed 32D cell lines.

In order to determine whether other Bcr/Abl-transformed cell lines are also sensitive to POH, we tested the effects of the monoterpenes on Bcr/Abl-transformed and nontransformed FDC.P1 cells. These experiments confirmed that treatment with up to 800 μm limonene had no detectable effect on either Bcr/Abl-transformed or nontransformed cell lines (Figure 3a). In contrast to limonene, treatment with POH inhibited growth of both nontransformed and transformed FDC.P1 cells. However, Bcr/Abl-transformed cells were reproducibly more sensitive than nontransformed cells. Whereas, after a 24 h treatment with POH the nontransformed FDC.P1 cells were inhibited with an IC_{50} of approximately 900 μm, Bcr/Abl-transformed cells were inhibited with an IC_{50} of 400 μm POH (Figure 3b). When the factor-independent Bcr/Abl-transformed cells were treated with POH in the absence of WEHI CM, the dose-response curve was shifted downward so that the IC_{50} was 200–300 μm (Figure 3b) indicating that in the absence of exogenous growth factor the transformed cells were even more sensitive to the effects of POH. Data in Figure 3c demonstrate that the sensitivity of FDC.P1 cells to POH increases with longer exposure to the monoterpene. Thus, 72 h incubation with 100 μm POH inhibited Bcr/Abl-transformed FDC.P1 cells by about 80%, while the nontransformed cells were inhibited about 20%. In the absence of WEHI CM the transformed cells were inhibited by about 90% with 100 μm POH which confirms that their sensitivity increases when exogenous growth factor is withdrawn. These data have been confirmed in multiple experiments and with three different Bcr/Abl-transformed FDC.P1 clones and three different control cell lines infected with the 'empty' virus (data not shown).

Bcr/Abl-transformed FDC-P1 cells are more resistant than nontransformed cells to various chemotherapy agents and irradiation

The greater sensitivity of Bcr/Abl-transformed cells to POH shown in Figures 2 and 3 contrasts with reports that the oncogene protects cells from growth inhibition and apoptosis induced by a variety of cytotoxic agents.[17–21,23,24,27,28,50] Therefore, it was important to confirm that, as previously reported, our Bcr/Abl-transformed cells were more resistant than the nontransformed cells to these cytotoxic agents. In order to test this, transformed and nontransformed FDC.P1 cells were treated with hydroxyurea (Figure 3d) or melphalan (Figure 3e). From the MTT assay, it is evident that the transformed cells had a three- to four-fold higher IC_{50} than the nontransformed cells and were, therefore, more resistant to these chemotherapy agents (Figure 3d, e, and data not shown).

Both Bcr/Abl and IL-3 reportedly protect hematopoietic cells from apoptosis induced by ionizing irradiation by causing a cell cycle delay at the G2/M restriction point. Therefore, the radiation response of the Bcr/Abl-transformed FDC.P1 cells was also compared to the response of the nontransformed parental cells in order to determine whether the cells responded to radiation as reported previously.[28] The cells were incubated in the presence or absence of 10% WEHI CM and either left unirradiated or irradiated with 5 Gy from a ^{137}Cs source. Twenty hours later, cell viability was quantitated

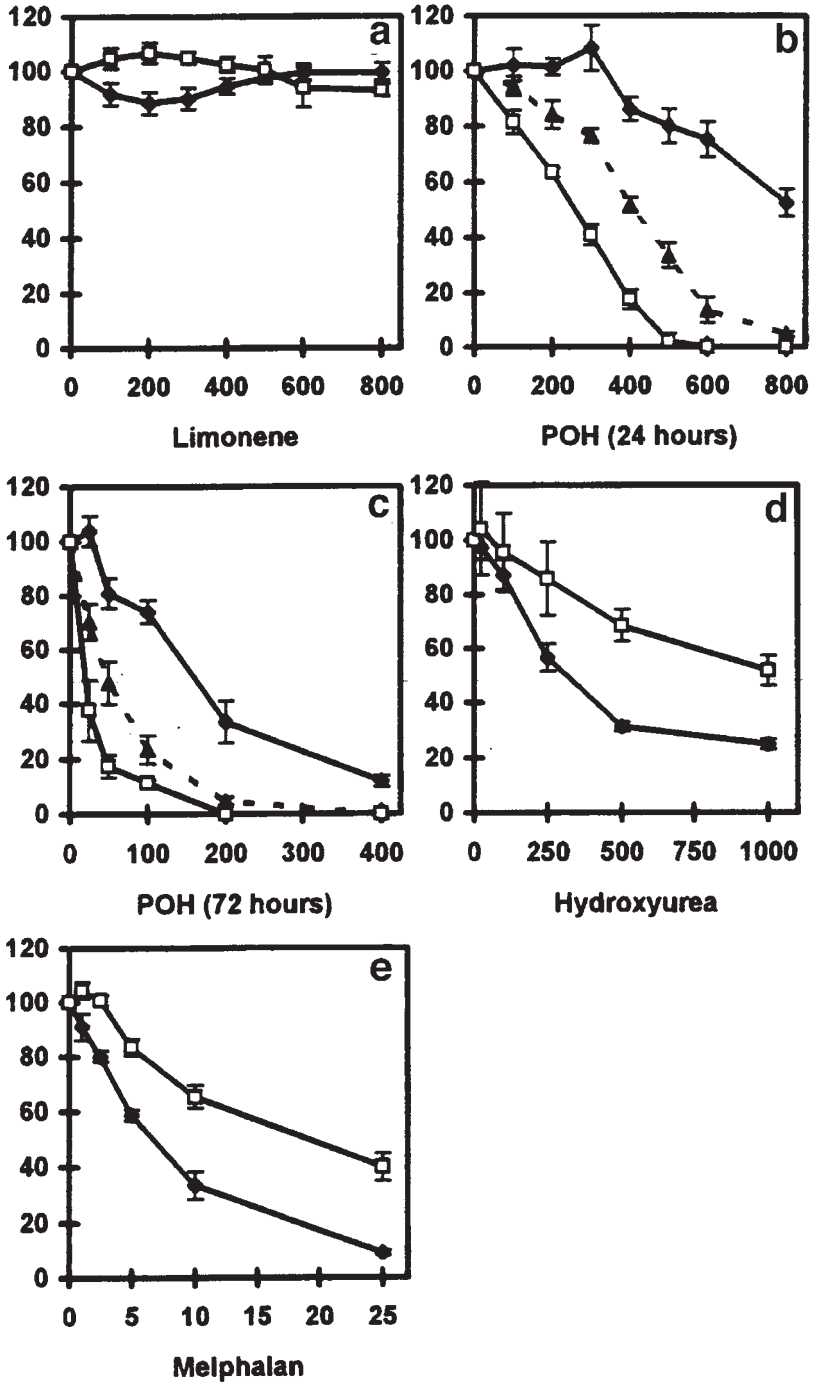

Figure 3 POH selectively inhibits proliferation of Bcr/Abl-transformed FDC.P1 cells. Either 1×10^4 (panels a, b, d and e), or 1×10^3 cells (panel c) from a Bcr/Abl-transformed or nontransformed cell line were plated in quadruplicate wells of 96-well plates and treated with the indicated concentrations of the different compounds. The transformed cells were treated with the different compounds in the presence of 10% WEHI CM as a source of IL-3, except in panels b and c, in which the transformed cells were treated with POH in both the presence and absence of WEHI CM. Cells were treated for 24 h (panels a, b, d, e) or for 72 h (panel c) with the indicated agent and their survival and growth was assayed using the MTT assay as described in Figure 2. Similar results were obtained in two to five separate experiments for three independent Bcr/Abl-transformed and nontransformed control cell lines. (◆) Nontransformed cells; (□) Bcr/Abl transformed cells in the presence of WEHI CM; (▲) Bcr/Abl transformed cells in the absence of WEHI CM.

by trypan blue exclusion and DNA content was measured by staining with propidium iodide. The data in Table 1 show that after 20 h without WEHI CM, the unirradiated nontransformed cells (group 1), as expected, showed reduced survival compared to the same cells incubated in the presence of WEHI CM (group 3). After irradiation, the nontransformed cells cultured without WEHI CM (group 2) showed further loss of viability and a significant increase in apoptotic cells with <2 n DNA content. As reported previously, both IL-3 (WEHI CM) and Bcr/Abl protected the viability of the cells after irradiation (compare group 4 to group 2 and group 6 to group 5). Furthermore, after irradiation both the WEHI CM-treated

Table 1 Bcr/Abl and IL-3 protect FDC.P1 cells from irradiation-induced apoptosis[a]

Group	Bcr/Abl-transformed	IL-3	Irradiation	% Viable	Cell cycle distribution			
					% G0/G1	% S	% G2/M	% <2 N
1	No	–	–	26.3	75.6	4.7	11.0	8.0
2	No	–	+	2.2	38.9	13.1	25.0	23.8
3	No	+	–	92.6	62.1	22.1	14.9	0.9
4	No	+	+	69.7	55.9	6.7	33.7	3.7
5	Yes	–	–	91.4	53.3	21.1	23.9	1.7
6	Yes	–	+	89.1	50.0	6.0	42.1	2.0

[a]Protection from apoptosis is associated with an accumulation of cells in G2/M (eg groups 4 and 6) which presumably allows cells time to repair DNA prior to cell division, thereby protecting them from mitotic catastrophe. Cell viability was measured by trypan blue exclusion and cell cycle distribution was measured by propidium iodide incorporation and flow cytometry. The cells were irradiated with 5 Gy and assayed 20 h later.

nontransformed cells (group 4) and the Bcr/Abl-transformed cells (group 6) showed a reduced fraction of cells in S phase, a concomitant increase of cells in G2/M, but only a minimal change in the number of apoptotic cells with <2 n DNA content. Thus, the radiation response of our nontransformed and Bcr/Abl-transformed FDC.P1 cells was similar to what was reported previously.

POH causes Bcr/Abl-transformed cells to undergo G0/G1 arrest and apoptosis

The POH-induced inhibition of Bcr/Abl-transformed cells as measured by the MTT assay in Figures 2 and 3, could reflect an effect of the compound on cell division and/or viability. In experiments where the effect of POH was measured by directly counting viable and nonviable cells, growth inhibition directly correlated with cytotoxicity measured by failure to exclude trypan blue. In general, a net loss in the number of viable Bcr/Abl-transformed FDC.P1 cells was observed after treatment with as little as 100–200 μm POH for 16–24 h. Microscopic examination of Wright-stained cytocentrifuge preparations of Bcr/Abl-transformed FDC.P1 cells showed that while POH-untreated cells displayed normal morphology (Figure 4A, B), cells that had been treated for 16 h with 400 μm POH (Figure 4C, D) showed nuclear condensation and cytoplasmic blebbing, both of which are consistent with apoptosis.[51,52] Other cells in the POH-treated group that did not show this morphology nevertheless demonstrated increased cytoplasmic vacuolization suggestive of early apoptotic changes.[51]

The above observation implies that a primary effect of POH on Bcr/Abl-transformed cells may be to induce apoptosis, similar to earlier reports on POH-induced regression of rodent carcinomas.[34–37] However, apoptosis often follows cell cycle arrest, which would also implicate inhibition of cell generation in the antileukemia effect. In order to address this question, the effect of POH on the cell cycle was examined by flow cytometric quantitation of DNA content that can also identify apoptotic cells with <2 n DNA content. Treatment of the nontransformed FDC.P1 cells with 500 μm POH for 16 h did not cause significant changes in cell cycle distribution (Figure 5A, panels *a* and *c*). In contrast, POH treatment caused a significant increase in the fraction of Bcr/Abl-transformed cells that were in G0/G1 and a concomitant decrease in the proportion of cells in the S and G2/M stages of the cell cycle (Figure 5B, panels *a* and *c*). Treatment with POH also left about 20% of the transformed cells with <2 n DNA content (Figure 5B, panels *a* and *c*).

In order to confirm independently that the POH-treated Bcr/Abl-transformed cells in the experiment shown in Figure 5 were undergoing apoptosis, nuclei were also analyzed using the TUNEL assay and internucleosomal DNA fragmentation was examined by agarose gel electrophoresis of genomic DNA. Treatment of nontransformed FDC.P1 cells with 500 μm POH for 16 h did not cause significant fluorescent labeling of cell nuclei in the TUNEL assay (Figure 5A, panels *b* and *d*) or DNA fragmentation (data not shown). However, similar treatment of Bcr/Abl-transformed FDC.P1 resulted in about 20% of cell nuclei being labeled in the TUNEL reaction (Figure 5B, panels *b* and *d*). DNA laddering after agarose gel electrophoresis was also observed in these POH-treated cells (data not shown). Thus, a similar fraction of POH-treated Bcr/Abl-transformed cells were identified as apoptotic after labeling nuclei with the TUNEL assay and by measuring DNA content.

In order to compare further the differential sensitivity to POH of the nontransformed and Bcr/Abl-transformed cells, the effect of increasing concentrations of POH on cell cycle distribution was examined. FDC.P1 and 32D cells were treated with different concentrations of POH then stained with PI in order to quantitate by flow cytometry, the number of cells at each stage of the cell cycle. Treatment of nontransformed FDC.P1 cells with up to 500 μm POH for 16 h resulted in only minimal changes in cell cycle distribution (Figure 6a). In contrast, after treatment with as little as 250 μm POH, Bcr/Abl-transformed FDC.P1 cells displayed obvious changes in cell cycle distribution and showed an increase in the fraction of apoptotic cells that have a subdiploid DNA content (Figure 6b). In both transformed and nontransformed FDC.P1 cells we observed a POH concentration-dependent decrease in the number of cells in S phase, and this correlated with a proportional increase in cells in G0/G1. However, in Bcr/Abl-transformed FDC.P1 cells but not in the nontransformed controls, the percentage of cells in G0/G1 declined sharply between 500 and 750 μm POH, and this correlated with a dramatic increase in the fraction of cells with a subdiploid DNA content. These data were reproduced in three different Bcr/Abl-transformed FDC.P1 cell lines (data not shown).

Similar results to increasing concentrations of POH were obtained with 32D cells. Thus, nontransformed 32D cells treated with increasing concentrations of POH showed only modest changes in their cell cycle distribution (Table 2). In contrast, Bcr/Abl-transformed 32D cells treated with 200–600 μm POH showed a simultaneous increase in cells in

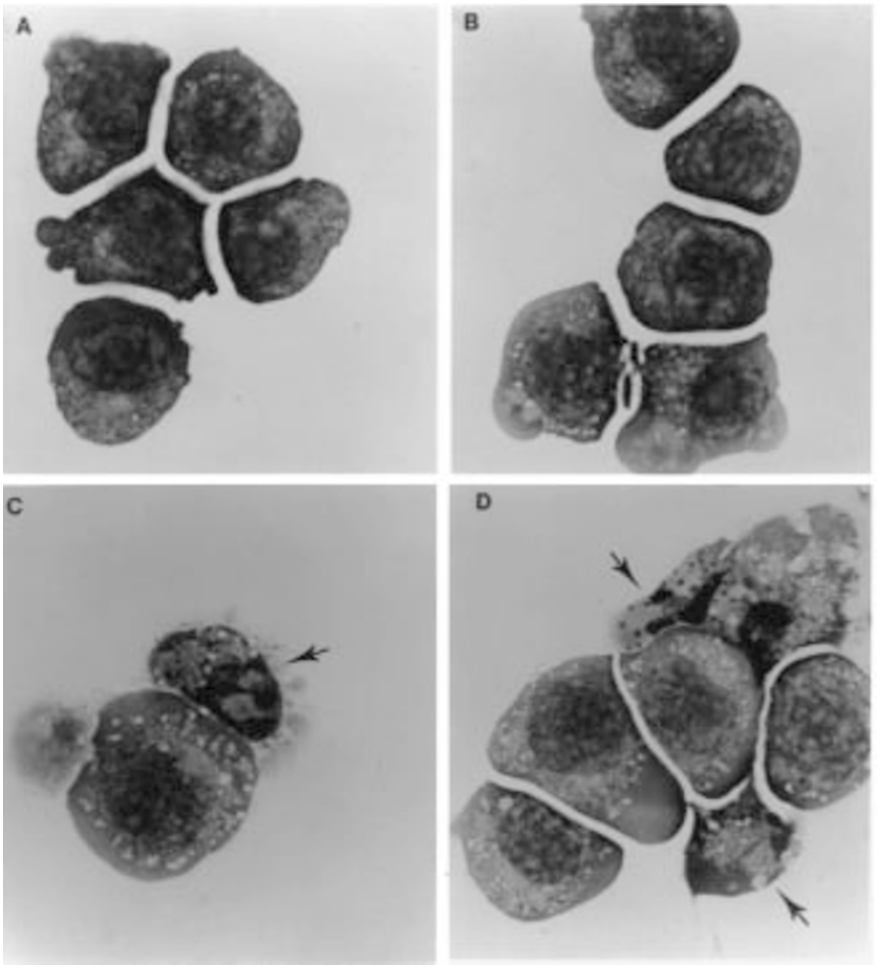

Figure 4 POH induces apoptosis in Bcr/Abl-transformed FDC.P1 cells. Transformed cells that were either untreated (panels A and B) or treated for 24 h with 400 μm POH (panels C and D) were cytocentrifuged and processed by Wright's stain. Two separate microscopic fields for each treatment group are shown. In the POH-treated group, some cells show significant nuclear condensation and cytoplasmic blebbing (arrows) while other cells demonstrated increased cytoplasmic vacuolization when compared to the untreated cells. Original magnification × 400.

G0/G1 and loss of cells in S phase. A significant reduction in the G0/G1 stage of the cell cycle occurred at 800 μm POH, and this was accompanied by an equivalent increase in the fraction of cells undergoing apoptosis (Table 2). These observations demonstrate that POH treatment preferentially causes Bcr/Abl-transformed 32D and FDC.P1 cells to accumulate in G0/G1 and to undergo apoptosis. This response occurs in a POH dose-dependent fashion.

Differential effects of POH on cell cycle kinetics of Bcr/Abl-transformed and nontransformed cells

The timing of the effects of POH on cell cycle distribution and apoptosis was examined by measuring the DNA content of cells treated with POH for increasing lengths of time. Beginning 3–6 h after POH treatment, Bcr/Abl-transformed FDC.P1 cells showed a steady accumulation in G0/G1 and a concomitant decrease in the fraction of cells in S phase (Figure 7). The number of cells with subdiploid DNA content did not change until after 12 h of POH treatment, at which time there was a sharp increase in apoptotic cells which correlated with a similarly sharp decrease in cells in G0/G1. These observations were reproduced in each of the different Bcr/Abl-transformed FDC.P1 cell lines. Furthermore, Bcr/Abl-transformed 32D cells also showed similar temporal changes in cell cycle distribution after exposure to POH. Within 12 h after POH treatment, the proportion of cells in G0/G1 increased from 40% to 75%. Between 12 and 24 h, the fraction of cells in G0/G1 then declined dramatically and this correlated with an increase in the proportion of cells with <2 n DNA content from 0% to 12% (data not shown).

The observation that cells accumulate in G0/G1 after POH treatment suggests that POH causes a cell cycle delay at G0/G1. However, this observation is also consistent with a POH-accelerated exit of cells from S and G2/M stages of the cell cycle. In order to evaluate the effects of POH on the ability of Bcr/Abl-transformed and nontransformed cells to traverse the cell cycle, cells were pulse-labeled with BrUdR then incubated for different lengths of time with or without POH. Cells were then harvested, fixed and processed for simultaneous analysis of DNA content and BrUdR staining. Since BrUdR is only incorporated into the DNA of cells in S phase, this enables quantitation of the effects of POH on the ability of the labeled cells to leave S phase, proceed through G2/M, G1 and to then reenter S-phase. Up to 6 h after treatment with POH, both nontransformed (Figure 8a) and Bcr/Abl-transformed FDC.P1 cells (Figure 8b) steadily emigrated out of early S phase (indicated by the box in the 0-h time point). By 9 h after POH treatment, cells in the nontransformed group

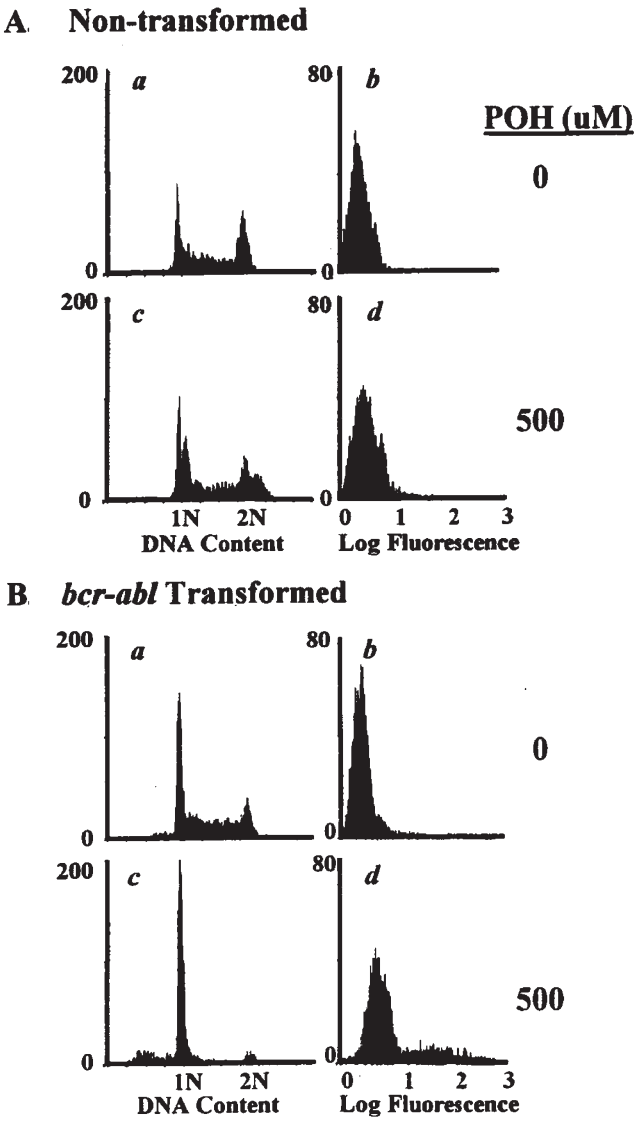

Figure 5 Bcr/Abl-transformed FDC.P1 cells are more sensitive than nontransformed cells to growth arrest and apoptosis induced by POH. Nontransformed (A) and Bcr/Abl-transformed (B) FDC.P1 cells were either untreated (panels *a* and *b*) or treated with 500 μm POH for 16 h (panels *c* and *d*). The cell cycle distribution (panels *a* and *c*) was determined by measuring the DNA content of propidium iodide stained nuclei using flow cytometry. The 1 n and 2 n DNA peaks point out cells in G0/G1 and G2/M, respectively. Apoptotic cells with a subdiploid DNA content are to the left of the 1 n peak. Apoptosis was also quantitated by flow cytometry after fluorescence labeling by the TUNEL assay (panels *b* and *d*). Both techniques revealed that 20% of the cells were undergoing apoptosis in the Bcr/Abl-transformed population treated with POH.

reentered early S phase, and this continued through the 12 and 15 h times. In contrast, Bcr/Abl-transformed cells failed to reenter S phase and, instead, accumulated in G0/G1.

The effects of POH on the fraction of cells in early S phase shown in Figure 8, are plotted in Figure 9, which also includes data from cells that were not treated with POH and that were not shown in Figure 8. In the absence of POH, both transformed and nontransformed cells showed the expected loss of the early S phase population 3 h after the BrUdR pulse. These transformed and nontransformed cells simultaneously began to reaccumulate in early S phase 9–12 h after labeling which

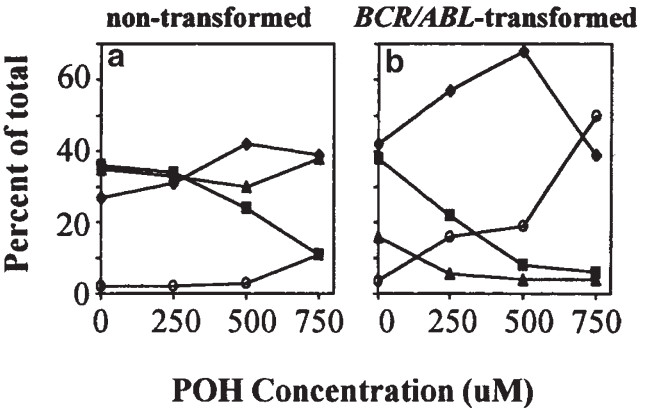

Figure 6 Effect of increasing concentration of POH on cell cycle distribution of nontransformed (a) and Bcr/Abl-transformed (b) FDC.P1 cells. Cells were treated for 16 h with the indicated concentrations of POH, then labeled with propidium iodide and DNA content quantitated by flow cytometry. The percent of cells in G0/G1 (◆), S (■), G2/M (▲) and with subdiploid DNA content (○) are plotted as a function of POH concentration.

indicates that they transit the cell cycle with similar kinetics. This second early S phase peak, seen in Figure 9, is prolonged because the cells that first reappear in early S phase after 9 h represent those that were in late S phase during the BrUdR pulse. Cells that were in early S phase during the BrUdR pulse reenter the early S phase compartment at a later time. In both Bcr/Abl-transformed and nontransformed cells, treatment with POH slowed the emigration from early S phase. This suggests that besides the selective G0/G1 delay in Bcr/Abl-transformed cells, the monoterpene also caused a general delay in S phase in both transformed and nontransformed cells. Since reentry into S phase does not seem to be affected in the POH-treated nontransformed cells, it appears that this POH-mediated S phase delay may occur in cells that are in early but not late S phase.

Discussion

While it is well documented that monoterpenes exhibit potent antitumor activity against a variety of rodent carcinomas,[34–37] this is the first report to show that the compounds may be effective against leukemia as well. Similar to what was observed in rat carcinoma models,[40,45] we found that POH was significantly more active than limonene against Bcr/Abl-transformed cells. Although the concentration of POH required to inhibit growth of the leukemia cells is fairly high (IC_{50} = 200–400 μm) it is well within the range of serum monoterpene levels that can be reached in rats, dogs, mice and humans fed POH (Refs 33, 36; Sahin and Clark, unpublished observations). We also found that POH concentrations that did not markedly affect nontransformed cells caused Bcr/Abl-transformed cells to undergo a G0/G1 cell cycle arrest that was followed by apoptosis. This ability of POH to selectively cause G0/G1 arrest and apoptosis in Bcr/Abl-transformed cells is significant since the Bcr/Abl-transformed hematopoietic cells are resistant to apoptosis induced by conventional chemotherapy agents and ionizing radiation. Bcr/Abl reportedly suppresses the apoptotic response to these agents by causing a pronounced delay at the G2/M cell cycle restriction point.[28] Therefore, the fact that in Bcr/Abl-transformed cells, POH induces apoptosis that is associated with a

Table 2 Bcr/Abl-transformed 32D cells are more sensitive than nontransformed cells to G0/G1 arrest and apoptosis induced by POH[a]

Group	Bcr/Abl-transformed	POH conc (μM)	Cell cycle distribution			
			% G0/G1	% S	% G2/M	% <2 N
1	No	0	61.4	24.6	13.7	0.3
2		200	57.3	26.9	15.5	0.4
3		600	64.0	20.8	14.1	0.8
4		800	67.7	17.8	13.1	1.4
5	Yes	0	54.9	24.9	18.9	1.1
6		200	60.1	21.6	13.7	3.5
7		600	76.9	6.4	9.2	7.1
8		800	56.1	7.8	10.4	25.6

[a]Bcr/Abl-transformed and nontransformed 32D cells were treated with the indicated concentration of POH for 24 h. Cell cycle distribution was measured by PI staining and flow cytometry.

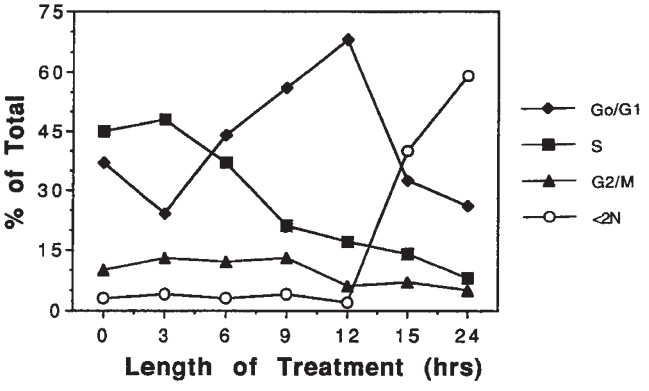

Figure 7 Timing of the POH effect on cell cycle distribution. A Bcr/Abl-transformed FDC.P1 cell line was treated with 400 μm POH, harvested at the indicated times, labeled with propidium iodide then analyzed by flow cytometry for DNA content. The fraction of cells in G0/G1 (◆), S (■), G2/M (▲) and with subdiploid DNA content (○) are plotted as a function of time.

G0/G1 arrest indicates that POH activates an apoptotic pathway that is not protected by the G2/M delay that is enforced by the oncogene.

Our observations suggest that the selective antileukemia effect of POH may operate by a process that both inhibits leukemia cell generation and induces leukemia cell death. At first glance, this antileukemia effect of POH appears to be similar to the effects of the compound on mammary tumors that are also characterized by inhibition of tumor cell generation and accelerated tumor cell death via apoptosis. However, in POH-induced regressing mammary tumors, apoptosis was evident prior to growth arrest,[39] which is distinct from the results in our leukemia model in which G0/G1 arrest preceded apoptosis. The effects of POH on Bcr/Abl-transformed cells are also different from the reported antitumor effects of POH on hepatic carcinomas that show increased apoptosis without an appreciable effect on tumor cell proliferation.[34] These distinct effects of POH on different malignancies indicate that monoterpenes either affect different oncogenic pathways in different types of malignancy, or that monoterpenes affect a common oncogenic activity, the consequence of which may differ in distinct types of malignancies.

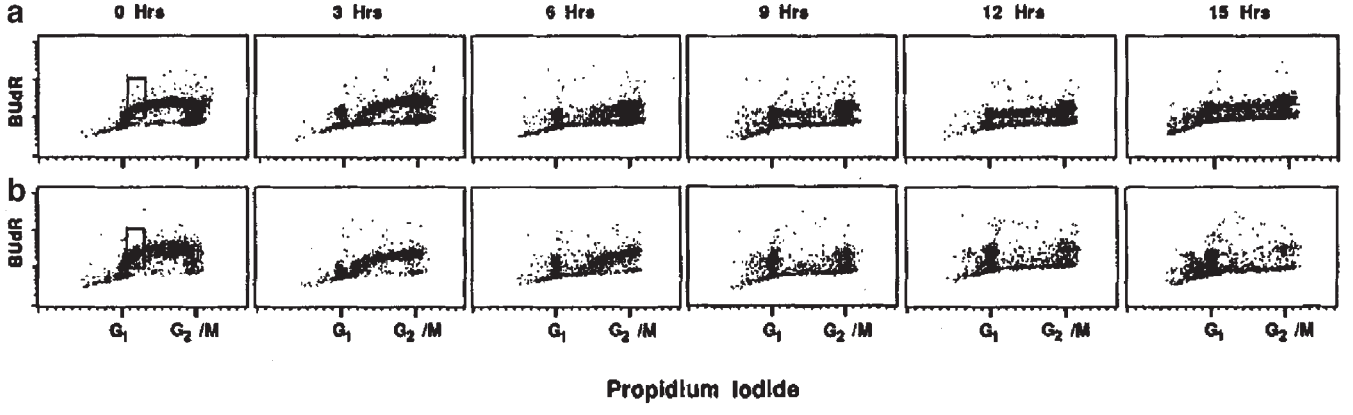

Figure 8 Effect of POH on cell cycle progression. Both nontransformed (a) and Bcr/Abl-transformed FDC.PI (b) cells were pulse labeled with BrUdR then incubated with 400 μm POH for the indicated time periods. At each time point cells were harvested and labeled with anti-BrUdR monoclonal antibody and PI, then analyzed by flow cytometry. The box in the 0 h panels indicates the early S phase population. The data reveal that both nontransformed and Bcr/Abl-transformed cells treated with POH exit the S phase of the cell cycle with approximately similar kinetics. However, while the Bcr/Abl-transformed cells undergo G0/G1 arrest and fail to reenter S phase, the nontransformed cells reenter S phase beginning 9 h after the BrUdR pulse.

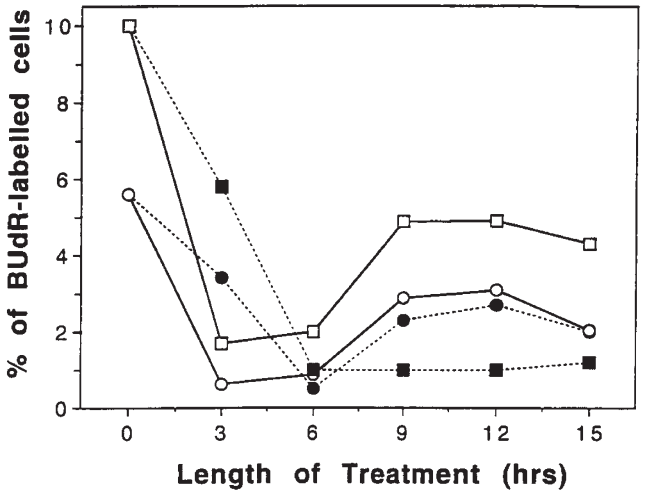

Figure 9 The percent of BrUdR-labeled cells in early S phase (ie within the boxes in the 0 h time point shown in Figure 8) is plotted as a function of time. Data are from the experiment in Figure 8, and are shown for both POH treated (●, ■) and untreated (○, □) populations. Bcr/Abl-transformed cells (□, ■); nontransformed cells (○, ●).

The fact that POH treatment caused Bcr/Abl-transformed cells to first accumulate in G0/G1 and then to undergo apoptosis raises the possibility that the primary antileukemia effect of POH may be to induce G0/G1 arrest with apoptosis being a secondary consequence of the growth arrest. However, the results presented here do not allow us to formally exclude the possibility that POH directly contributes to apoptosis as well. Nevertheless, since POH seems to induce apoptosis in a cell cycle-dependent manner in Bcr/Abl-transformed cells, then the selective antileukemia effect could be accounted for by faster cell cycle transit of the Bcr/Abl-transformed cells compared to the nontransformed controls. However, the fact that both transformed and nontransformed FDC.P1 cells pulse labeled with BrUdR proceeded through the cell cycle at a similar pace indicates that different cell cycle kinetics may not necessarily be the reason for their differential sensitivity to POH.

The observation that compared to nontransformed cells, POH preferentially induces G0/G1 arrest and apoptosis in Bcr/Abl-transformed cells makes it reasonable to think that the Bcr/Abl kinase sensitizes cells to the effects of POH. However, since both the Bcr/Abl tyrosine kinase and normal growth factor receptor tyrosine kinases can interact with common cell regulatory pathways it may seem counterintuitive that POH could selectively inhibit transformed but not nontransformed cells by blocking such pathways. On the other hand, our demonstration that POH selectively inhibits Bcr/Abl-transformed cells is consistent with the possibility that POH either interacts with different regulatory mechanisms in Bcr/Abl-transformed *vs* nontransformed cells, or that in both transformed and nontransformed cells, POH affects similar regulatory pathways, albeit in different ways. More information on the mechanism by which POH preferentially inhibits growth and causes apoptosis in Bcr/Abl-transformed cells is needed in order to discern between these alternatives.

Even though the mechanism of the POH antileukemia effect is not known, POH-induced regression of rat carcinomas has been associated with several activities that may be relevant to this anticarcinogenic activity as well as the antileukemia effect reported here. One of these activities includes induction of TGF-β1 and/or M6P/IGFII receptors.[34,46] This activity of POH is potentially relevant to its anticancer and antileukemia effects because the latent form of TGF-β1 has two mannose residues that also bind to M6P/IGFII receptors, which facilitates activation of the cytokine.[53,54] Therefore, since most cells can secrete TGF-β1, this receptor-mediated activity of monoterpenes was postulated to be responsible for the observed carcinoma regression via a cytostatic/differentiation mechanism.[34,46] This mechanism could also play a role in the antileukemia effect since hematopoietic cells express the M6P/IGFII receptor[53,54] and can activate latent TGF-β1.[54] Additionally, hematopoiesis can be regulated by TGF-β1.[55,56] However, we found that TGF-β1 neutralizing antibodies do not protect Bcr/Abl-transformed cells from the antileukemia effects of POH (Jenkins and Clark, unpublished observations). For this reason, we do not believe that the antileukemia activity of POH operates through a TGF-β1-dependent mechanism.

POH also inhibits isoprenylation of small G proteins, including p21 Ras, and this was postulated to be related to its anticarcinoma activity.[45] Interestingly, Ras is necessary for Bcr/Abl-mediated transformation[8,12] and resistance to apoptosis.[26] Since farnesylation is necessary for membrane localization and function of the Ras molecule,[41,57] it seems reasonable that inhibiting this post-translational modification could play a role in the POH-mediated G0/G1 arrest and apoptosis seen in Bcr/Abl-transformed cells. However, growth inhibition of v-Ha-Ras-transformed rat liver epithelial cells by monoterpenes does not correlate with a change in the subcellular distribution of Ras.[58] Furthermore, the antileukemia response to POH is rapid with G0/G1 arrest apparent within 3–6 h after POH treatment. This rapid response to POH was also reported in a differentiation model in which Neuro-2A cells were induced to form neurites within 4 h after exposure to POH.[59] In distinct contrast to these relatively rapid effects of POH, farnesylated Ras reportedly has a half-life of >24 h,[60] making it unlikely that POH could exert such rapid effects on proliferation and differentiation by inhibiting Ras farnesylation.

In summary, this report documents the cellular response of Bcr/Abl-transformed and nontransformed cells to POH. Although the precise mechanism by which POH preferentially induces growth arrest and apoptosis in Bcr/Abl-transformed cells is not yet known, the mechanism behind this activity may point to new strategies for treating Ph^+ leukemia. How POH selectively induces G0/G1 arrest and apoptosis in Bcr/Abl-transformed cells is under investigation.

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