

# Anti-Leukemia Effect of Perillyl Alcohol in Bcr/Abl-Transformed Cells Indirectly Inhibits Signaling through Mek in a Ras- and Raf-Independent Fashion

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## ABSTRACT

**Purpose:** Perillyl alcohol (POH) displays preventive and therapeutic activity against a wide variety of tumor models, and it has been suggested that this might be associated with the ability of POH to interfere with Ras prenylation. POH also selectively induces G<sub>1</sub> arrest and apoptosis in Bcr/Abl-transformed hematopoietic cells. Because signaling through Ras is necessary for Bcr/Abl transformation, we examined whether POH induces its anti-leukemia effect by inhibiting Ras signaling.

**Experimental Design:** The ability of POH to inhibit posttranslational farnesylation and signaling from Ras as well as signaling through the Raf-Mek-Erk cascade was examined in Bcr/Abl-transformed and mock-transformed cells and related to the anti-leukemia effect of POH.

**Results:** POH does not affect Ras prenylation or Ras activity, but it blocks signaling downstream of Ras by reversing the state of activation of the Erk kinase, Mek. POH affects Mek activity only when it is added to intact cells. Treatment of either cell lysates or of purified Mek with POH has no effect on Mek activity. Inhibition of the Mek-Erk pathway seems to be related to the POH anti-leukemia effect for the following reasons: (a) the concentration of POH needed to block the Erk pathway, as well the kinetics with which POH inhibits this signaling cascade, both correlate with the anti-leukemia effect of POH; (b) both U0126 (a specific Mek inhibitor) and POH induce similar anti-leukemia effects; and (c) mock-transformed hematopoietic cells are simultaneously resistant to POH anti-leukemia effects and inhibition of the Mek-Erk pathway.

**Conclusion:** Blocking Mek is sufficient to induce growth arrest and apoptosis in Bcr/Abl-transformed cells; therefore, POH represents a novel small molecule inhibitor of Mek that might be effective for treating Bcr/Abl leukemias.

## INTRODUCTION

In response to mitogenic or differentiation signals, the p21 Ras small G protein complexes with and activates a MAP3K,<sup>2</sup> such as Raf-1, which in turn phosphorylates and activates the MAP kinase kinase (Mek1/2), which does the same to the p42/44 MAPK, Erk. Phosphorylated Erk then translocates into the nucleus where it phosphorylates and activates transcription factors, thereby completing the circuit between the cell membrane and the nucleus (1). Oncogenic signals are also delivered through this cascade and play an important role in the pathogenesis of human cancer (2) and leukemia (3–9). In malignant cells, Ras can be activated in several ways including by point mutations that leave Ras constitutively activated (2, 4, 10, 11), which is the most common genetic abnormality detected in human hematopoietic malignancies (2). Aberrant Ras signaling also arises because of mutations that alter the activities of different proteins that regulate the activity of Ras (4, 12–14). Most relevant to this report is the fact that Ras can be constitutively activated by oncogenic tyrosine kinases, such as Bcr/Abl (10, 11, 15–19) that is expressed from the t(9;22) Philadelphia chromosome translocation (20). Bcr/Abl signals that are transmitted through Ras and the Erk cascade (15, 19) play an important role in transformation by Bcr/Abl (15, 21, 22). Therefore, it is not surprising that Bcr/Abl leukemias are sensitive to both loss of Ras expression (16) and activity (23) and to inhibition of the MAPK pathway downstream of Ras (21, 24, 25).

Signaling pathways that are stimulated by the Bcr/Abl kinase may provide targets for new anti-leukemia therapies. This principle has been proven in studies with the Bcr/Abl tyrosine kinase inhibitor STI571 (26–28), which can induce remission in patients with Ph<sup>+</sup> leukemia (29, 30). However, some patients do not respond well to STI571, and many who do, eventually relapse (29, 31) with STI571 unresponsive leukemia. The lack of a response to STI571 is attributable to reactivation of Bcr/Abl signaling caused by point mutation in the Abl kinase domain or by Bcr/Abl gene amplification (32). For this reason, it would be useful to identify new inhibitors of critical Bcr/Abl signaling pathways that might be useful for treating Ph<sup>+</sup> leukemias.

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<sup>2</sup> The abbreviations used are: MAP3K, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; POH, perillyl alcohol; RBD, Ras-binding domain; IL-3, interleukin 3; MBP, myelin basic protein; OA, okadaic acid; WEHI, Walter and Eliza Hall Institute.

Monocyclic monoterpenes represent a new class of anticancer compounds that are able to both prevent and treat a wide variety of rodent malignancies, including mammary, stomach, pancreatic, colon, lung and liver cancers (33–38). Monoterpenes affect several cell functions, some of which may be related to their anticancer activities. For instance, monoterpenes block intermediate activities of the mevalonate metabolic pathway that are responsible for the production of ubiquinone, cholesterol, and precursors of protein prenylation (farnesyl- and geranylgeranyl-pyrophosphates; Refs. 39–43). Some monoterpenes are also weak but specific inhibitors of farnesyl and geranylgeranyl transferases (42). Monoterpene anticarcinoma activity correlates with inhibition of prenylation of small G proteins such as Ras (39, 41, 43–46), and it has been proposed that this might explain their broad antitumor activity (47).

Because posttranslational prenylation is necessary for anchoring Ras in the plasma membrane where it becomes functional (48), the ability of monoterpenes to inhibit Ras prenylation could feasibly interfere with signaling from the Bcr/Abl tyrosine kinase through Ras. We previously reported that Bcr/Abl-transformed cells are more sensitive than nontransformed cells to growth arrest and apoptosis that are induced by the monoterpene POH (49). This finding is important because Bcr/Abl-transformed hematopoietic cells are more resistant than nontransformed cells to most chemotherapy agents and ionizing radiation (50, 51). It is reasonable to think that inhibition of Ras prenylation may be responsible for the anti-leukemia effect of POH in Bcr/Abl-transformed cells. However, we report here that the monoterpene does not affect Ras farnesylation or activity. Rather, POH rapidly and indirectly inhibits signaling through the Erk kinase pathway at the level of Mek.

## MATERIALS AND METHODS

**Reagents.** All monoclonal antibodies and antisera were obtained from commercial sources, except the anti-Abl rabbit antisera (anti-pEX5), which was described previously (52), and the antiphosphotyrosine monoclonal antibody 4G10, which was a gift from Brian Druker (Oregon Health Sciences University, Portland, OR). The specific Mek inhibitor U0126 (53, 54) was purchased from Promega (Madison, WI). POH was obtained from Aldrich Chemicals (Milwaukee, WI) and used as described previously (49). Recombinant Raf-1, Mek1, and Erk1 kinases (both active and inactive forms) and RBD agarose were purchased from Upstate Biotechnology (Lake Placid, NY), as was the recombinant Elk transcription factor, which was used as an Erk kinase substrate. Recombinant IL-3 was a gift from Immunex (Seattle, WA).

**Cells.** Bcr/Abl-transformed murine FDC.P1 and 32D cell lines and their mock transformation controls have been described (49, 55). The human Ph<sup>+</sup> chronic myelogenous leukemia-derived cell lines K562 and Bv173 were obtained from Owen Witte (University of California, Los Angeles, CA). Cells were grown in RPMI media containing 5–10% fetal bovine serum. IL-3-dependent cell lines were maintained in this media plus 10% WEHI-3-conditioned media as a source of IL-3.

**Analysis of Cell Growth and Apoptosis.** Cell cycle distribution and apoptosis were measured by propidium iodide staining of cell nuclei and flow cytometry (49, 55). For

[<sup>3</sup>H]thymidine uptake, 5–20 × 10<sup>5</sup> cells were treated in triplicate for either 0.5 or 2 h with POH or U0126 in 1.0 ml of medium in 24-well plates. For the last 15–30 min of treatment, the cells were pulse labeled with 5 μCi of [<sup>3</sup>H]thymidine, and the radioactivity present in trichloroacetic acid insoluble material was measured by scintillation counting. Data are presented as the mean of three to four experiments ± SD.

**Cell Lysis.** For immunoblot analyses of whole cell lysates, cells were suspended in cold PBS containing 100 μM Na<sub>3</sub>VO<sub>4</sub> and kept on ice for 10 min. The cells were then washed twice in PBS-vanadate. Total cell lysates were prepared by adding boiling SDS-lysis buffer containing 1% SDS, 10 mM Tris (pH 6.8), and 5 mM EDTA (100 μl/5 × 10<sup>6</sup> cells) while gently vortexing. The cells were placed in a boiling water bath for 15 min with occasional vigorous vortexing to shear DNA. After this, the cells were chilled on ice, and leupeptin (1 μg/ml), antipain (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and vanadate (1 mM) were added. Lysates were clarified in a microcentrifuge at high speed for 15 min at 4°C.

For immunoprecipitation of Raf, Mek, Erk, and Bcr/Abl kinases, 10<sup>7</sup> cells were washed in PBS-vanadate, lysed in 1 ml of ice-cold lysis buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM vanadate, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium PP<sub>1</sub>, 10 mM sodium glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mg/ml antipain, and 1 μM microcystin]. Lysates were clarified for 15 min in a microcentrifuge at 4°C. Cellular proteins were immunoprecipitated for 2–18 h at 4°C, and antibody complexes were collected for 1–2 h with protein A-coupled agarose. Immunoprecipitates were processed as described below for analysis of kinase activities. For the RBD pull-down assay, cells were lysed in 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40, 0.25% deoxycholate, 10% glycerol, and the protease and phosphatase inhibitors described above.

**Immunoblots.** Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes that were then washed with TBST [50 mM Tris (pH 7.5), 200 mM NaCl, and 0.1% Tween 20] for 5 min at room temperature. Membranes were blocked with TBST containing either 5% nonfat dry milk or 3% BSA for 1–3 h at room temperature or overnight at 4°C. Blocked membranes were probed with a primary antibody, diluted according to the supplier's instructions, in TBST containing either 5% milk or BSA, overnight at 4°C on a rocker. Membranes were then washed three times for 15 min in TBST (containing 1% Tween 20). Hybridization with the horseradish peroxidase-conjugated secondary antibody and membrane washes were performed as with the primary antibody, except that the hybridization was for 1 h at room temperature. Proteins were detected using the enhanced chemiluminescence kit from Amersham (Piscataway, NJ) and detected by exposure to X-ray film. Bands were digitally quantitated using ImageJ (version 1.24o), a public domain image analysis program that was developed at the NIH. The signals from experimental bands were normalized to actin-loading controls and compared with similarly normalized bands from untreated controls.

**Biochemical Analyses.** Active (GTP-loaded) Ras was collected with the RBD of Raf-1 that was covalently coupled to

agarose (Upstate Biotechnology). RBD agarose was mixed with cell lysates no longer than 30 min at 4°C to minimize spontaneous Ras hydrolysis of GTP to GDP. For control, lysates were also immunoprecipitated with an agarose-coupled anti-Ras monoclonal antibody that recognizes H-, N-, and K-Ras (anti-H-Ras259; Santa Cruz Biotechnology, Santa Cruz, CA). Ras proteins were separated through 12% SDS-PAGE and detected by immunoblotting with an anti-Ras monoclonal antibody (Ras10; Upstate Biotechnology), which recognizes the three forms of Ras.

To evaluate the effects of POH on the mevalonate biosynthesis pathway and on protein prenylation, cells were treated overnight with 30 μM lovastatin (an inhibitor of β-hydroxy-β-methylglutaryl coA reductase) to deplete the endogenous pool of mevalonate. The cells were then placed, for 6 h, in fresh media containing POH and lovastatin, and for the last 3 h of POH treatment, cells were labeled with (*R,S*)[2-<sup>14</sup>C]mevalonolactone (50 mCi/mmol; New England Nuclear, Boston, MA), which converts to <sup>14</sup>C-mevalonate. For analyses of <sup>14</sup>C-labeled small G proteins, cells were either lysed in SDS sample buffer to examine all labeled cellular proteins, or cells were lysed in ice-cold radioimmunoprecipitation assay buffer (PBS, 1% NP40, 0.5% deoxycholate, and 0.1% SDS, plus protease and phosphatase inhibitors) for immunoprecipitation with antibodies specific for Ras, RhoA, and Rab6, as described by Ren *et al.* (56). <sup>14</sup>C-labeled lipids were analyzed by extracting neutral lipids with acetone and resolving them on TLC plates that were developed with hexane/diethyl ether/acetic acid (70:30:2, v/v) and analyzed for radioactivity using a phosphoimager (40, 43).

Bcr/Abl kinase activity was measured as described previously (52). Erk kinase assays were performed using reagents and instructions from the p44/42 MAP Kinase Immunoprecipitation Kinase Assay kit (Upstate Biotechnology), except that we used recombinant Elk protein as substrate. Phosphorylated Elk was measured by Western blot using rabbit anti-phospho-Elk (ser383) antisera (Cell Signaling Technology, Beverly, MA). Mek assays were performed in a cascade reaction in which immunoprecipitated Mek phosphorylated Erk. Erk activity was then measured by its ability to phosphorylate MBP. This assay was performed using the MEK1 Immunoprecipitation Kinase Assay kit (Upstate Biotechnology). <sup>32</sup>P-labeled MBP was measured by phosphoimaging after SDS-PAGE. To measure MAP3K activity, Raf-1, A-Raf, B-Raf, MEKK, and Mos were immunoprecipitated from POH-treated and untreated cells, and the kinase reactions were set up using reagents from the Raf-1 Immunoprecipitation Kinase Cascade Assay kit (Upstate Biotechnology). However, after the first reaction (phosphorylation of Mek1), SDS-PAGE sample buffer was added to the reaction mix, and phosphorylated Mek1 was detected by immunoblot using anti-phospho-Mek1/2 (ser217/221) antisera (Cell Signaling Technology).

## RESULTS

### POH Rapidly Induces Growth Arrest and Apoptosis.

We previously reported that POH first causes G<sub>0</sub>-G<sub>1</sub> arrest and that this is followed by exit from G<sub>0</sub>-G<sub>1</sub> via apoptosis (49), which suggests that the primary effect of POH is to induce growth arrest. In those experiments, G<sub>1</sub> arrest was measured 3 h

Table 1 POH rapidly inhibits DNA synthesis

Cell type <sup>a</sup>	POH (μM)	% activity <sup>b</sup>
FDC.P1(C13) <sup>c</sup>	1200	25 ± 7
	800	50 ± 5
	400	72 ± 11

<sup>a</sup> Cells were treated in triplicate with POH for 30 min and labeled with 5 μCi of <sup>3</sup>H-TdR for the last 15 min.

<sup>b</sup> Percentage of <sup>3</sup>H-TdR uptake compared with untreated control ± SD. Values are from three experiments.

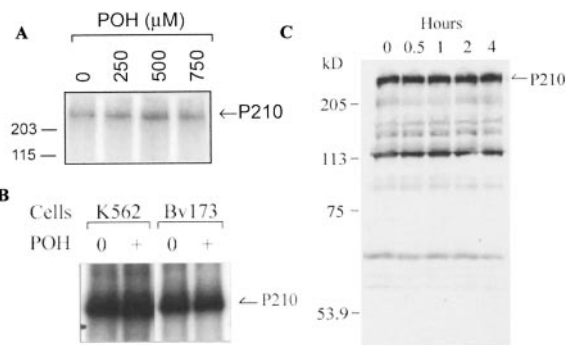
<sup>c</sup> P210 Bcr/Abl-transformed FDC.P1 cells (clone 3).

after treatment with POH. To determine whether monoterpenes can cause growth arrest more quickly than this, Bcr/Abl-transformed FDC.P1 cells were treated for 30 min with POH and labeled with [<sup>3</sup>H]thymidine for the last 15 min of the treatment period. Significant inhibition of DNA synthesis occurred within this short treatment period, and the extent of inhibition depended on the concentration of POH (Table 1). However, the IC<sub>50</sub> for this short treatment was 800 μM, which is greater than the IC<sub>50</sub> reported in 24- or 72-h assays, which was approximately 400 μM and 30 μM, respectively (49). Thus, although the anti-leukemia effect of POH is rapid, this is only seen at higher concentrations of the monoterpene. In other experiments, we found that an early event in apoptosis, the proteolytic cleavage of poly(ADP-ribose) polymerase, first became noticeable 2 h after POH treatment and was complete by 4 h.<sup>3</sup> Hence, POH induces growth arrest and apoptosis rapidly; therefore, the biochemical mechanisms by which POH induces these biological effects must also occur rapidly.

**POH Does Not Affect Bcr/Abl Kinase Activity.** POH-mediated growth arrest and apoptosis could be attributable to inhibition of Bcr/Abl kinase activity. However, data in Fig. 1A show that in Bcr/Abl-transformed FDC.P1 cells, up to 8 h of treatment with concentrations of POH as much as 750 μM did not affect the autokinase activity of Bcr/Abl. Likewise, treatment with 800 μM POH did not affect Bcr/Abl autokinase activity in the human Ph<sup>+</sup> cell lines K562 and Bv173 (Fig. 1B). We also examined whether POH treatment affects the overall pattern of tyrosine phosphorylated proteins in Bcr/Abl-transformed FDC.P1 cells. Cells were treated with 800 μM POH for 0.5–4 h, and total cell lysates were processed for immunoblotting with antiphosphotyrosine antibody. The Western blot data in Fig. 1C show several detectable bands of tyrosine phosphorylated proteins in addition to the tyrosine-phosphorylated p210 Bcr/Abl kinase. Treatment with POH did not appreciably alter the detection of any of these bands.

**POH Does Not Inhibit Protein Prenylation or Ras Activation.** We next tested whether the anti-leukemia effect of POH, such as its anticarcinoma effect (39, 41, 43–47), is related to its ability to inhibit prenylation of small G proteins. Bcr/Abl-transformed FDC.P1 cells were treated with increasing amounts of POH and labeled with (2-<sup>14</sup>C)mevalonolactone, as reported by Ren *et al.* (40, 56). Total <sup>14</sup>C-labeled proteins were separated

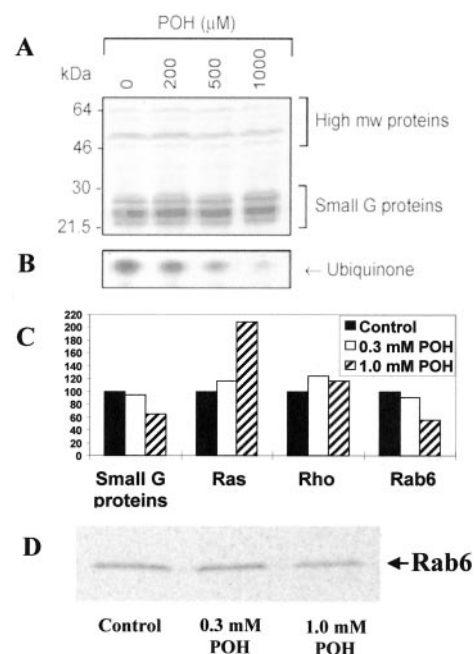
<sup>3</sup> X. Yang and S. S. Clark, The antileukemia agent, perillyl alcohol, inhibits signals through STAT5 in Bcr/Abl-transformed cells.



**Fig. 1** POH does not affect Bcr/Abl kinase activity. The anti-pEX-5 antibody was used to immunoprecipitate P210 from Bcr/Abl-transformed FDC.P1 cells that were treated for 8 h with increasing concentrations of POH (A) or from the human Ph<sup>+</sup> cell lines K562 and Bv173 (B). Bcr/Abl autokinase activity was measured as described in "Materials and Methods." C, cells were treated up to 4 h with 500  $\mu$ M POH and lysed in boiling SDS sample buffer and 200  $\mu$ g of protein run/lane for Western blotting with an antiphosphotyrosine antibody.

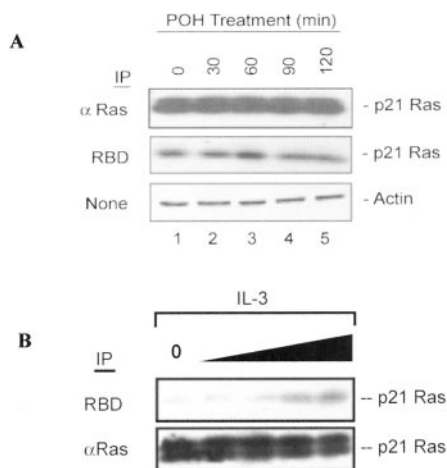
by 12% SDS-PAGE and visualized by phosphoimaging. Data in Fig. 2A reveal that that up to 1000  $\mu$ M POH did not appear to affect prenylation of either high molecular weight proteins or small G proteins that are found in the  $M_r$  21,000–26,000 range. In contrast, Fig. 2B shows that increasing concentrations of POH impairs cellular synthesis of ubiquinone, which indicates that the POH treatment inhibited mevalonate metabolism in the Bcr/Abl-transformed cells, even though overall protein prenylation was not noticeably affected. Quantitation of the radioactivity in each spot revealed that ubiquinone production was inhibited by 46, 77, and 93% with 200, 500, and 1000  $\mu$ M POH, respectively.

Although POH does not discernibly affect the prenylation of the total pool of small G proteins in Bcr/Abl-transformed cells, an effect of POH on the prenylation of specific small G proteins could be masked by the lack of an effect on the majority of proteins in the  $M_r$  21,000–26,000 range. To evaluate whether POH specifically affected any of the three known prenyl-protein transferases, H-Ras, RhoA, and Rab6 proteins were immunoprecipitated from (2-<sup>14</sup>C)mevalonolactone-labeled cells treated with increasing concentrations of POH. H-Ras is posttranslationally modified by FPTase, whereas RhoA and Rab6 are modified by GGPTase, type I and type II, respectively. The amount of radiolabel incorporated into each immunoprecipitated band was measured by phosphoimaging and is depicted graphically in Fig. 2C. Representative data for immunoprecipitated Rab6 is shown in Fig. 2D. Although 300  $\mu$ M POH is sufficient to induce growth arrest and apoptosis in Bcr/Abl-transformed FDC.P1 cells (49), prenylation of these small G proteins is not markedly affected at this concentration of POH. At high monoterpene concentrations (1000  $\mu$ M), there were variable effects on the prenylation of these small G proteins that were not seen at lower concentrations that are sufficient to cause the anti-leukemia effect. However, rather than inhibiting Ras farnesylation as predicted, the high concentration of POH increased Ras farnesylation. Conversely, treatment with 1000  $\mu$ M POH inhibited geranylgeranylation of Rab6 by approximately 40%. Similar observations were seen in both of two separate experiments.



**Fig. 2** POH does not inhibit farnesylation of Ras. Cells were labeled with [2-<sup>14</sup>C]mevalonolactone, then treated for 6 h with POH as described in "Materials and Methods." A, total <sup>14</sup>C-labeled cellular proteins. Small G proteins are found in the  $M_r$  21,000–26,000 range. B, <sup>14</sup>C-labeled cellular lipids were extracted from cells treated as described above and analyzed by TLC. The effect of POH on ubiquinone synthesis is shown. In C and D, specific small G proteins were immunoprecipitated from the <sup>14</sup>C-labeled cells described in A that had been treated with or without POH and analyzed by SDS-PAGE and phosphoimaging. Cell lysates were immunoprecipitated with antisera to H-Ras, RhoA, and Rab6, which are prenylated by farnesyltransferase and geranylgeranyl transferase type I and type II, respectively. Data in C show the percentage of change in detection of each small G protein, relative to POH-untreated controls after quantitation by phosphoimaging. Representative immunoprecipitation data of Rab6 are shown in D. Similar observations were seen in two independent experiments.

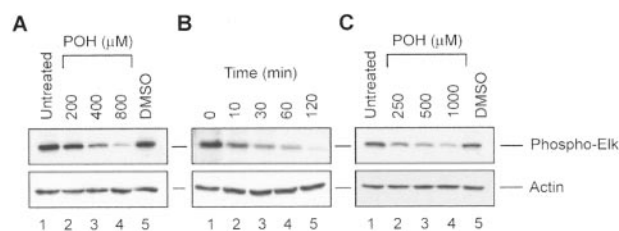
To further evaluate whether monoterpenes inhibit signaling through Ras in Bcr/Abl-transformed FDC.P1 cells, the cells were treated with POH, then lysed and split into two groups. Total Ras was immunoprecipitated from one group while GTP-loaded-activated Ras was collected from the other group using the agarose-coupled RBD of Raf. This was followed by immunoprecipitation of actin to control for protein loading. Data in Fig. 3A show no change in the level of expression of total Ras protein or activated Ras in the POH-treated cells. Identical observations were seen in both of two experiments using Bcr/Abl-transformed FDC.P1 cells, as well as in one experiment using K562 cells (data not shown). In contrast to the lack of an effect of POH on the level of active Ras, RBD-precipitated Ras levels decreased when IL-3 was removed from the IL-3-dependent, nontransformed FDC.P1 cells. The level of active Ras then increased when the IL-3 starved cells were refed with increasing concentrations of recombinant IL-3 (Fig. 3B). This shows that the RBD pull-down assay can detect changes in the level of active Ras, which strengthens the conclusion that POH does not affect Ras activation in Bcr/Abl-transformed cells. The lack of



**Fig. 3** POH does not affect the level of activated Ras that binds to the RBD of Raf. **A**, Bcr/Abl-transformed FDC.P1 cells were treated with or without 800  $\mu\text{M}$  POH for the indicated time and divided into two groups for collection of total Ras or activated RBD-binding Ras. Ras proteins were detected by immunoblot. After immunoprecipitation for total Ras, lysates were reimmunoprecipitated with antiactin, which was also detected by immunoblot to control for protein loading. Identical results were seen with K562 cells (data not shown). **B**, in mock-transformed cells, the level of active Ras that associates with RBD is affected by IL-3. Mock-transformed, IL-3-dependent FDC.P1 control cells were cultured overnight in RPMI containing 1% fetal bovine serum and 0.5% WEHI-3-conditioned media. The next morning, the cells were washed in RPMI containing 0.3% fetal bovine serum and incubated in the same media for 3 h. Recombinant IL-3 (30–1000 units/ml) was then added for 30 min, after which Ras proteins were collected by immunoprecipitation with anti-Ras antisera or by RBD pull-down. Ras proteins were detected by immunoblot.

an effect of POH on RBD-precipitable Ras is consistent with the absence of an effect of biologically active concentrations of POH on Ras farnesylation. Together, these observations indicate that, contrary to what we and others (47) predicted, biologically relevant concentrations of POH that cause growth arrest and apoptosis in Bcr/Abl-transformed cells do not disrupt signaling through Ras.

**POH Inhibits Signaling through p42/44 MAPK (Erk) and Mek in Bcr/Abl-transformed Cells.** Although the POH anti-leukemia effect works in a Ras-independent manner, the biological effects of POH are still consistent with inhibition of signaling downstream of Ras. We, therefore, examined the effect of POH on Erk kinase activity in Bcr/Abl-transformed FDC.P1 cells. Erk was immunoprecipitated from POH-treated and untreated cells, and its kinase activity was measured by phosphorylation of its natural substrate, Elk. Phospho-Elk was then detected by immunoblotting. Data in Fig. 4A show that POH inhibits Erk kinase activity in a concentration-dependent manner. In this experiment, Erk activity was inhibited approximately 5, 50, and 85% at 200, 400, and 800  $\mu\text{M}$  POH, respectively. POH inhibition of Erk activity also is rapid and time dependent. Within 10 min, 800  $\mu\text{M}$  POH inhibited Erk activity by 46%, which increased to 95% inhibition by 120 min (Fig. 4B). POH also inhibits Erk activity in Bcr/Abl-transformed mouse 32D and BaF3 cells (data not shown), as well as in the human Ph<sup>+</sup> EM2 and Bv173 cell lines (data not shown) and in



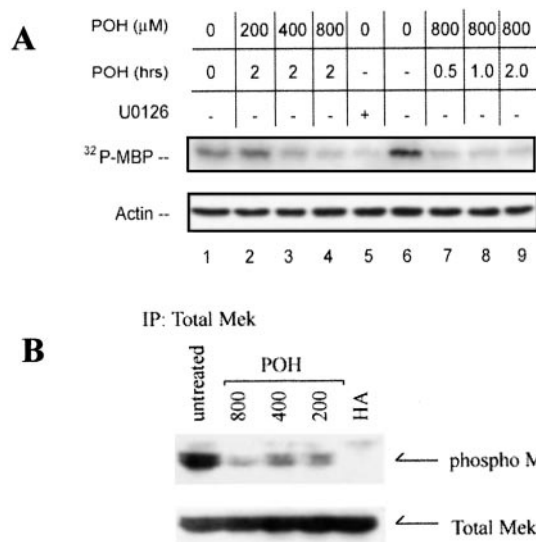
**Fig. 4** POH inhibits Erk kinase activity in Bcr/Abl-transformed cells. Both murine Bcr/Abl-transformed FDC.P1 cells (**A** and **B**) and human Ph<sup>+</sup> K562 cells (**C**) were treated for 2 h with the indicated concentrations of POH (**A** and **C**) or were treated with 800  $\mu\text{M}$  POH for the indicated length of time (**B**). After POH treatment, cells were lysed and immunoprecipitated with anti-Erk. Erk kinase activity was measured using recombinant Elk as a substrate, and phosphorylated Elk was detected by Western blot. Lysates were reimmunoprecipitated with antiactin, which was detected by Western blot to control for protein loading.

the K562 cell line (Fig. 4C). In K562 cells, Erk activity was inhibited 30, 58, and 88% with 250, 500, and 1000  $\mu\text{M}$  POH, respectively.

Because the Erk kinase is activated on phosphorylation by Mek, it is possible that POH blocks Erk activity by inhibiting Mek. To test this, Mek was immunoprecipitated from Bcr/Abl-transformed cells that had been treated for different lengths of time or with increasing concentrations of POH, and Mek kinase activity was measured in a cascade reaction. Similar to the effect of POH on Erk kinase activity, Mek kinase activity is quickly inhibited by POH concentrations greater than 200  $\mu\text{M}$  (Fig. 5A, Lanes 1–5). Thus, a 2-h treatment with 200, 400, or 800  $\mu\text{M}$  POH resulted in Mek inhibition of 0, 59, and 64%, respectively, compared with untreated controls. Mek inhibition was complete after a 30-min treatment with 800  $\mu\text{M}$  POH (70% inhibition; Fig. 5A, Lanes 6–9). Thus, Mek and Erk kinases are blocked with similar kinetics and with similar monoterpene concentrations, and this correlates with G<sub>1</sub> arrest, which is the initial anti-leukemia effect of POH (49).

To understand the mechanism of Mek inhibition by POH, cells were treated with increasing concentrations of monoterpene, and whole cell lysates were separated by SDS-PAGE for immunoblot analysis. The blots were first probed with an antiserum that recognizes phosphorylated (activated) Mek, then stripped and reprobed with an antiserum against total Mek. As shown in Fig. 5B, a 4-h treatment with 200  $\mu\text{M}$  POH significantly reduced the level of phospho-Mek, and this effect increases with the POH concentration. In contrast, the level of total Mek was unaffected by POH. For control, cells were also treated with herbimycin, a tyrosine kinase inhibitor that blocks Bcr/Abl kinase activity (57). As expected, inhibiting Bcr/Abl activity also reduced the level of phosphorylated, but not total, Mek protein. Thus, POH inhibition of Mek activity is associated with a reduction in the level of the activated form of the enzyme.

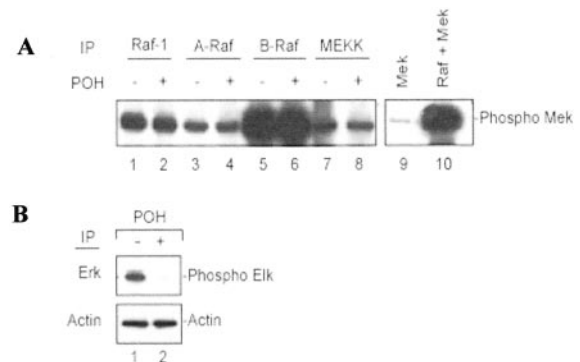
**POH Does Not Affect MAP3K (Raf) Activity.** The family of MAP3Ks that includes Raf-1, A-Raf, B-Raf, MEKK (Mek kinase-1), and Mos are immediate upstream activators of Mek and are logical targets for monoterpene-mediated inactivation of Mek. Because multiple MAP3K gene products can be expressed in cells, POH-treated and untreated FDC.P1 cells



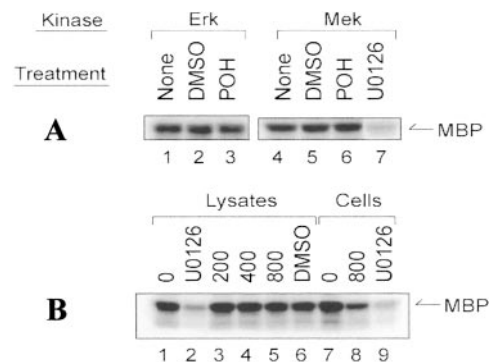
**Fig. 5** POH inhibits MAP2K (Mek) kinase activity. **A**, Mek kinase activity was measured in a cascade reaction in which Mek phosphorylated Erk, which then phosphorylated MBP. Cells were treated with POH or U0126, as indicated. U0126 is a Mek kinase inhibitor.  $^{32}\text{P}$ -labeled MBP was detected by phosphoimaging. **B**, cells were treated with the indicated concentrations of POH for 4 h, and active (phosphorylated) Mek was detected by Western blot using an anti-phospho-Mek antibody. The blot was stripped and reprobbed with anti-Mek to measure the effect of POH on total Mek protein levels. For control, cells were also treated with the tyrosine kinase inhibitor herbimycin to block signals from Bcr/Abl.

were lysed, then split into separate groups and immunoprecipitated with antisera to the different MAP3K proteins. Kinase activities were then measured by *in vitro* phosphorylation of recombinant Mek, which was detected by immunoblotting with phospho-Mek antiserum. Data in Fig. 6A show that although Raf-1, A-Raf, B-Raf, and MEKK are expressed and active in the cells, B-Raf is the most active. In other experiments, only negligible Mos activity was detected (data not shown) and was not studied further. Importantly, POH treatment did not affect the activity of any of these MAP3Ks (Fig. 6A and data not shown). As a positive control for the POH effect, lysates from the same POH-treated and untreated cells that were immunoprecipitated with Raf-1 in Fig. 6A were reprecipitated with anti-Erk antisera, and the Erk kinase activity was tested (Fig. 6B). As seen before, POH treatment profoundly inhibited Erk kinase activity, even though there was no effect on MAP3K activity in the same lysate. The cell lysates were again reimmunoprecipitated with antiactin antisera to demonstrate equivalent protein levels in the POH-treated and untreated groups (Fig. 6B). Identical results were observed in two separate experiments (data not shown). We conclude that POH inhibits signaling through the MAPK pathway downstream of Raf or other MAP3Ks.

**POH Only Inhibits Mek and Erk Kinase Activities in Intact Cells.** Because POH inhibition of Mek and Erk kinase activities was not associated with inhibition of the upstream MAP3K effector, we tested whether POH could directly inhibit Mek and/or Erk kinase activity. To do this, we first examined



**Fig. 6** POH does not affect MAP3K activity. Bcr/Abl-transformed cells were treated with 600  $\mu\text{M}$  POH, and the different MAP3Ks were immunoprecipitated and their kinase activities measured using Mek as a substrate. Phospho-Mek was detected by Western blot. *Lane 9*, recombinant Mek substrate alone as negative control; *Lane 10*, Mek substrate was phosphorylated by recombinant Raf-1 as a positive control. The *bottom panel* shows that Erk kinase activity in the cells was inhibited under the POH treatment conditions that showed no effect on MAP3K activity. Erk kinase activity was measured as described in Fig. 4.



**Fig. 7** POH does not directly inhibit Mek or Erk kinase activities. **A**, Recombinant, active Erk and Mek were suspended in kinase buffer (without ATP or  $\text{Mg}^{++}$ ) from the Erk kinase kit purchased from Upstate Biotechnology. The indicated agents were added to the enzyme preparations and incubated for 1 h at 30°C. ATP/ $\text{Mg}^{++}$  was added to initiate the kinase reactions that ran for 30 min at 30°C. Erk kinase activity was evident by  $^{32}\text{P}$ -phosphorylation of MBP, whereas Mek kinase activity was evaluated in a cascade reaction in which inactive Erk was first phosphorylated, which then was tested using MBP as substrate. POH (800  $\mu\text{M}$ ) was used. U0126 (20  $\mu\text{M}$ ) was used as positive control for Mek inhibition. **B**, POH or U0126 (20  $\mu\text{M}$ ) was added either to cell lysates or to intact cells for 2 h at room temperature, after which immunoprecipitated Mek kinase activity was tested in the cascade reaction as described above.  $^{32}\text{P}$ -labeled MBP was detected by phosphoimaging.

whether adding POH to recombinant, phosphorylated Mek and Erk kinases would inhibit their activities. In Fig. 7A, POH was added to purified enzyme in kinase buffer lacking ATP and  $\text{Mg}^{++}$ . After incubation,  $^{32}\text{P}$ -ATP and  $\text{Mg}^{++}$  were added to initiate the kinase reaction. The data show that POH does not directly affect the kinase activities of the purified enzymes. In contrast, the Mek antagonist U0126 strongly inhibited Mek activity. We also compared the effect of adding POH and U0126 to cells before and after they had been lysed. The data in Fig. 7B

Table 2  $^3\text{H}$ -TdR uptake in Bcr/Abl-transformed cells treated with POH and U0126

Experiment	Cell type <sup>a</sup>	POH		U0126	
		( $\mu\text{M}$ )	% <sup>b</sup>	( $\mu\text{M}$ )	% <sup>b</sup>
1	K562 <sup>a</sup>	1200	39 $\pm$ 3	90	19 $\pm$ 10
		800	56 $\pm$ 9	60	34 $\pm$ 10
		400	81 $\pm$ 11	30	71 $\pm$ 6
2	FDC.P1(C13) <sup>c</sup>	900	37 $\pm$ 7	60	24 $\pm$ 18
		600	48 $\pm$ 8	30	68 $\pm$ 24
		300	60 $\pm$ 13	10	75 $\pm$ 13

<sup>a</sup> Cells were treated in triplicate for 2 hr with the indicated concentration of either POH or U0126. They were labeled with 5  $\mu\text{Ci}$  of  $^3\text{H}$ -TdR for the last 30 min of the treatment period.

<sup>b</sup> Percentage of  $^3\text{H}$ -TdR uptake compared with untreated control  $\pm$  SD. Values are from three experiments.

<sup>c</sup> P210 Bcr/Abl-transformed FDC.P1 cells, clone 3.

show that there was no effect on Mek activity when 200–800  $\mu\text{M}$  POH were added after cell lysis (Lanes 1 and 3–5). Yet, when 800  $\mu\text{M}$  POH were added to intact cells, Mek activity was inhibited (Lanes 7 and 8). POH also failed to block Erk kinase activity in cell lysates (data not shown). In contrast to POH, U0126 significantly blocked Mek kinase activity when added either before or after cell lysis (Fig. 7B). Thus, unlike U0126, POH does not directly inhibit Mek kinase activity and only inhibits Mek (and Erk) when added to intact cells.

**Inhibition of Mek Is Sufficient to Induce Both Growth Arrest and Apoptosis in Bcr/Abl-transformed Cells.** Data presented here suggest a cause and effect relationship between monoterpene inhibition of Mek and Erk and induction of  $G_0$ - $G_1$  arrest and apoptosis in Bcr/Abl-transformed cells. If true, then other Mek inhibitors should cause similar anti-leukemia effects. This was tested by comparing the effects of the Mek inhibitors U0126 and POH on Bcr/Abl-transformed FDC.P1 and K562 cells. The cells were treated for 2 h with POH or U0126 and pulsed with [ $^3\text{H}$ ]TdR over the last 30 min. Data in Table 2 show that both POH and U0126 inhibited DNA synthesis in a dose-dependent fashion. Bcr/Abl-transformed and mock-transformed FDC.P1 cells also were treated overnight with increasing concentrations of U0126 and POH, and the effect on cell cycle distribution was examined by propidium iodide staining. Data in Fig. 8 show a dose-dependent increase of apoptotic cells that is typically seen in POH-treated Bcr/Abl-transformed cells. Thus, apoptosis is first apparent with 400  $\mu\text{M}$  POH (Fig. 8r) and increases with the concentration of POH. In contrast to the Bcr/Abl-transformed cells, mock-transformed FDC.P1 control cells were less sensitive to POH and only showed a marginal increase in apoptotic cells at the highest concentration of POH (Fig. 8u), which is consistent with what we reported previously (49). The same cells responded in similar fashion to the Mek inhibitor U0126. Apoptotic cells became apparent with 5  $\mu\text{M}$  of the inhibitor (Fig. 8f), and the fraction of apoptotic cells increased along with the concentration of U0126. In contrast to the transformed cells, U0126-treated mock-transformed cells did not undergo apoptosis induced by the Mek inhibitor but did begin to show loss of cells in S phase with 5–10  $\mu\text{M}$  U0126 (Fig. 8e).

Data in Fig. 8 confirm that Bcr/Abl-transformed cells are more sensitive to POH than mock-transformed cells, as we

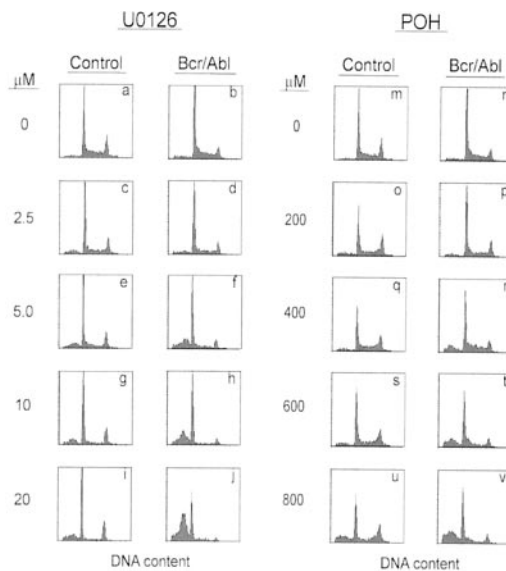


Fig. 8 Blocking Mek is sufficient to induce selective growth arrest and apoptosis in Bcr/Abl-transformed cells. Bcr/Abl-transformed and mock-transformed FDC.P1 cells were treated overnight with the indicated concentrations of either POH or the Mek inhibitor U0126 and processed for propidium iodide staining and flow cytometry. Mock-transformed cells were treated in the presence of a minimal concentration (0.5%) of WEHI-3-conditioned media to avoid apoptosis that would be caused by withdrawal of IL-3 from the cells.

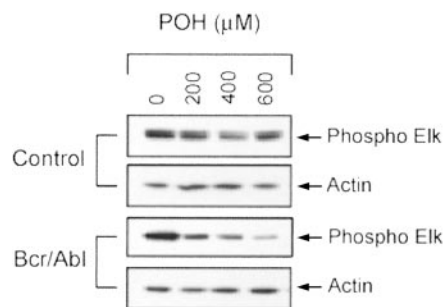


Fig. 9 POH preferentially inhibits Erk kinase activity in Bcr/Abl-transformed FDC.P1 cells compared with mock-transformed (control) cells. Cells were treated for 2 h with the indicated concentrations of POH, then lysed for immunoprecipitation of Erk as well as actin as a control for protein loading. Erk kinase activity was measured as described in "Materials and Methods." The mock-transformed cells were treated with POH in the presence of 100 units/ml recombinant IL-3 to avoid changes in the level of Erk activity caused by withdrawal of IL-3.

reported previously (49). If this selective anti-leukemia effect is mechanistically related to inhibition of Mek-Erk signaling, then POH should more effectively inhibit this signaling pathway in Bcr/Abl-transformed cells compared with the mock-transformed controls. This was tested by treating transformed and mock-transformed cells with increasing concentrations of POH, then measuring Erk kinase activities. Data in Fig. 9 show that in mock-transformed cells, the Erk kinase was not inhibited with as much as 600  $\mu\text{M}$  POH. In contrast, in Bcr/Abl-transformed cells, Erk inhibition was evident with 200  $\mu\text{M}$  POH and increased with the concentration of POH used (Fig. 9).

## DISCUSSION

Several reports have documented that POH exhibits anti-tumor activity *in vitro* and *in vivo* against many different rodent carcinomas (33–38). Studies have also shown that the anticarcinoma effect of monoterpenes closely correlates with their ability to inhibit prenylation of small G proteins (39–43, 58). It was, therefore, proposed that monoterpene anticancer activity might be related to their ability to block signaling from small G proteins, particularly p21 Ras (47). We previously reported that monoterpenes also exert an anti-leukemia effect on Bcr/Abl-transformed leukemia cells by causing G<sub>0</sub>-G<sub>1</sub> arrest that is followed by apoptosis (49). However, in contrast to the anticarcinoma effect, the monoterpene anti-leukemia effect does not correlate with inhibition of Ras farnesylation. Concentrations of POH sufficient to induce the anti-leukemia effect failed to reduce the level of GTP-loaded Ras or to inhibit MAP3K activity that is immediately downstream of Ras. Furthermore, the anti-leukemia response to POH is rapid because significant inhibition of DNA synthesis and induction of apoptosis are seen within 30–120 min after treatment with POH. In contrast, farnesylated Ras has a long half-life >24 h (59), which makes it unlikely that POH could exert such a rapid anti-leukemia effect by inhibiting Ras prenylation. Thus, the monoterpene anti-leukemia effect does not work by inhibiting Ras, which raises the possibility that monoterpenes induce anti-leukemia and anticarcinoma effects by distinct mechanisms. This could explain why the *in vitro* anti-leukemia activity is evident at much lower concentrations (200–400  $\mu\text{M}$ ) of POH than are needed to see anticarcinoma effects *in vitro* (1–5 mM).

We reported previously that in an overnight *in vitro* survival assay, the POH IC<sub>50</sub> was 300–400  $\mu\text{M}$ , whereas the IC<sub>50</sub> was approximately 30  $\mu\text{M}$  in a 72-h assay (49). These concentrations correlate well with the concentration of monoterpene needed to inhibit Mek/Erk signaling. Furthermore, this concentration range is attainable *in vivo*. After a single oral gavage of 1 mg/kg, rats achieved a serum monoterpene level of 1400–1600  $\mu\text{M}$  (39, 60), whereas rats fed a 2% POH diet accumulated 800  $\mu\text{M}$  in their serum (60). In humans, serum monoterpene levels of 400–700  $\mu\text{M}$  are obtained with minimal toxicity (61, 62). Hence, the concentrations of POH that cause the *in vitro* anti-leukemia effects that we report here and those reported previously (49) appear to be pharmacologically relevant.

Given the inability of POH to inhibit Ras signaling, it was surprising to learn that in Bcr/Abl-transformed cells monoterpene concentrations sufficient to induce growth arrest and apoptosis also quickly blocked the Erk signaling pathway at the level of Mek. For the following reasons, this effect of POH appears to be relevant to its anti-leukemia activity. First, inhibition of Mek and Erk kinase activities is detectable within 30 min after monoterpene treatment, and this closely coincides with the kinetics of POH-induced G<sub>1</sub> arrest, which is the earliest manifestation of the POH anti-leukemia effect. Second, similar concentrations of POH are needed to both exert an anti-leukemia effect and to block Erk signaling *in vitro*. Third, like POH, the specific Mek inhibitor U0126 also was able to induce G<sub>1</sub> arrest and apoptosis, confirming that inhibiting Mek is sufficient to cause both of these effects in Bcr/Abl-transformed cells. Finally, mock-transformed FDC.P1 cells, which are relatively

resistant to monoterpene-induced growth arrest and apoptosis (49), are also resistant to POH-mediated inhibition of signaling through the Erk pathway.

The fact that POH and U0126 cause both growth arrest and apoptosis in the leukemia cell lines is consistent with reports that Bcr/Abl kinase signaling through the Erk cascade drives G<sub>1</sub>-S transition (63) and protects cells from apoptosis (24, 25, 64). However, other reports claim that the Bcr/Abl antiapoptotic program is independent of this signaling pathway (65, 66). These conflicting reports raise the question as to how POH and U0126 inhibition of Mek causes apoptosis in Bcr/Abl-transformed cells. We previously suggested that apoptosis is secondary to POH-mediated growth arrest in Bcr/Abl-transformed cells (49). This was based on cell cycle kinetic findings, which showed that POH-treated Bcr/Abl-transformed cells first accumulate in G<sub>0</sub>-G<sub>1</sub>, then undergo apoptosis (49). This model is also consistent with the observation that when treated with POH, nontransformed cells, which are more resistant to POH, continue to cycle through G<sub>1</sub> and do not undergo apoptosis (49). If this notion is correct, then POH and U0126 inhibition of Mek would likely be responsible for causing growth arrest, whereas apoptosis would be an indirect consequence of Mek inhibition. However, at this stage, we cannot formally rule out that in addition to inducing growth arrest, monoterpenes might also directly cause apoptosis in Bcr/Abl-transformed cells. Regardless of the mechanism involved, it is significant that treatment with POH or U0126 leads to apoptosis in cells that are generally resistant to programmed cell death.

How POH inhibits Mek kinase activity is not known. However, it is clear that POH and U0126 block Mek kinase activity by different mechanisms. U0126 is a noncompetitive antagonist that can directly inhibit the activity of phosphorylated and even constitutively active mutants of Mek (67). U0126 also inhibits Mek activity in intact cells without interfering with the state of Mek activation (68). In contrast to U0126, POH is an indirect inhibitor that causes a reduction in the level of phosphorylated, active Mek. This could occur if POH blocked an upstream activator of the Mek kinase; however, we found that POH has no effect on any of the MAP3Ks that were examined. It is also unlikely that POH directly reverses the state of Mek activation, because POH was unable to affect Mek activity when added to phosphorylated, recombinant Mek or to cell lysates containing active Mek. In other words, POH only reverses Mek phosphorylation and activity in the context of intact cells.

These results raise the possibility that POH affects a regulator of Mek that, on the disruption of cells, is no longer able to affect Mek activity. This indirect inhibition of Mek by POH could feasibly operate in a number of different ways. For instance, the monoterpene could activate a phosphatase that would reverse the state of Mek activation. Mek can be regulated by phosphatases PP2A (69–71) and PP1 (72), both of which are specifically inhibited by OA (73, 74). We found that OA protects the state of Mek phosphorylation in monoterpene-treated cells,<sup>4</sup> which indicates that an OA-sensitive phosphatase does, in fact, down-regulate Mek phosphorylation after treatment with

<sup>4</sup> L. Zhong and S. S. Clark, unpublished data.

POH. However, we were unable to determine whether this reversal of Mek phosphorylation is caused by an effect of the monoterpene on a Mek phosphatase activity or whether POH interference with Mek phosphorylation simply allows normal phosphatase activity to reduce the cellular pool of phosphorylated Mek. This question is under further investigation.

POH also could block the activation of Mek without affecting MAP3K activity by interfering with the ability of MAP3K to phosphorylate Mek. This process is tightly regulated by a scaffold complex that transports inactive Mek from the cytoplasm to the membrane, where it is activated by MAP3K (75, 76). POH interference with scaffold function could, therefore, leave Mek inaccessible for activation by MAP3K. Hence, the scaffold complex represents a potential molecular target for monoterpene-mediated anti-leukemia activity. Experiments are under way to learn how POH affects Mek phosphorylation independent of an effect on MAP3K activity.

The fact that POH more effectively blocks signaling through the Erk pathway in Bcr/Abl-transformed cells than in mock-transformed parental cells seems counterintuitive because the Bcr/Abl tyrosine kinase uses signaling pathways in common with normal cytokine receptors. However, there is precedence for such selectivity. Farnesyltransferase inhibitors selectively suppress Ras-transformed cell lines while exerting minimal effects on normal cells, even though normal growth factor receptors also signal through farnesylated Ras (48, 77). Thus, transformed cells seem to be more sensitive than nontransformed cells to inhibition of signaling through Ras. It is, therefore, possible that quantitative or qualitative differences in signaling through Mek from the Bcr/Abl kinase *versus* the IL-3 receptor might explain their distinct sensitivities to POH.

POH represents a novel small molecule inhibitor of the Erk signaling cascade. Because elevated signaling through the Erk pathway is found in a wide variety of human cancer cell lines and primary tumor samples (78–80), as well as in a number of different leukemia cell lines and primary acute leukemias (4), it will be interesting to see whether the ability of monoterpenes to inhibit Mek kinase activity might account for the broad anticancer effect of POH that has been reported. At the very least, results presented here suggest that the Mek/Erk signaling pathway represents a potential target for developing new chemotherapy regimens for treating Ph<sup>+</sup> leukemias.

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