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Antileukemia activity of perillyl alcohol (POH): uncoupling apoptosis from G0/G1 arrest suggests that the primary effect of POH on Bcr/Abl-transformed cells is to induce growth arrest

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In hematopoietic cells, the Bcr/Abl tyrosine kinase that is encoded by the Philadelphia chromosome translocation both stimulates proliferation and activates an anti-apoptotic program that is associated with a G2/M delay upon exposure to various apoptotic stimuli. We recently reported that the monocyclic monoterpene, perillyl alcohol (POH) selectively induces in Bcr/Abl transformed cells, G0/G1 arrest and apoptosis. Therefore, POH activates anti-proliferative and apoptotic pathways against which the Bcr/Abl kinase does not protect. In this report, we show that in Bcr/Abl-transformed cells, POH induces cytoplasmic acidification, redistribution of phosphatidylserine in the plasma membrane along with DNA fragmentation, all of which can be prevented by the phorbol ester, TPA. The ability of TPA to protect against POH-induced cytotoxicity was blocked by inhibitors of protein kinase C (PKC) and the Na^+/H^+ antiport. In contrast, TPA does not protect the cells from POH-mediated G0/G1 arrest. While POH inhibits a distal step in the mevalonate biosynthesis pathway, lovastatin, also a potential anticancer agent, inhibits the initial step in this pathway. Not surprisingly, lovastatin also induces G0/G1 arrest and apoptosis in Bcr/Abl-transformed cells, however, TPA protects cells from both apoptosis and G0/G1 arrest caused by lovastatin. Thus, in Bcr/Abl-transformed cells, POH and lovastatin cause growth arrest by different mechanisms. Together, these observations demonstrate that POH-mediated cell cycle arrest precedes apoptosis and raises the possibility that that the primary effect of POH is to induce G0/G1 arrest with apoptosis being a consequence of the growth arrest.
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Introduction

The Philadelphia chromosome (Ph) is a balanced reciprocal translocation that joins the c-Abl protooncogene on chromosome 9 with the c-Bcr gene on chromosome 22. The resulting Bcr/Abl oncogene encodes a fusion protein that has constitutive tyrosine kinase activity.[1] One consequence of Bcr/Abl kinase expression is to circumvent diverse receptor-mediated signaling pathways that regulate hematopoiesis.[2–15] In some cases, expression of Bcr/Abl may be associated with low-level expression of cytokines or their receptors which is thought to set up an autocrine growth loop.[8,14] However, in other situations in which autocrine growth cannot be demonstrated, the Bcr/Abl kinase appears to interact with signaling pathways that drive cell growth and that activate an antiapoptotic program that allows prolonged cell survival in the absence of growth factors.[2–7,14,16] Bcr/Abl-transformed cells that resist apoptosis upon removal from growth factors also demonstrate cross-resistance to apoptosis induced by ionizing irradiation and various chemotherapy agents that have different mechanisms of action.[2–5,17–19] This suppression of the apoptotic response by the Bcr/Abl kinase does not appear to be associated with an effect on p53-dependent, WAF1/CIP1-mediated G1 arrest, but rather, is associated with a pronounced delay at the G2/M cell cycle restriction point.[9,20] Thus, while genotoxic agents cause apoptotic deletion of nontransformed hematopoietic cells at G2/M, Bcr/Abl-transformed cells exposed to these drugs undergo G2/M delay.[20] This delay presumably allows the cells time to repair and complete DNA replication before chromosome segregation, thereby preventing mitotic catastrophe. These observations indicate that Bcr/Abl plays an important role in the pathogenesis of Ph^+ leukemias by inhibiting the normal rate of cell death and enabling Ph^+ cells to resist therapeutic regimens that cure many Ph-negative leukemias.[21] The successful treatment of Ph^+ leukemias, therefore, requires strategies to circumvent Bcr/Abl-mediated resistance to apoptosis.

It is not surprising that the pleiotropic consequences of Bcr/Abl expression requires an active tyrosine kinase that interacts with numerous cellular regulatory pathways.[22] For instance, the Bcr/Abl kinase activates NF-κB,[13] the Ras[23–26] and Rac[12] small G proteins, mitogen-activated signaling pathways,[15] p27 cyclin-dependent kinase,[10] signaling through STAT-5,[11] and phosphatidylinositol-3′ kinase (PI-3 kinase).[27–29] Bcr/Abl also induces constitutive expression of c-Myc[30,31] and perhaps Bcl-2.[32,33] The activation of several of these mechanisms by Bcr/Abl is necessary for transformation of hematopoietic cells by the oncogene. Thus, suppression of c-Myc[31] or p21 Ras[25] with dominant negative mutants, blocking either PI-3 kinase or Bcl-2 expression with antisense oligonucleotides,[32,34] or inhibiting NF-κB[13] activity reportedly arrests cell growth and diminishes cell viability in Bcr/Abl-transformed cell lines. It is, therefore, reasonable to assume that signaling pathways downstream of the Bcr/Abl kinase may provide targets for developing novel therapeutic regimens designed to inhibit the growth of Bcr/Abl-transformed cells and reverse resistance to apoptosis. This principle has been proven in studies with the new Bcr/Abl tyrosine kinase inhibitors, STI-571[35–39] and PD180970,[16] and by lowering Bcr/Abl expression with ansamycin antibiotics[40] or antisense oligonucleotides.[41]

Monocyclic monoterpenes are produced in the mevalonate pathway of plants, but not mammalian cells, and represent a new class of anticancer compounds that can both prevent cancer and induce regression of advanced carcinomas in rats. Thus, dietary administration of the monoterpenes, limonene or perillyl alcohol (POH) can cure advanced mammary, stomach, lung, skin and liver cancers in rats, mice or hamsters[42–51] and they do not cause significant toxicity in rats, dogs or humans.[42–45,52–56] The anticancer activities of these monoter-

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penes are associated with a selective induction of apoptosis in the neoplastic tissue but not in adjacent normal tissues.[43,50] Although the specific anticancer mechanisms are poorly understood, the broad anticancer activity of monoterpenes, coupled with their negligible toxicity prompted us to test limonene and its hydroxylated derivative, POH, against Bcr/Abl-transformed and nontransformed cell lines. We recently reported that POH, but not limonene, induced G1 arrest followed by apoptosis in Bcr/Abl-transformed cells.[51] Importantly, while Bcr/Abl-transformed hematopoietic cells are more resistant than nontransformed cells to cytotoxicity caused by various chemotherapy drugs and ionizing radiation, the Bcr/Abl transformants were more sensitive than their nontransformed counterparts to these effects of POH. Thus, the fact that in Bcr/Abl-transformed cells, POH induces apoptosis that is associated with a G1 arrest indicates that POH activates an apoptotic pathway that is not protected by the G2/M delay seen in the response of Bcr/Abl-transformed cells to other cytotoxic agents. In the current report, we further characterize the cellular response of Bcr/Abl-transformed cells to POH.

Materials and methods

Cell culture

IL-3-dependent murine cell lines, FDC.P1 and 32D, were transformed by infection with helper-free p210 Bcr/Abl virus produced as described previously.[51] For control, nontransformed cell lines were infected with the retrovirus that only carried the *tk-neo* selectable marker and no Bcr/Abl. Infected cells were selected in G418 and multiple independently transformed clones were isolated. Clones infected with the Bcr/Abl expressing virus were shown to express a high level of kinase-active p210. These cells also efficiently formed agar colonies in the absence of IL-3 and developed hematopoietic malignancy in syngeneic mice (data not shown). Transformed FDC.P1 and 32D cells were maintained in RPMI-1640 that was supplemented with 5% fetal bovine serum. In order to evaluate the effects of POH on cell growth and resistance to apoptosis that are mediated by Bcr/Abl and not by factors present in serum, cells were washed and plated in RPMI-1640 media containing a minimal amount of FBS (0.3%) with the indicated concentration of POH and/or other agents as described in the text.

Pharmacologic agents

POH (Aldrich Chemical, Milwaukee, WI, USA) was mixed with DMSO to give a 1.0 M solution that was then added to 10 ml of RPMI containing 0.3% FBS. This was incubated at 37°C for 1 h with occasional mixing before being added to cell cultures. PKC inhibitors and TPA were purchased from Sigma Chemical (St Louis, MO, USA) and suspended in DMSO. Calphostin C stock solution (0.1 mg/ml in DMSO,126 mM) was diluted in culture media, added to cell cultures, which were placed under a lamp in a CO_2 incubator for 30 min,[57] then overnight in a dark incubator. Staurosporine stock solution (1 mM in DMSO) was diluted in culture media before adding to cell cultures. Amiloride analogs, DMA · HCl, and EIPA were purchased from Alexis Biochemicals (San Diego, CA, USA). The water-soluble DMA · HCl was dissolved to 10 mM in PBS at 37°C and EIPA dissolved in DMSO to make a 100 mM stock. Both stocks were diluted in culture media prior to adding to cell cultures overnight.

Analysis of cell growth, viability and apoptosis

The effect of the different pharmacological agents on cell growth and viability was measured either by directly counting the number of cells that exclude trypan blue, or by the MTT assay using a 'Cell Growth Determination Kit, MTT Based' (Sigma). To analyze the cell cycle distribution and to quantitate apoptotic cells with subdiploid DNA content, cells were fixed with 90% ethanol, permeabilized with 0.1% Triton-X-100, treated with 5 μg/ml RNase and stained with propidium iodide (50 μg/ml). Nuclear DNA content was measured by flow cytometry using a FACScan Plus flow cytometer. Apoptosis was also measured by simultaneously staining cells with propidium iodide and fluoresceinated annexin V (Sigma).[58]

Measurement of intracellular pH

Approximately 10^6 cells were washed twice in PBS buffer then incubated for 30 min at 37°C in 1 ml of PBS containing 2 μl of the pH-sensitive dye, BCECF-AM (Sigma) (from a 1 mg/ml stock solution dissolved in DMSO). BCECF-loaded cells were then collected by centrifugation and resuspended in 0.1 ml of PBS buffer for flow cytometry. The intracellular pH was estimated from the ratio of the emission fluorescence signals at 525 nm (pH dependent) and 640 nm (pH independent) following excitation at 488 nm using an argon laser on a FacStar flow cytometer. The cytoplasmic pH of cells treated with various agents was measured by comparison to standards of BCECF-loaded cells permeabilized with nigericin (Sigma) in a high K^+ buffer of known pH.

Statistical analysis

Data were analyzed using Student's *t*-test for unpaired samples.

Results

TPA protects Bcr/Abl-transformed cells from POH-induced apoptosis but not from cell cycle arrest

We recently reported that either the Bcr/Abl tyrosine kinase or IL-3 can protect factor-dependent FDC.P1 cells from apoptosis induced by either withdrawal of growth factor or ionizing radiation.[51] Here, we further demonstrate that like Bcr/Abl and IL-3, the phorbol ester, TPA, also can protect the IL-3-dependent FDC.P1 (Figure 1) and 32D (data not shown) hematopoietic cell lines from apoptosis caused by removal of growth factors. The data in Figure 1 show that the viability of FDC.P1 cells is severely reduced within 12 h, and that virtually all cells are dead 24 h after withdrawal from IL-3. In contrast, in the presence of IL-3 or TPA, cells maintained equal viability at 12 h and demonstrated proliferation at 24 h. Combining IL-3 and TPA did not obviously alter the survival or proliferation of the cells. These, along with our earlier observations[51] suggest that there may be some overlap in the signaling pathways that can be affected by Bcr/Abl, IL-3 and TPA. Therefore, we reasoned that if POH blocks signals downstream from Bcr/Abl

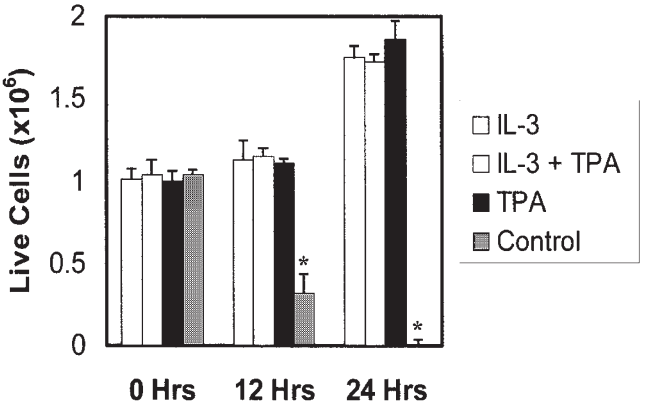

Figure 1 TPA protects nontransformed, factor-dependent FDC.P1 cells from apoptosis induced by withdrawal of IL-3. Cells (1×10^6/ml) were incubated for the indicated time with or without 10% WEHI-3 supernatant as a source of IL-3, TPA, both or neither (control). Viable cells were determined by trypan blue exclusion. Average values and standard deviations for triplicate cultures are shown. Asterisks indicate $P < 0.05$.

that confer resistance to apoptosis,[51] then TPA, which can protect cells from apoptosis following removal of growth factors, might also protect Bcr/Abl-transformed cells from POH-induced apoptosis. In order to test this hypothesis, the effects of increasing concentrations of POH, with or without TPA, were evaluated on different murine hematopoietic cells lines that had been transformed with Bcr/Abl. These cell lines were selected because TPA alone does not inhibit their proliferation. In contrast, TPA causes growth arrest and differentiation in the human Ph^+ cell lines, K562 and Bv173 (data not shown).[59,60]

As expected, the viability of Bcr/Abl-transformed FDC.P1 cells, measured by trypan blue exclusion, was greatly reduced in a dose-dependent fashion by overnight treatment with POH, while 100 nM TPA partially protected the cells from POH cytotoxicity (Figure 2). A similar level of protection was

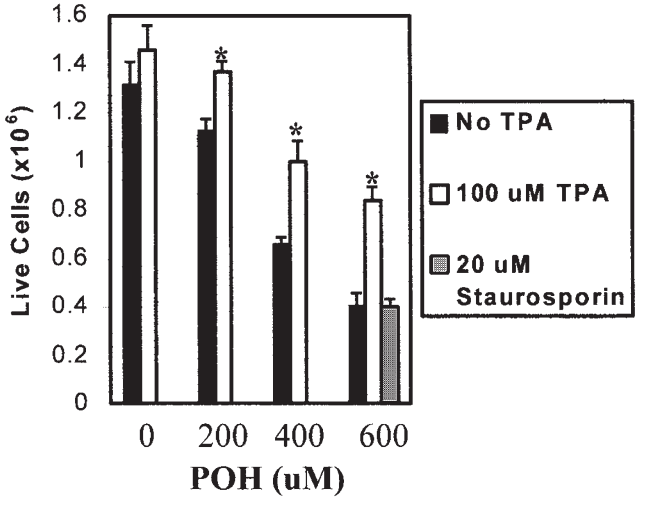

Figure 2 TPA protects Bcr/Abl-transformed FDC.P1 cells from POH-mediated cytotoxicity. Cells were plated in triplicate in 24-well tissue culture plates (6×10^5/well) in the presence of increasing concentrations of POH ± 100 nM TPA. Staurosporine (20 nM) was also included with TPA in one group treated with 600 μM POH. After 16 h the cells were harvested and viable cells were counted microscopically by trypan blue exclusion. Average values and standard deviations for triplicate cultures are shown. Asterisks indicate $P < 0.05$.

seen with TPA concentrations as low as 5 nM (data not shown). This protection by TPA against POH-induced apoptosis has been repeated in multiple experiments using three different Bcr/Abl-transformed cell lines (data not shown). In cells treated with 600 μM POH, the protective effect of TPA was completely reversed by the PKC inhibitor staurosporine (Figure 2). Therefore, we further tested the potential role of PKC in the protective effect seen with TPA. Table 1 shows the results of two independent experiments in which Bcr/Abl-transformed FDC.P1 cells were treated with POH ± TPA and with or without the PKC inhibitors, staurosporine (experiment 1) or calphostin (experiment 2). Both PKC inhibitors reversed, in a dose-dependent fashion, the TPA-mediated protection from POH. Importantly, the same concentrations of either PKC inhibitor, alone, were not toxic to the cells. These results were also repeated using a Bcr/Abl-transformed murine 32D cell line (data not shown). Hence, it appears that TPA protects cells from POH-mediated cytotoxicity through a PKC-dependent fashion.

In order to determine whether the protection afforded by TPA correlated with reversal of POH-induced cell cycle arrest, apoptosis, or both, Bcr/Abl-transformed 32D and FDC.P1 cells were incubated overnight with POH ± TPA and their nuclei labeled with propidium iodide in order to measure DNA content by flow cytometry. Figure 3 shows that treating Bcr/Abl-transformed 32D cells overnight with increasing concentrations of POH resulted in a corresponding increase of apoptotic cells with subdiploid (<2 N) DNA content. However, the fraction of subdiploid cells was reduced when TPA was also added to the cells (compare panels e to f and g to h). The fraction of cells in S phase also was reduced in cells treated with 600 μM POH and this was not affected by TPA (compare panels g and h).

Table 2 shows similar PI staining results obtained using Bcr/Abl-transformed FDC.P1 cells. In addition, cell viability was measured by trypan blue exclusion. Compared to the untreated control group, POH caused a decrease in the frac-

Table 1 Protein kinase C inhibitors reverse TPA protection from POH-induced cytotoxicity[a]

Experiment	*Treatment*	*Viability (% of control)*[b]
1	POH	49
	POH + TPA	76
	POH + TPA + St 6.25[c]	63
	POH + TPA + St 12.5	42
	St 6.25	94
	St 12.5	104
2	POH	27
	POH + TPA	58
	POH + TPA + Cal 25[d]	39
	POH + TPA + Cal 50	29
	Cal 25	115
	Cal 50	100

[a]Bcr/Abl-transformed FDC.P1 (Cl 3) cells were plated in duplicate wells in a 24-well plate (6×10^5/well), treated for 20 h as indicated with DMSO carrier (control), 600 μM POH, 100 nM TPA, and PKC inhibitor.
[b]Viable cells were counted by trypan blue exclusion and the data represent the percent of viable cells relative to the DMSO treated control. Similar data for each PKC inhibitor were collected in at least three independent experiments.
[c]PKC inhibitor, staurosporine (6.25 or 12.5 nM).
[d]PKC inhibitor, calphostin (25 or 50 nM).

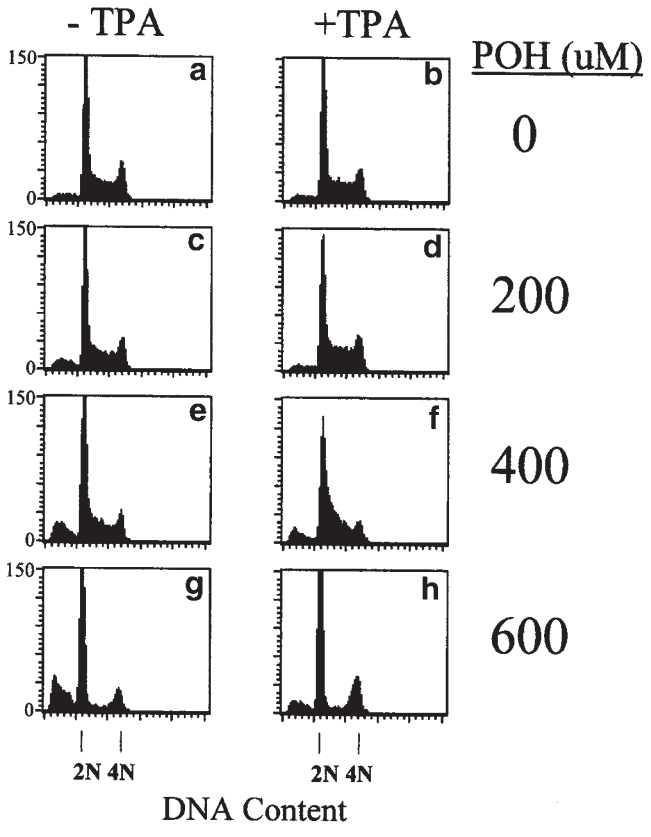

Figure 3 TPA protects against POH-induced apoptosis but not POH-induced cell cycle arrest. Bcr/Abl-transformed 32D cells were treated with increasing concentrations of POH ± TPA (100 nM) for 18 h. Cell nuclei were stained with propidium iodide and the DNA content evaluated by flow cytometry. Populations with 2 N (G0/G1) and 4 N (G2/M) DNA content are indicated. Apoptotic cells with <2 N DNA content are found to the left of the 2 N peak.

Table 2 TPA protects against POH-induced apoptosis, but not G0/G1 arrest[a]

Treatment group	*% Viable*	*Cell cycle distribution (% of cells)*			
		G0/G1	*S*	*G2/M*	*<2 N*
Untreated	80	36	32	9	6
TPA	85	36	38	8	3
POH	28	44	9	9	32
POH + TPA	55	61	8	16	11
POH + TPA + Staurosporine	33	41	10	13	30
Lovastatin	19	63	9	8	21
Lovastatin + TPA	74	29	38	7	3

[a]Bcr/Abl-transformed FDC.P1 cells (Cl 3) were treated for 17 h as indicated with TPA (100 nM), POH (300 μM), lovastatin (10 μM), staurosporine (20 nM), or a combination of these. Viability was determined by trypan blue exclusion and cell cycle distribution was measured by flow cytometry of propidium iodide-stained nuclei.

tion of cells in S phase from 32% to 9% as well as a concomitant increase from 6% to 32% in apoptotic cells with <2 N DNA content (Table 2). However, TPA protected the cells from POH-induced apoptosis by reducing the fraction of cells with <2 N DNA content to almost the background level of apoptosis seen in untreated cells. In the POH-treated cells, TPA also caused an increase in the number of cells in G0/G1, but did not affect the POH-induced reduction of cells in S phase, similar to what was seen in Figure 3. Importantly, TPA alone had no obvious effect on either the cell cycle distribution or viability of the cells. As in Figure 2, staurosporine reversed the ability of TPA to inhibit POH-induced apoptosis, and the TPA-mediated accumulation of cells in G0/G1. It was, therefore, concluded that TPA protects against POH-induced cytotoxicity by activating a PKC-dependent mechanism that protects cells from POH-induced apoptosis, but not from the POH-induced reduction of cells in S phase.

POH inhibits a distal step in the mevalonate biosynthesis pathway while lovastatin is an inhibitor of HMG-CoA reductase, which converts HMG-CoA to mevalonate in the first step of the pathway.[61,62] Thus, it is not surprising that like POH, lovastatin also induced apoptosis and an accumulation of Bcr/Abl-transformed FDC.P1 cells in G0/G1. TPA also protected the cells from lovastatin-induced cytotoxicity in that 74% of the cells treated with both lovastatin and TPA (Table 2) were viable, compared to only 19% of the cells treated with lovastatin alone. But, unlike its activity in concert with POH, TPA also protected the cells from the lovastatin-induced growth arrest. Thus, the percent of cells in G0/G1 increased from 36% in untreated cells to 63% in lovastatin-treated cells, and this was reduced back to 29% in cells treated with both lovastatin and TPA. Therefore, while TPA may exert similar protective effects against the cytotoxic activity of POH and lovastatin, TPA clearly induces different effects on the cytostatic activities of these mevalonate pathway inhibitors. This indicates that POH and lovastatin exert their cytostatic effects through different mechanisms.

POH-induced apoptosis is associated with cytoplasmic acidification

Apoptosis is often associated with intracellular acidification;[63–69] however, this is not a uniform observation since apoptosis may occur at normal cytoplasmic pH.[70,71] In order to determine whether the POH-induced apoptosis in Bcr/Abl-transformed FDC.P1 cells is accompanied by a change in cytoplasmic pH, cells treated overnight with POH and untreated controls were loaded with the pH-sensitive dye, BCECF, and intracellular pH was analyzed by flow cytometry. In both experiments, the untreated controls (Figure 4a and e) contained cell populations with a single cytoplasmic pH peak that was measured by comparing it to standards of known pH to be about 7.5. In experiment 1, nontransformed FDC.P1 cells cultured with and without IL-3 were also examined. The pH profile of the nontransformed cells in the presence of IL-3 (data not shown) was identical to the profile of the Bcr/Abl-transformed cells shown in panel a. However, when cultured overnight without IL-3, FDC.P1 cells underwent apoptosis[51] (data not shown) and also demonstrated an acidic pH shift (Figure 4b). Treatment of Bcr/Abl-transformed cells with POH gave rise to a distinct subpopulation of cells that had a similar acidic pH (Figure 4c and f) as the nontransformed cells cultured without IL-3 (Figure 4b). Using a control series of BCECF-loaded cells that had different known cytoplasmic pH, it was determined that untreated cells maintained a peak cytoplasmic pH of about 7.5 (Figure 4e) while the POH-induced population had a peak intracellular pH of 6.7 (Figure 4f).

Intracellular acidification that is associated with apoptosis can often be reversed by activating the Na^+/H^+ antiport[63,70–73]

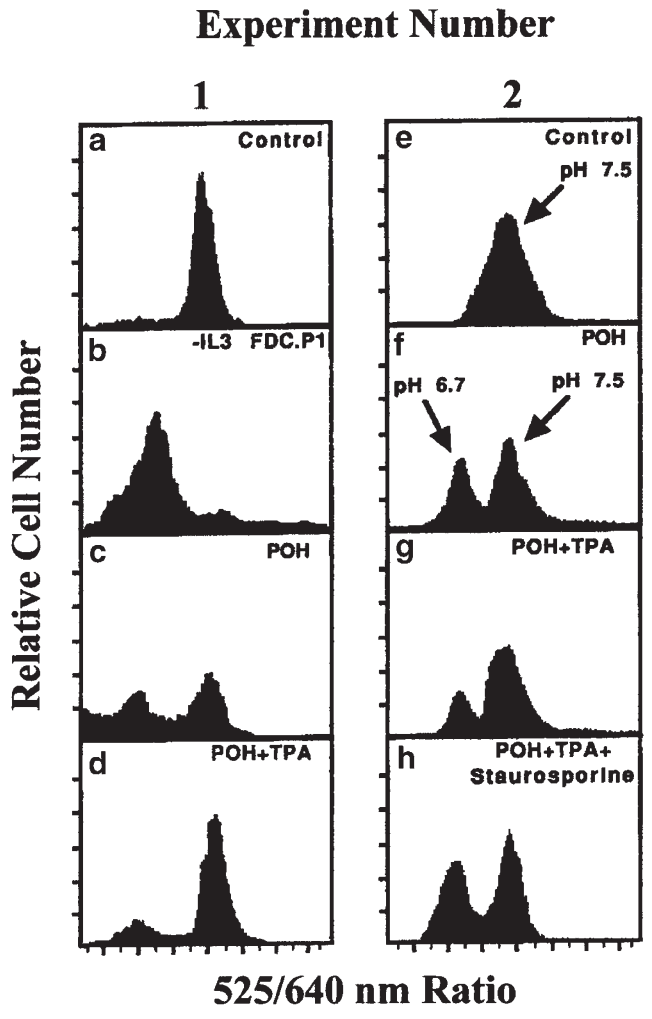

Figure 4 POH-induced apoptosis is associated with intracellular acidification, which is inhibited by treatment with TPA. In two different experiments, nontransformed and Bcr/Abl-transformed FDC.P1 cells were treated with 300 μM POH ± TPA (100 nM) ± staurosporine (20 nM) for 16 h. Cells were loaded with the pH-sensitive dye, BCECF, and the intracellular pH was analyzed by flow cytometry. Untreated Bcr/Abl-transformed controls (a, e) demonstrated a single population of cells with a peak pH of 7.5. Both transformed and nontransformed cells that were untreated showed an identical pH profile (data not shown). Upon removal of IL-3 from nontransformed FDC.P1 cells (b), a decrease in the intracellular pH was observed as a shift to the left of the pH peak. Treatment of the Bcr/Abl-transformed cells with POH alone resulted in the appearance of a distinct subpopulation of cells with a cytoplasmic pH of 6.7 (c and f). Treatment with POH + TPA caused a reduction in the acidic peak (D and G) and this was prevented by staurosporine in experiment 2 (h).

which exports protons from the cells.[74] PKC phosphorylation of the Na^+/H^+ antiport increases its affinity for H^+, which in turn stimulates exchange activity.[63,67,68,73,74] Therefore, we examined whether the previously demonstrated ability of TPA to protect against POH-induced apoptosis correlated with a protection against POH-induced acidification. In the presence of TPA, the fraction of cells undergoing the POH-induced pH shift was obviously reduced (compare panels c *vs* d and f *vs* g in Figure 4). Thus, in experiment 1, 52% of the cells treated with POH alone were acidic and TPA reduced this to 25%. In experiment 2, 41% of POH-treated cells were acidic, whereas, in the presence of POH + TPA 25% of the cells became acidic. Staurosporine blocked the ability of TPA to inhibit this pH shift (compare panels g *vs* h), so that 48% of the cells were acidic.

Since POH simultaneously induces apoptosis and cellular acidification, both of which are inhibited by TPA, we predicted that cells induced by POH to undergo apoptosis would be found in the acidic subpopulation. To test this, cells treated with POH were loaded with BCECF, and the subpopulations with different cytoplasmic pH (Figure 4f) were sorted by flow cytometry. Nuclei from the sorted and unsorted populations were then labeled with propidium iodide for analysis of DNA content. The data in Figure 5a demonstrate that the unsorted POH-treated population displayed the typical enrichment of cells in G0/G1, reduction of cells in S phase, and an apoptotic subpopulation with <2 N DNA content. The data also show that the sorted acidic subpopulation almost totally consisted of cells with <2 N DNA (Figure 5b), whereas the subpopulation with the normal pH was devoid of apoptotic cells (Figure 5c). Together, these observations demonstrate that POH-induced apoptosis is accompanied by cytoplasmic acidification that can be blocked by TPA, and that this likely is PKC dependent.

In order to determine whether the antiport plays a role in TPA-mediated protection against POH-induced apoptosis, we tested the ability of two different analogs of amiloride, a specific inhibitor of the Na^+/H^+ pump, to block TPA-mediated protection from POH-induced apoptosis. Data in Table 3 demonstrate that 20–50 μM of a water-soluble form of dimethylamiloride (DMA · HCl) alone had a minimal effect on the viability of the Bcr/Abl-transformed cells. As expected, POH was cytotoxic (11% viability relative to the DMSO control) and this was reversed upon treatment with TPA (44% viability); however, this TPA protection was blocked by DMA · HCl (Table 3). We also examined the effect of a differ-

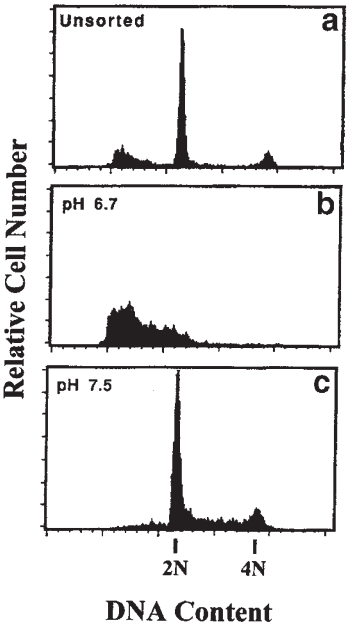

Figure 5 Apoptotic cells are found within the POH-induced acidic subpopulation. The Bcr/Abl-transformed cells treated with POH in Figure 4f were sorted by flow cytometry into acidic and basic subpopulations. Sorted and unsorted populations were labeled with propidium iodide and analyzed for DNA content. The figure shows the cell cycle distribution of unsorted cells (panel a), and the sorted, acidic (panel b), and basic (panel c) populations. Virtually all of the cells found within the pH 6.7 subpopulation had <2 N DNA content.

Table 3 The Na^+/H^+ antiport inhibitor, dimethylamiloride (DMA · HCl), reverses TPA protection from POH-induced cytotoxicity[a]

Group	*POH*	*TPA*	*DMA · HCl*	*Viability (% of control)*
1	–	–	–	100
2	+	–	–	11
3	+	+	–	44
4	+	+	50 μM	14
5	+	+	30 μM	19
6	+	+	20 μM	19
7	–	–	50 μM	113
8	–	–	30 μM	93
9	–	–	20 μM	93

[a]Bcr/Abl-transformed FDC.P1 (Cl 3) cells were plated in duplicate wells in a 24-well plate (6×10^5/well), treated for 20 h as indicated with DMSO carrier (control), 600 μM POH, 100 nM TPA and the indicated concentrations of DMA · HCl. Viable cells were counted by trypan blue exclusion and the data represent the percent of viable cells relative to the DMSO control (group 1).

ent amiloride analog, EIPA, on the cell cycle distribution of cells treated with or without POH + TPA. Figure 6 shows that 2.5 μM EIPA alone had little effect on the cell cycle distribution of the Bcr/Abl-transformed cells (Figure 6, panel d), while a reduction in the number of cells in S and G2/M phases was seen in cells treated with 5.0 μM EIPA (Figure 6c). At 10.0 μM, EIPA was toxic as seen by the appearance of apoptotic cells with subdiploid DNA content (Figure 6b).[75] As shown before, POH treatment reduced the proportion of cells in S phase and caused apoptosis (Figure 6e), while TPA reversed the POH-mediated apoptosis (Figure 6f). The ability of TPA to protect the cells from POH-mediated apoptosis was abrogated by EIPA. This is especially evident in Figure 6i, where 2.5 μM EIPA, which by itself was not toxic to the cells, caused an increase in the fraction of cells with subdiploid DNA content. Interestingly, 5 and 10 μM concentrations of EIPA seemed to enhance the apoptosis effect of POH (panels g and h). From these observations, we conclude that the ability of TPA to protect against POH-induced apoptosis requires a functional Na^+/H^+ antiport and that inhibiting the antiport enhances POH cytotoxicity.

Cytoplasmic acidification is an intermediate event in apoptosis[63] and precedes DNA fragmentation.[65] Thus, the PKC and Na^+/H^+ antiport-dependent ability of TPA to protect cells from POH cytotoxicity suggests that this protection may represent an effect on an early-to-intermediate step in the apoptotic sequence. An earlier event in the apoptotic sequence is the redistribution of plasma membrane phosphatidylserine[58] that can be detected by staining cells with FITC-conjugated annexin V. Bcr/Abl-transformed FDC.P1 cells were treated overnight with and without POH in the absence or presence of TPA, then stained with FITC-annexin V and propidium iodide (Figure 7). Both untreated cells and cells treated only with TPA, showed good viability with relatively few apoptotic and necrotic cells (Figure 7a and b). Overnight treatment with POH caused all the cells to simultaneously stain positive with annexin and PI, indicating that POH caused apoptosis and cell death in 99% of the treated population (Figure 7c and Table 4). However, in the presence of TPA (Figure 7d and Table 4), 44% of the POH-treated cells remained fully viable while other cells appeared to be in an early stage of apoptosis. Thus, TPA seems to protect cells from entering apoptosis when measured by redistribution of plasma membrane phosphatidyl serine.

Discussion

The observation that TPA protects cells from POH-induced apoptosis extends previous observations that PKC agonists are able to protect cells from apoptosis induced by a variety of stimuli.[63,64,67,73] However, we also show here that the protective effect of TPA is limited to POH-induced apoptosis, since the phorbol ester did not reverse the POH-induced G0/G1

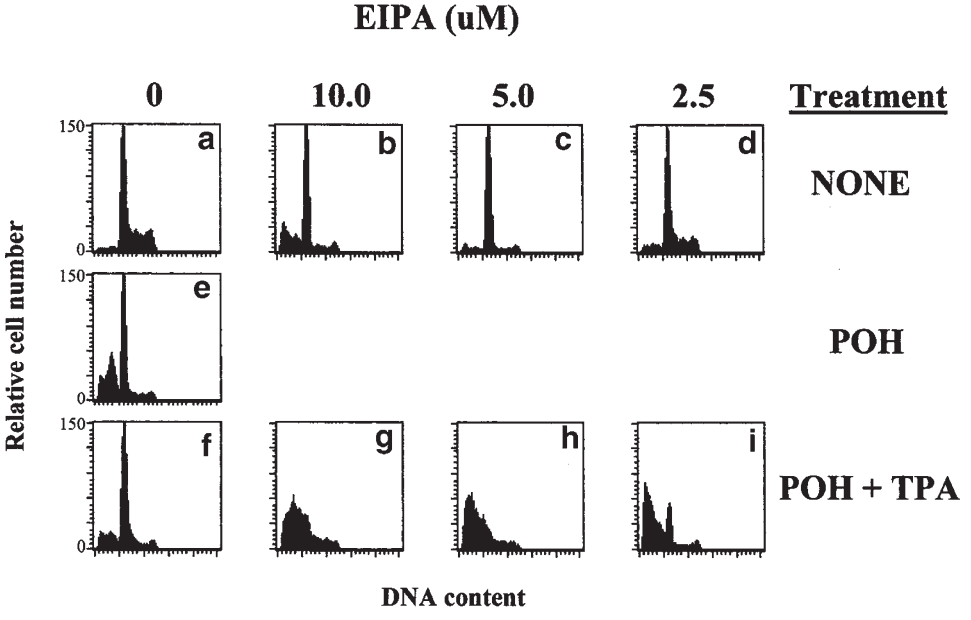

Figure 6 The Na^+/H^+ antiport inhibitor, EIPA, reverses TPA protection from POH-induced apoptosis. Bcr/Abl-transformed FDC.P1 cells were treated for 18 h with 600 μM POH ± TPA (100 nM) ± the indicated concentrations of EIPA. Cells were stained with PI and DNA content was analyzed by flow cytometry.

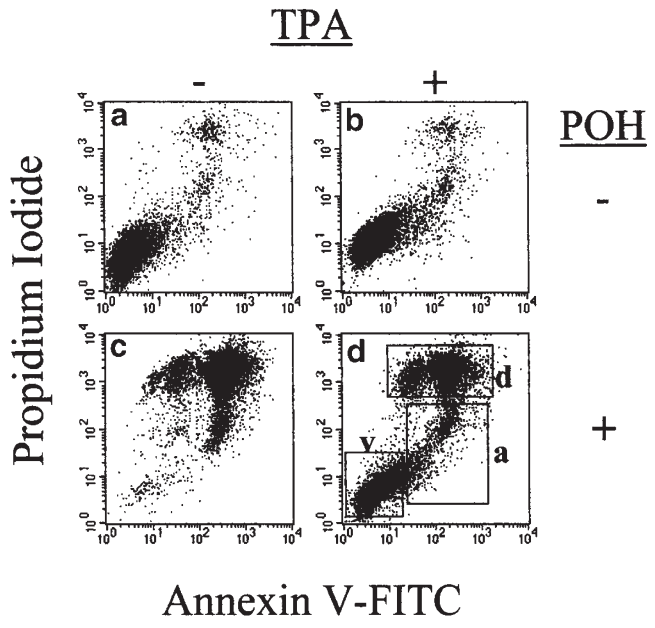

Figure 7 TPA protects cells at an early stage of POH-induced apoptosis. Transformed FDC.P1 cells were treated ± POH (600 μM) ± TPA (100 nM) for 18 h, after which, cells were labeled with FITC-annexin V and PI and analyzed by flow cytometry. In panel d, the different populations of cells are labeled so that v = viable cells (negative for both annexin and PI), a = early and late apoptotic populations (positive for annexin and negative to weakly positive for PI) and d = dead cells that stain brightly with both annexin and PI. The percentage of cells in each population for each group is given in Table 4.

Table 4 TPA protects cells from an early stage of POH-induced apoptosis[a]

Group	*POH*	*TPA*	*% of total population*		
			v	*a*	*d*
A	–	–	92	4	3
B	–	+	89	6	3
C	+	–	1	9	83
D	+	+	44	10	40

[a]Bcr/Abl-transformed FDC.P1 cells were treated as described in the legend to Figure 7. The percent of cells in each subpopulation (v = viable, a = apoptotic, and d = dead) were determined as shown in Figure 7d. The groups, a–d correspond to panels a–d in the figure.

arrest. In contrast, TPA did protect lovastatin-treated cells from both apoptosis and G0/G1 arrest. Thus, the effect of TPA on cell cycle arrest seems specific to the stimuli that induces the cytostasis. This also indicates that while POH and lovastatin both interfere with the mevalonate pathway, albeit at different points, they induce G0/G1 arrest through distinct mechanisms that are differently affected by TPA. It will be interesting to compare how POH and lovastatin cause cell cycle arrest in Bcr/Abl-transformed cells.

One way that TPA protects against apoptosis involves activating PKC, which then phosphorylates and stimulates the Na^+/H^+ antiport to inhibit apoptosis-associated cellular acidification.[63,76] The Na^+/H^+ antiport is the primary mechanism for removal of excess H^+ ions from cells, and it is regulated by cytoplasmic pH so that under neutral or alkaline conditions the exchanger has a low affinity for H^+ and is inactive. Under acidic conditions the antiport has a high affinity for H^+ and becomes active. However, phosphorylation by PKC also enhances the antiport's affinity for H^+, which helps maintain intracellular alkalinization.[77] Since, in the present study, PKC antagonists reversed TPA inhibition of both POH-mediated apoptosis and intracellular acidification, it is likely that TPA-mediated protection from POH is due to a PKC-dependent mechanism. However, care must be taken in interpreting the association between phorbol ester protection from apoptosis and PKC-mediated cellular alkalinization since TPA-induced protection from apoptosis has also been attributed to a PKC-dependent mechanism other than activation of the antiport.[67,71] Nevertheless, because two different amiloride analogs inhibited the ability of TPA to protect against POH-mediated cytotoxicity, we conclude that TPA protection in our model requires a functional antiport. In fact, when the antiport is inhibited, cells appear to be even more sensitive to POH-mediated cytotoxicity. Together, these observations are consistent with TPA protecting Bcr/Abl-transformed cells from POH-mediated apoptosis through PKC activation of the Na^+/H^+ antiport. Thus, TPA protection seems to work at a stage within the apoptotic sequence (ie cellular acidification) rather than at the initiation of apoptosis.

When apoptosis is associated with intracellular acidification, the drop in pH precedes DNA fragmentation,[65] which suggests a role for an acid endonuclease in DNA fragmentation.[65,67–71] Thus, preventing cytoplasmic acidification is expected to protect against DNA fragmentation, but not to affect other events that precede DNA digestion, such as cell cycle arrest.[65] Our data showing (1) that apoptotic cells are totally found in the acidic subpopulation of POH treated cells, (2) that TPA can protect against both POH-mediated cell acidification and apoptosis, (3) that TPA protection from POH-mediated apoptosis requires a functional Na^+/H^+ antiport and is PKC-dependent and (4) that TPA does not affect POH-mediated G1 arrest, together, are consistent with the TPA protective effect operating prior to cytoplasmic acidification and DNA fragmentation but after cell cycle arrest. Interestingly, TPA also protected cells from the membrane redistribution of phosphatidyl serine that is reported to be a fairly early event in apoptosis.[58] This raises the possibility that membrane redistribution actually may occur subsequent to cell acidification in POH-induced apoptosis. Alternatively, it is also possible that TPA may interfere with membrane redistribution through a distinct mechanism that is not related to the TPA effect on cytoplasmic pH.

In Bcr/Abl-transformed cells, TPA only protects against POH-mediated apoptosis and not against POH-mediated arrest in G0/G1. In fact, TPA enhanced the accumulation of cells in G0/G1 in POH-treated cells. These observations raise the possibility that the primary effect of POH may be to arrest Bcr/Abl-transformed cells in G0/G1 and that apoptosis may be secondary to this cell cycle arrest. This is consistent with our earlier observation that nontransformed cells, which are less sensitive than Bcr/Abl-transformed cells to POH, continue to cycle through G1 while the transformed cells are arrested at G1.[51] This is also consistent with our earlier cell cycle kinetic findings, which showed that upon POH treatment, Bcr/Abl-transformed cells accumulate in G0/G1 for 12 h, after which the number of apoptotic cells with subdiploid DNA content sharply increases, which corresponds to an equally sharp decrease in the number of cells in G0/G1.[51] Together, these observations mean that by preventing apoptotic elimination with TPA, the POH-treated cells arrested in G0/G1 are unable

to enter either S phase or the apoptotic pathway and simply accumulate in G0/G1. This raises the possibility that POH anticancer activity will be most effective against malignancies with a high mitotic index and least effective against those with a low mitotic index.

Cytokine-mediated cell proliferation and resistance to apoptosis are associated with the active maintenance of an alkaline cytoplasmic pH.[76–78] On the other hand, withdrawing necessary growth factors often results in intracellular acidification and apoptosis.[73,79,80] PKC agonists, such as bryostatin-1 and TPA, can mimic the ability of cytokines to maintain cytoplasmic pH as well as to stimulate proliferation and block apoptosis.[63,64,81–84] It is not surprising, therefore, that PKC inhibitors often block factor-dependent cell proliferation[84] and induce apoptosis.[85] In light of these similar activities of cytokines and PKC agonists, it is interesting that the ability of POH to induce growth arrest, cytoplasmic acidification and apoptosis in Bcr/Abl-transformed cells mimics the effects of both PKC antagonists and withdrawing growth factor from factor-dependent hematopoietic cells. This suggests a model in which POH disrupts signaling pathways downstream of Bcr/Abl that are critical for maintaining cell growth and viability of the leukemic cells.

In conclusion, it is clear that in Bcr/Abl-transformed cells, POH induces apoptosis by an atypical mechanism that does not activate the Bcr/Abl anti-apoptotic response that is characterized by a G2/M arrest.[20] How POH causes apoptosis in these cells remains unknown, however, the evidence presented in this article and in our previous report[51] support the notion that POH treatment of Bcr/Abl-transformed cells first induces growth arrest that is then followed by apoptosis. We propose a model in which the primary effect of POH on Bcr/Abl-transformed cells is to induce growth arrest by blocking signals downstream of the Bcr/Abl tyrosine kinase. According to this model, apoptosis may simply be a consequence of this growth arrest rather than a direct response of the cells to POH. In other words, these data suggest that in order to learn how apoptosis is initiated by POH in our model, we need to understand how POH interferes with Bcr/Abl-driven cell proliferation. This hypothesis is under investigation.

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