Induction of Apoptosis in Malignant B Cells by Phenylbutyrate or Phenylacetate in Combination with Chemotherapeutic Agents

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ABSTRACT

Phenylacetate (PA) and phenylbutyrate (PB) are aromatic fatty acids that are presently undergoing evaluation as potential antineoplastic agents. In vitro, PA and PB cause differentiation or growth inhibition of malignant cells. Clinical trials of these drugs as single agents indicate that they are not myelosuppressive; therefore, combinations with other chemotherapy agents may be possible. The goals of this study were to determine whether PA and PB (a) are cytotoxic to malignant B cells from patients with non-Hodgkin’s lymphoma and B-cell chronic lymphocytic leukemia and (b) exhibit additive or synergistic induction of apoptosis when administered to myeloma cell lines in combination with conventional drugs. In the clinical specimens, cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and percent apoptosis was measured using 7-aminoactinomycin D and flow cytometry. Viability was decreased by >50% in 7% (1/15) of non-Hodgkin’s lymphoma samples treated with 5 mM PA, 27% treated with 1 mM PB, and 60% treated with 2 mM PB. Likewise, viability was decreased by >50% in 44% (4/9) of chronic lymphocytic leukemia samples treated with 5 mM PA, 67% treated with 1 mM PB, and 100% treated with 2 mM PB. Studies in the myeloma cell lines demonstrated that PB treatment induced activation of caspases 3, 7, and 9 accompanied by cleavage of their substrates and internucleosomal DNA degradation. Combinations of PA or PB with conventional drugs (cytarabine, topotecan, doxorubicin, etoposide, chlorambucil, melphalan, fludarabine, carboplatin, and cisplatin) were examined for synergism (combination index <1 in median effect analysis) in inducing apoptosis of both the MY5 and 8226 human myeloma cell lines. At concentrations that killed >50% of cells, most combinations were additive; however, PB was synergistic with cytarabine, etoposide, and topotecan, with the combination index <1 at each of the 50, 75, and 95% apoptosis levels. These observations indicate that PA and PB can induce apoptosis in malignant B cells and enhance the cytotoxicity of agents used in the treatment of these malignancies.

INTRODUCTION

PA3 and PB, two closely related aromatic fatty acids presently in use for the treatment of patients with urea cycle abnormalities or portal systemic encephalopathy (1–4), are now undergoing evaluation as potential antineoplastic agents. A number of observations have provided the impetus to test PA and PB in humans with cancer. Samid et al. (5) demonstrated that treatment of HL-60 human acute promyelocytic leukemia cells with PA led to a dose-dependent reduction in cell proliferation, a rapid (within 4 h) down-regulation of myc expression, and subsequent granulocytic maturation. PA decreased cell viability and induced differentiation in an acute myelocytic cell line (6). PA and PB were subsequently shown to cause growth arrest of glioma cells in vitro (7, 8) and to prolong the survival of rats with malignant brain tumors by inducing tumor differentiation (9). Recent studies in prostate cancer cell lines (10) and primary neoplastic myeloid cells (11) indicated that both PA and PB can induce growth inhibition and apoptosis, with PB being more potent than PA. In vitro studies of PA in colon carcinoma cells demonstrated a longer inhibition of growth after sequential use of fluorodeoxyuridine and PB than with either drug as a single agent (12).

The mechanism(s) by which PA and PB cause apoptosis in malignant cells is presently under investigation. PA is an inhibitor of mevalonic acid PPi decarboxylase (13). In addition, PA and PB activate human peroxisome proliferator-activated receptors, which are ligand-activated transcription factors that up-regulate the expression of several genes that code for lipid-metabolizing enzymes (14). The net result of these changes is a decreased conversion of mevalonic acid to farnesyl PPi (15), decreased cholesterol production, decreased protein prenylation (7, 16), and decreased activation of the p21ras target p42/44 MAPK/ERK2 (17). Experiments with Ras-transformed tumor cells have documented that sensitivity to PA is associated with inhibition of p21ras prenylation.

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3 The abbreviations used are: PA, phenylacetate; CI, combination index; CLL, chronic lymphocytic leukemia; FCS, fetal calf serum; DMSO, dimethylsulfoxide; MNC, mononuclear cell; MTTR, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NHL, non-Hodgkin’s lymphoma; OD, optical density; PB, phenylbutyrate; 3H-TdR, tritiated thymidine; 7-AAD, 7-aminactinomycin D.
(18). Additional experiments in a MCF7ras breast cancer cell line have revealed that PA with and without tamoxifen can down-regulate Bcl-2 and induce apoptosis (19,20). PA-induced growth arrest in MCF7 cells has also been shown to be associated with up-regulation of p21Waf1/Cip1 (21).

The unique mechanism(s) of action of PA and PB, coupled with the lack of myelosuppression or other serious organ toxicity when these agents were used in patients without neoplastic diseases (22–26) and the insensitivity of PA and PB to P-glycoprotein-mediated resistance (27), make these agents attractive for use in patients with cancer. In a Phase I trial of PA by continuous i.v. infusion for 14 days, doses of 150–374 mg/kg/day produced PA blood levels of ~0.3–2.6 mM. The toxicities observed were dose-related mental confusion, lethargy, odor, and nausea, all of which resolved within 18 h of discontinuing the drug. Peak levels >5 mM were consistently associated with toxicity (28). In a Phase I trial of PB administered as a 30-min infusion in doses ranging from 600 to 2000 mg/m², the peak PB concentration ranged from 0.5 to 2 mM; no significant toxicities were reported (29). In a study of 19 g/day of oral PB in patients with cystic fibrosis, the highest blood PB concentration achieved was 2 mM (26). The lack of myelosuppression, nephrotoxicity, and peripheral neuropathy of PA and PB in the human studies completed to date suggests that it might be possible to combine these agents with conventional anticancer regimens.

The goals of this study were to (a) assess the effects of PA and PB in vitro on tumor samples from patients with B-cell malignancies and (b) combine PA and PB in vitro with agents commonly used to treat hematological malignancies. These studies have identified drug combinations that might be promising for future clinical development.

MATERIALS AND METHODS

Patient Samples.

Samples of malignant lymphomas represented excess tumor tissue from patients undergoing lymph node biopsy for clinical purposes. The samples of B-cell CLL cells were from blood samples obtained with each patient’s permission at the time of routine phlebotomy as part of a study approved by the Institutional Review Board of the Mayo Clinic/Foundation.

Cell Lines and Drugs.

The cell lines used for these experiments were: ARH, CESS, U266, MY-5, and 8226 (obtained from American Type Culture Collection); ANBL-6, KP-6, KAS-6/1, and DP-6 (kindly provided by Dr. Diane Jelinek, Mayo Clinic/Foundation, Rochester, MN); and Dox 6 and Dox 40 (generously provided by Dr. William S. Dalton, Moffitt Cancer Center, Tampa, FL). They were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and l-glutamine. Topotecan was provided by Dr. Randall K. Johnson (Smith Kline Beecham, King of Prussia, PA). Cytarabine, doxorubicin, etoposide, chlorambucil, melphanal, fludarabine, carboplatin, and cisplatin were from Sigma Chemical Co. (St. Louis, MO). PA and PB were provided by Elan Pharmaceuticals (Gainesville, GA).

Cell Viability, Proliferation, and Apoptosis Assays.

Viability of PA- or PB-Treated Malignant B Cells from Patients. Lymphoma samples were prepared for testing as previously described (30). Briefly, lymphoma tissue samples were minced over a fine mesh screen to obtain a single-cell suspension, then centrifuged over Ficoll-Hypaque to isolate MNC. For CLL, MNCs were isolated from whole blood by centrifugation over Ficoll-Hypaque. The effect of PA and PB on viability of fresh NHL or CLL cells was determined using an MTT tetrazolium dye reduction assay (31,32). Fresh MNCs (5 × 10⁶) were plated into each well of a 96-well round-bottomed tissue culture plate. Culture medium lacking (control) or containing 5 mM PA, 1 mM PB, or 2 mM PB was added to 12 wells (one row of the plate) for each experimental condition. On day 6, the MTT was added to each well. After overnight incubation at 37°C, the plate was centrifuged, the culture medium was aspirated from each well, the precipitated formazan was dissolved in DMSO, and the OD was read on a plate reader at 550 nm with a 620 nm blank. Based on 12 wells/data point, survival was calculated by the average OD (cells + drug) – OD (medium + drug) divided by the average OD (control cells) – OD (medium) and expressed as a percentage.

Effect of PA or PB on Proliferation of Malignant B-cell Lines. The effect of PA and PB on the proliferation of the cell lines was determined by measuring [³H]Tdr incorporation into trichloroacetic acid precipitable material or DNA content by flow cytometry using propidium iodide as previously described (33).

Measurement of Apoptosis by 7-AAD Staining. The ability of PA or PB to induce apoptosis was determined by using 7-AAD and flow cytometry (34). Control and drug-treated cells were washed with cold PBS and centrifuged for 5 min at 300 × g. The supernatant was decanted, and 500 µl of 20 µg/ml 7-AAD (Calbiochem, La Jolla, CA) were added. After incubation at 4°C for 20 min, cells were washed in PBS, centrifuged, resuspended in 500 µl of cold PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson) within 30 min. The control tube (without 7-AAD) was used to set the threshold for 7-AAD fluorescence. Cells that are 7-AAD negative are alive and effectively exclude the dye; cells that exhibit bright 7-AAD fluorescence are dead and are readily permeable to 7-AAD; cells undergoing apoptosis have 7-AAD staining intermediate between these two gates (34). The percentages of live, apoptotic, and dead cells were calculated by use of the Cell Quest software program.

Apoptosis induced by treatment with PB was also demonstrated by the detection of characteristic internucleosomal DNA fragmentation upon agarose gel electrophoresis. MY5 human myeloma cells were incubated with 0 (control), 0.5 mM, 1 mM, 3 mM, or 5 mM PB in culture medium for 48 h. DNA was isolated from the cultured cells, and gel electrophoresis was performed using a commercial kit (Boehringer Mannheim, Indianapolis, IN).

Immunoblotting. Samples from MY-5 cells treated with 250 ng/ml anti-Fas (clone CH-11 Upstate Biotechnology Incorporated, Lake Placid, NY) for 3 h or 8226 cells treated with 2 mM PB for 0–48 h were sedimented at 200 × g, washed once with ice-cold serum-free medium, and solubilized in 6 mM guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.5 at
4°C), 10 mM EDTA, 150 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After sonication, samples were treated with iodoacetamide to block free sulphydryl groups and then dialyzed sequentially into 4 M urea and 0.1% (w/v) SDS as previously described (35). After an aliquot was removed for the determination of protein (36), each sample was lyophilized to dryness; resuspended at a concentration of 5 mg protein/ml in a SDS sample buffer consisting of 4 M deionized urea, 2% (w/v) SDS, 62.5 mM Tris–HCl (pH 6.8 at 21°C), and 1 mM EDTA; and heated to 65°C for 20 min. Aliquots containing 50 μg of total cellular protein were subjected to SDS-PAGE on gels with 5–15% (w/v) acrylamide gradients, transferred to nitrocellulose or polyvinylidene difluoride, and probed with antibodies using techniques previously described in detail (37). Mouse monoclonal antibodies against poly (ADP-ribose) polymerase, lamin A, topoisomerase I, and heat shock protein 90 were provided by Guy Poier (Laval University, St. Foy, Quebec), Frank McKeon (Harvard University, Cambridge, MA), Y-C. Cheng (Yale University, New Haven, CT), and David Toft (Mayo Clinic), respectively. Rabbit antisera that recognize procaspase-7 and procaspase-8 as well as the epitopes IETD and PEPD generated upon activation of caspase-3 and caspase-9, respectively, were raised as recently described (38). Peroxidase-coupled affinity-purified secondary antibodies were from Kirkegaard & Perry (Gaithersburg, MD).

**Examination of the Effect of Drug Combinations.**

The effect of simultaneous exposure to PA or PB and other agents was assessed in the MY5 and 8226 human myeloma cell lines. Cells were incubated with one or both drugs simultaneously for 4 days and then analyzed for apoptotic and dead cells. Before the combinations were tested, the LD_{50} was determined from single-agent exposure of the cells to each of the drugs for 96 h. PA or PB was then combined with each of the cytotoxic drugs simultaneously at a fixed ratio of doses that usually corresponded to 1/2, 5/8, 3/4, 7/8, 1, and 1.5 times the individual LD_{50}. The range of concentrations of drug used and the concentrations typically achievable in humans are summarized in Table 1. Fractional survival (f) was calculated by dividing the percent survival (percentage cells not apoptotic or dead) in drug-treated wells by the percent survival in control wells. Data were subsequently analyzed by the median effect method of Chou and Talay (39, 40). For each level of cytotoxicity, the CI was calculated under the assumption that the agents are mutually nonexclusive, i.e., the action of one does not affect the action of the other. This assumption was based on what is known about the mechanisms of the various agents. A CI value <1 indicates synergy between the drugs, a CI of 1 suggests that the drug effects are additive, and a CI value >1 indicates antagonism. The assays were repeated until the correlation coefficients (R) were equal to 0.9 for all three median effect lines (PA or PB alone, drug alone, and the combination). In the 8226 cell line, the analyses were repeated up to four times; the results of the multiple CI plots are summarized by providing the mean ± SE.

**RESULTS**

**Effect of PA and PB on Samples of Malignant B Cells from Patients.** Early clinical trials of PA and PB have used prolonged oral (weeks to >1 year; Refs. 23, 26, and 41) or i.v. (up to 2 weeks) (28, 42, 43) infusion schedules. To examine the effect of this treatment on clinical samples of malignant B cells, 15 tissue biopsy samples of NHL and 9 blood samples from patients with B-CLL were exposed to PA or PB for 6 days (Table 2). The short-term cultures of NHL or CLL cells were treated with 5 mM PA, 1 mM PB, or 2 mM PB, all of which are clinically achievable concentrations (28, 29, 42). When the NHL cells were treated with 5 mM PA for 6 days, a >50% reduction in viability (by MTT assay) was found in 7% (1/15) of the samples. Larger effects were observed when the cells were treated with PB. When 1 mM PB was used, 27% (4/15) had a >50% reduction in viability. This increased to 60% (9/15) with 2 mM PB. The results for the nine CLL cases showed a similar trend but greater sensitivity. In particular, a >50% reduction in viability was found in 44% (4/9) of samples treated with 5 mM PA, 67% treated with 1 mM PB, and 100% treated with 2 mM PB. Because CLL lymphocytes are not proliferating, these effects appear to reflect an induction of cell death rather than an inhibition of proliferation.

**Effects on Cell Proliferation of Myeloma Cell Lines.** To examine the effects of PA and PB on malignant B cells in greater detail, human myeloma cell lines were exposed to the same concentrations of PA and PB used in the lymphoma and B-CLL experiments. The ARH, U266, MY-5, DOX 6, and DOX 40 cell lines were resistant (>80% of control) to 5 mM PA, whereas the other cell lines showed some evidence of inhibition.

**Table 1** Range of concentrations of the drugs combined with phenylacetate and phenylbutyrate in these experiments with the 8226 and MY5 human myeloma cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>8226 (μM)</th>
<th>MY5 (μM)</th>
<th>Concentrations in μM achievable in humans (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.01–0.12</td>
<td>0.01–0.16</td>
<td>0.01–7 (59–61)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.05–1.8</td>
<td>0.05–0.35</td>
<td>10–92 (62)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.05–0.5</td>
<td>0.1–2</td>
<td>0.01–0.1 (63, 64)</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>0.2–4</td>
<td>0.05–1</td>
<td>10–150 (65)</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>5–120</td>
<td>10–125</td>
<td>2–6.3 (66, 67)</td>
</tr>
<tr>
<td>Melphalan</td>
<td>1–80</td>
<td>2–30</td>
<td>1–13 (68, 69)</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>5–180</td>
<td>3–40</td>
<td>0.63–0.76 (70)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.5–12</td>
<td>1–20</td>
<td>8–30 (71–73)</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>8–130</td>
<td>10–160</td>
<td>1.4–13.5 (74)</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>2000–20,000</td>
<td>2000–20,000</td>
<td>1360–4600 (28, 42, 43)</td>
</tr>
<tr>
<td>Phenylbutyrate</td>
<td>250–3000</td>
<td>250–3000</td>
<td>1120–2020 (23, 29, 75)</td>
</tr>
</tbody>
</table>

*Also included are concentrations of these drugs achievable in humans.
of proliferation as measured by [3H]TdR incorporation into DNA (Fig. 1). ARH was also resistant to PB; however, the other 10 cell lines were sensitive to PB in a dose-dependent manner.

To study these antiproliferative effects in greater detail, 2 of the 10 sensitive cell lines (MY5 and 8226) were arbitrarily selected for additional experiments. These cells were treated with PA or PB for up to 96 h, stained with propidium iodide, and subjected to flow cytometry. PB induced a dose-dependent decrease in the percentage of cells in the S phase in both cell lines (Fig. 2). The percent S phase at 96 h. However, in the MY5 cells, the decrease in the S phase of PB-treated cells was also time-dependent. In the MY5 cells, the percent S phase of PB-treated cells dropped substantially at 24 h, and the percent S phase of the 2 mM PB-treated cells remained relatively stable between 24–96 h. However, in the 1 mM PB-treated MY5 cells, we consistently observed a slight increase in the percent S phase at 48 and 72 h (although still lower than control) followed by a reduction in the percent S phase at 96 h.

**Induction of Apoptosis by PA or PB.** To further explore the possible induction of apoptosis in malignant B cells, MY5 and 8226 human myeloma cell lines were incubated with PA or PB for 48 h and then stained with 7-AAD and analyzed by flow cytometry (Fig. 3). As previously reported (34), healthy cells do not take up 7-AAD, necrotic cells are freely permeable to 7-AAD, and apoptotic cells take up intermediate amounts of this dye. Treatment with PA or PB resulted in a dose-dependent increase in the number of cells taking up intermediate amounts of 7-AAD. Both cell lines had similar sensitivities to PA; however, the 8226 cell line was more sensitive to PB than the MY5 cells (Fig. 4). A 96-h incubation with PA induced apoptosis with LD50 s of ~13 and 15 mM for the MY5 and 8226 cell lines, respectively. These concentrations of PA are not achievable in humans without substantial neurotoxicity. In contrast, the LD50 s for PB in the MY5 and 8226 cell lines were 1.9 mM and 0.8 mM, respectively, concentrations that are achievable in humans using present infusion schedules (see “Introduction”).

To provide further evidence that the PB-treated cells were undergoing apoptosis, two sets of experiments were performed. First, MY5 and 8226 cells treated with varying concentrations of PB for 48 h were lysed so that DNA could be analyzed by agarose gel electrophoresis. A nucleosomal ladder of DNA fragments, one of the cardinal features of apoptosis, was detected after treatment with PB in both cell lines (Fig. 3, right panel).

In a complementary set of experiments, 8226 cells were treated with 2 mM PB for 4–48 h and examined by immunoblotting for signs of caspase activation, another hallmark of apoptosis (44, 45). MY-5 cells subjected to Fas-mediated apoptosis served as a positive control. As indicated in Fig. 5A, the caspase-3 substrate poly (ADP-ribose) polymerase and the caspase-6 substrate lamin A (46) were cleaved to 89-kDa and 45-kDa fragments, respectively (Fig. 5A, arrowheads) in PB-treated cells. These cleavages have previously been reported to reflect the appearance of active caspases in nuclei (37). Likewise, topoisoasmerase I, which can be cleaved by either caspase-3 or caspase-6 (47), was partially degraded in PB-treated cells. Cleavage of all three of these polypeptides was faintly detectable at 24 h and readily detectable at 48 h. In contrast, heat shock protein 90 and the nucleolar protein B23, two polypeptides that are not caspase substrates, were unaffected by PB.

To identify some of the caspases that are activated in PB-treated 8226 cells, the blots shown in Fig. 5A were reprobed with anticaspase antisera (Fig. 5B). Treatment of 8226 cells with 2 mM PB resulted in activation of caspase-3 and caspase-8 as demonstrated using antibodies that recognize only the active forms of these enzymes (38). This activation was evident at 24 h but became more pronounced at 48 h (Fig. 5B, Lanes 7 and 8, top two panels). A decrease in procaspase-7 was detected.
accompanied by the appearance of active species of this enzyme (Fig. 5B, third and fourth panels). In contrast to MY-5 cells treated with anti-Fas antibodies, in which procaspase-8 levels decreased as caspase-8 was activated (Fig. 5B, Lanes 1 and 2), procaspase-8 levels remained constant in PB-treated 8226 cells, suggesting that the death receptor/FADD/procaspase-8 pathway (44) was not activated.

Collectively, the results shown in Fig. 5, A and B indicate that one of the two well-characterized initiator caspases (caspase-9) and several downstream effector caspases are activated in PB-treated 8226 cells, thereby providing further support for the view that PB induces apoptosis.

**Interaction of PA and PB with Cytotoxic Agents.** In additional experiments, the effect of combining PA or PB with conventional chemotherapy agents was examined. For these studies, 7-AAD was used to quantitate the percentage of cells that were viable, apoptotic, or ruptured; and the method of Chou and Talay (40) was used to mathematically assess the effect of combining the agents. Results obtained using this approach are illustrated in detail in Fig. 6 for the combination of PB + cytarabine, and they are summarized in Figs. 7 and 8.

When 8226 cells were treated with PB and cytarabine, each of these agents by itself induced apoptosis (Fig. 6, A and B). When the cells were exposed to a fixed 1750:1 ratio of PB and cytarabine, more cells were killed than when cells were exposed to each agent alone (Fig. 6, A and B). After the log [(1/t) − 1] versus log (drug dose) was plotted for each treatment (Fig. 6C), the X intercept and slope determined for each line were used as described by Bible et al. (39), Chou et al. (40), and Kaufmann et al. (48) to calculate the CI (Fig. 6D), a parameter that indicates whether the doses of the two agents required to produce a given degree of cytotoxicity are greater than (CI > 1), equal to (CI = 1), or less than (CI < 1) the doses that would be required if the effects of the two drugs were strictly additive. Based on what is presently known about the actions of PB and cytarabine, we assumed that the two agents were mutually nonexclusive, i.e., that the action of PB did not preclude the action of cytarabine and vice versa (Fig. 6D, dashed line). At concentrations that induced apoptosis in 25% of the cells, the CI was 1.2, indicating that the effects were less than additive. In contrast, the CI decreased to 0.8, 0.6, and 0.4 at doses of the combination that induced apoptosis or death in 50%, 75%, and 90% of the cells. For the sake of completeness, the CI calculated under the assumption of mutually exclusive drug interactions is also shown (Fig. 6D, solid line).

The CI plots obtained when PB was combined with doxorubicin, etoposide, or topotecan are shown in Fig. 7. With doxorubicin + PB, the CI was >1 (indicating antagonism) at all levels of cytotoxicity. In contrast, with etoposide or topotecan + PB, less than additive or additive effects were observed at low levels of cytotoxicity (<40% apoptotic or dead cells), but synergism (CI < 1) was observed at all concentrations that induced apoptosis in >50% of the cells.

Results of multiple analyses are summarized in Fig. 8. The majority of combinations displayed a CI > 1 at doses that induced apoptosis or death in 25% of the cells. Less than additive (CI > 1) or additive (CI = 1) effects were observed over a wide range of concentrations of PA in combination with doxorubicin, chlorambucil, fludarabine, cisplatin, and carboplatin. Similar results were obtained with PB in combination with doxorubicin, chlorambucil, fludarabine, and cisplatin. In contrast, greater than additive effects were consistently observed with PA or PB in combination with topotecan or cytarabine at doses that induced apoptosis or death in >50% of the cells. In addition, synergism (CI < 1) was demonstrated with PB or PA + etoposide, melphalan, or carboplatin at doses that induced apoptosis or death in >50% of 8226 cells.

**DISCUSSION**

Patients with advanced stage low-grade NHL, B-CLL, and multiple myeloma have B-cell malignancies that are treatable, but...
not curable, with conventional agents. New anticancer drugs that are not myelosuppressive and can enhance the effectiveness of presently used chemotherapy agents are needed. Most of the recently developed new agents are myelosuppressive, making it difficult to add these drugs directly to chemotherapy regimens without alteration of the drug dosage. The findings that PA and PB, when used as single agents for benign or malignant disease (23, 26, 28, 29, 41–43, 49), are nonmyelosuppressive and without major organ toxicity makes the combination with conventional agents attractive. In this report, the cytotoxic actions of PA and PB on malignant B cells were studied. We observed that clinical samples of NHL and CLL are sensitive to these agents in vitro, that proliferation of myeloma cell lines can be inhibited, and that myeloma cell lines can be induced to undergo apoptosis with clinically achievable concentrations of these drugs. Most importantly, the results suggest that the induction of apoptosis is synergistic when PA or PB is combined with some of the cytotoxic agents presently used in the treatment of the B-cell malignancies. The most synergistic effects were observed with PB in combination with the S-phase active agents cytarabine or topotecan.

This is the first systematic examination of interactions between PA or PB and agents commonly used in the treatment of B-cell malignancies. Previous in vitro studies of PA or PB with other agents have been quite limited. Samid et al. (50) demonstrated that the effect of PA on K562 leukemia cells was enhanced by IFN-α and hydroxyurea. Likewise, low-dose PB (0.5 mM) potentiated the effect of hydroxyurea in prostate cell lines (51), and IFN-α improved the antiproliferative effects of PA in several human lung adenocarcinoma cell lines (52). Recently, Sidell et al. (53, 54) have shown PA to synergize with retinoic acid in inhibiting the proliferation and inducing the differentiation of neuroblastoma cells. The cytotoxic effects of PB and vincristine were additive in neuroblastoma cell lines (55). The combination of PA and cisplatin was also additive in inhibiting the growth of ovarian cancer cells (56).

In many previous studies that have examined drug combinations in malignant cells, drug effects were measured as a reduction in cellular survival.
of colony growth compared to the control. In this study, we used induction of apoptosis as the end point to measure drug effects on malignant cells. Recent studies raise the possibility that apoptosis might be a better measure of the ability to kill cancer cells than suppression of colony growth (57). Moreover, apoptotic cells are readily quantitated by flow cytometry (34), making this approach somewhat less labor intensive than enumerating colonies in soft agar. Which methodology is best able to predict drug effectiveness in patients is not known.

The method used for quantitating apoptosis (uptake of the fluorescent dye 7-AAD) has been extensively characterized (34). Studies in the myeloma cell lines confirmed that the enhanced uptake of 7-AAD was accompanied by internucleosomal DNA degradation (Fig. 3), caspase activation (Fig. 5B), and cleavage of a number of well-characterized caspase substrates (Fig. 5A). Previous studies (reviewed in ref. 44) have demonstrated that there are two canonical pathways of caspase activation. One involves a mitochondrial release of cytochrome c to the cytosol, where it binds the scaffolding protein Apaf-1, which in turn binds and activates the zymogen form of caspase-9. Once activated, caspase-9 can proteolytically activate caspases-3 and -7, the former of which activates caspase-6 (44). The other major pathway involves ligation of death receptors followed by binding of the adaptor molecule FADD, which in turn binds and activates the zymogen form of caspase-8. Once activated, caspase-8 can cleave caspase-3, thereby leading to downstream events. Alternatively, caspase-8 can cleave the Bcl-2 family member Bid, generating a fragment that facilitates mitochondrial release of cytochrome c and activation of caspase-9, which amplifies the direct effects of caspase-8 (44). Results presented in Fig. 5B help distinguish between these alternatives in PB-treated cells. In particular, we observed PB-induced activation of caspase-9, caspase-3, and caspase-7 without any alteration in procaspase-8. These observations not only demonstrate for the first time that PB is triggering apoptosis through a caspase-9-initiated pathway, but also provide confirmation that the enhanced uptake of 7-AAD truly reflects apoptosis in the cells used in the present studies.

To date, there have been no reports of clinical trials in humans in the United States combining PA or PB with other therapeutic agents. The only notable exception is a recent case report by Warrell et al. (58) demonstrating that the combination of i.v. PB and oral all-trans-retinoic acid was effective in restoring remission in a patient with relapsed acute promyelocytic leukemia. Infusion of PB was also reported to produce an increase in histone acetylation in marrow and blood MNCs. This single case report provides evidence that PB can synergize with other agents to provide a clinically significant benefit in vivo.
Although our in vitro results suggest that combining PB with a conventional regimen containing cytarabine, topotecan, etoposide, or alkylating agents such as melphalan may likewise be efficacious, further preclinical and clinical studies are required to determine whether the finding of synergy in vitro will translate into a more effective clinical treatment. In choosing combinations for further evaluation, it might be important to keep in mind that the PA doses required to kill malignant B cells will be difficult to achieve in patients without substantial neurotoxicity. In contrast, cytotoxic PB levels appear to be more readily achievable.

Although PA and PB have been reported to affect cell proliferation by inhibiting protein prenylation (see “Introduction”), we have not observed an accumulation of unfarnesylated prelamin A or Pxf (two markers of inhibited protein farnesylation) in PB-treated human myeloma cell lines. These results

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Fig. 8 CI values for each of the nine common chemotherapy agents combined with PA on the 8226 (A) and MY5 (B) cell lines or PB on the 8226 (C) and MY5 (D) cell lines. All CI values were calculated with the assumption that the agents were mutually nonexclusive in their mechanisms of action. In the case of the 8226 cell line, the results of multiple CI plots are the mean ± SEM.
raise the possibility that other biochemical effects might be responsible for the cytotoxicity observed in these cell lines. Because the mechanism of action of PA and PB as single agents is not well understood, it is difficult at this time to explain the synergy found with other chemotherapeutic agents. Additional studies are clearly required to further investigate the mechanism by which PA and PB induce apoptosis in malignant B cells. In the meantime, the present results identify some PB-containing combinations that exhibit synergistic cytotoxic effects in malignant B cells and might be worthy of further evaluation in Phase I and II studies.

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