

## N-(4-hydroxyphenyl)retinamide increases ceramide and is cytotoxic to acute lymphoblastic leukemia cell lines, but not to non-malignant lymphocytes

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**The retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR), mediates p53-independent cytotoxicity and can increase reactive oxygen species and ceramide in solid tumor cell lines. We determined changes in ceramide and cytotoxicity upon treatment with 4-HPR (3–12  $\mu\text{M}$ ) in six human acute lymphoblastic leukemia (ALL) cell lines: T cell (MOLT-3, MOLT-4, CEM), pre-B-cell (NALM-6, SMS-SB), and null cell (NALL-1). Exposure to 4-HPR (12  $\mu\text{M}$ ) for 96 h caused 4.7 (MOLT-3), 3.5 (MOLT-4), 3.9 (CEM), 2.9 (NALM-6), 4.7 (SMS-SB), AND 4.5 (NALL-1) logs of cell kill. The average 4-HPR concentration that killed 99% of cells ( $\text{LC}_{99}$ ) for all six lines was 4.8  $\mu\text{M}$  (range: 1.5–8.9  $\mu\text{M}$ ). Treatment with 4-HPR (9  $\mu\text{M}$ ) for 24 h resulted in an  $8.9 \pm 1.0$ -fold (range: 4.9–15.7-fold) increase of ceramide. Ceramide increase was time- and dose-dependent and abrogated by inhibitors of *de novo* ceramide synthesis. Concurrent inhibition of ceramide glycosylation/acetylation by *d,l*-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol) (PPMP) further increased ceramide levels, and synergistically increased 4-HPR cytotoxicity in four of six ALL cell lines. 4-HPR was minimally cytotoxic to peripheral blood mononuclear cells and a lymphoblastoid cell line, and increased ceramide <2-fold. Thus, 4-HPR was cytotoxic and increased ceramide in ALL cell lines, but not in non-malignant lymphoid cell types.**

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**Keywords:** 4-HPR; ceramide; acute lymphoblastic leukemia; PPMP; glucosylceramide; 1-O-acylceramide

### Introduction

Despite significant advancement in the treatment of acute lymphoblastic leukemia (ALL), overall survival in adult ALL is at most 50% and progression rates in high risk pediatric ALL remain between 20 and 30%.<sup>1</sup> Therefore, new therapies with novel mechanisms of action are needed to treat refractory or recurrent leukemia.

N-(4-hydroxyphenyl)retinamide (4-HPR; fenretinide) is a synthetic retinoid that mediates p53-independent cytotoxicity in solid tumor cell lines.<sup>2–5</sup> Previous studies have shown that 4-HPR is cytotoxic to a variety of cancer cell lines, including neuroblastoma, colorectal, head and neck, breast, prostate, small cell lung cancer, ovarian, cervical, and some leukemia and lymphoma cell lines.<sup>3,5–25</sup> 4-HPR has been studied clinically as a chemopreventative agent in breast, bladder, and oral cavity cancers, but only recently have pediatric and adult clinical trials begun to study 4-HPR as a chemotherapeutic agent.<sup>26–31</sup> While 4-HPR shows promise as a chemotherapeutic agent because of its limited systemic toxicity,<sup>32</sup> its activity in many cancer types, including T and B cell leukemias, is not well established.

The mechanism of action of 4-HPR has not been fully elucidated. It may involve generation of reactive oxygen species

(ROS), and ceramide, a sphingoid-based lipid second messenger molecule.<sup>5,21,33–35</sup> In our previous work, 4-HPR was shown to increase both ROS and ceramide during the induction of p53-independent mixed apoptosis/necrosis in neuroblastoma cell lines.<sup>5</sup> Evidence of a central role for ceramide in cell-death pathways has emerged in recent years, as ceramide activates caspases in apoptosis, causes mitochondrial electron transport disruption and subsequent generation of ROS, and inactivates Bcl-2, a pro-life molecule.<sup>36–41</sup> These observations provide the rationale for increasing and modulating ceramide levels in cancer cells as a therapeutic modality. Ceramide can be increased as the result of activation of various sphingomyelinases, or as a result of increased *de novo* synthesis.<sup>42</sup> It is not clear if ceramides derived from these separate processes exist in distinct pools or if they have equivalent messenger functions. Ceramide is metabolized to nontoxic sphingolipids through a variety of pathways (Figure 1). Two pathways of ceramide detoxification involve glycosylation to glucosylceramide via glucosylceramide synthase (GCS), and 1-O-acylation, via 1-O-acylceramide synthase (1-O-ACS).<sup>43,44</sup> PPMP (*d,l*-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol)) is an inhibitor of both GCS and 1-O-ACS enzymatic activities.<sup>45,46</sup> We have previously reported that PPMP can increase 4-HPR-induced ceramide levels and synergize cytotoxicity in certain solid tumor cell lines.<sup>34</sup>

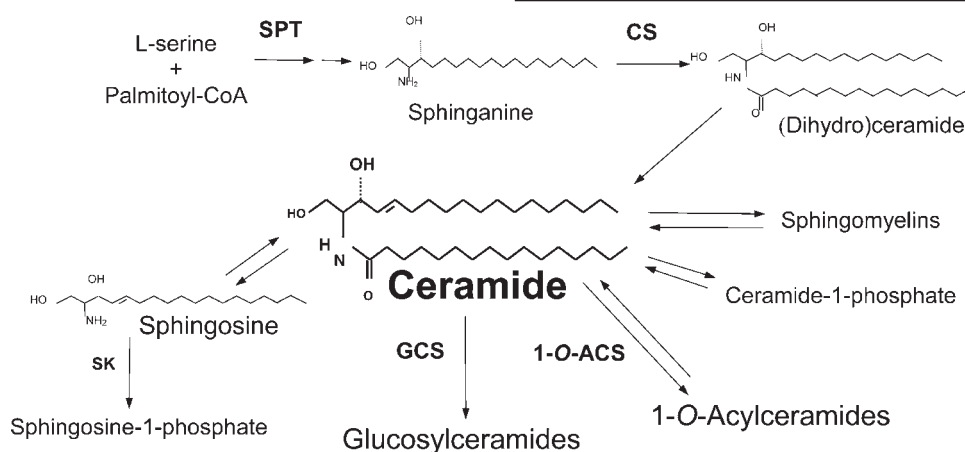
The current study had three specific aims: (1) to broadly determine the *in vitro* cytotoxicity of 4-HPR against ALL cell lines compared to non-malignant lymphocytes; (2) to determine if 4-HPR increased ceramide by *de novo* production in ALL cell lines; and (3) to determine whether a modulator of ceramide metabolism, PPMP, could increase 4-HPR-induced ceramide levels or cytotoxicity in ALL cell lines.

### Materials and methods

#### Materials

4-HPR was kindly provided by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI), the National Institutes of Health (NIH) (Bethesda, MD, USA).

*d,l*-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol) (PPMP) was purchased from Matreya (Pleasant Gap, PA, USA). Fluorescein diacetate (FDA) was from the Eastman Kodak Company (Rochester, NY, USA), and was dissolved in dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$ . Thin-layer chromatography (TLC)-grade organic solvents, eosin Y, L-cycloserine, and fumonisins B<sub>1</sub> were from Sigma Chemical Co (St Louis, MO, USA). Ecolume scintillation cocktail and Cap-pel lymphocyte separation medium (LSM) were from ICN Biomedicals (Costa Mesa, CA, USA). [9,10-<sup>3</sup>H(N)]-Palmitic acid (50 Ci/mmol) was from Dupont NEN Research Products (Boston, MA, USA). RPMI-1640 medium, fetal bovine serum (FBS), and L-glutamine were from Gemini BioProducts



**Figure 1** Abbreviated schematic of the pathways of *de novo* ceramide synthesis and metabolism. The first, and rate-limiting step, is the condensation of L-serine and palmitoyl-CoA to form ketosphinganine, which is reduced to sphinganine. Sphinganine is N-acylated using various chain lengths of fatty acyl-CoA to dihydroceramide, which is desaturated to ceramide. Ceramide is metabolized or catabolized to various products including sphingosine, glucosylceramides, and 1-O-acylceramides. SPT, serine palmitoyltransferase; CS, ceramide synthase; SK, sphingosine kinase; GCS, glucosylceramide synthase; 1-O-ACS, 1-O-acylceramide synthase. Fumonisin B<sub>1</sub> is an inhibitor of CS. *d,l*-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol) (PPMP) inhibits both GCS and 1-O-ACS.

(Calabasas, CA, USA). Glucosylceramide (purified from Gaucher's spleen) was from Matreya. Ceramide and other lipid standards were from Avanti Polar Lipids (Alabaster, AL, USA). Uniplate Silica Gel G 250 micron TLC plates were from Analtech (Newark, DE, USA). Falcon Brand plasticware was from Becton Dickinson and Co (Franklin Lakes, NJ, USA). 4-HPR and PPMP were dissolved in ethanol and stored at  $-20^{\circ}\text{C}$ .

### Cell culture

The human T-lymphoblast (MOLT-3, MOLT-4, CEM), pre-B-lymphoblast (NALM-6, SMS-SB), and null cell (NALL-1) acute lymphoblastic leukemia (ALL) cell lines were studied.<sup>47–52</sup> MOLT-3 and MOLT-4 were established from a patient in relapse after multidrug chemotherapy.<sup>53</sup> The MOLT-4 cell line possesses a p53 gene mutation.<sup>54</sup> The immortalized, B-lymphoblastoid cell line (SMS-SAL) was established by exposing peripheral blood mononuclear cells from a neuroblastoma patient to the Epstein-Barr virus (EBV).<sup>55</sup> Normal peripheral blood mononuclear cells (PBMC) were obtained by carbonyl iron treatment (to deplete monocytes) and Cappel lymphocyte separation medium (LSM) separation of peripheral blood cells harvested for peripheral blood stem cell collection. Cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS at  $37^{\circ}\text{C}$  in a humidified incubator containing 95% room air +5%  $\text{CO}_2$  atmosphere.

### Cytotoxicity assay

Cytotoxicity in cell lines was determined using a digital imaging fluorescence-based microscopy assay (DIMSCAN).<sup>34,56,57</sup> DIMSCAN quantifies viable cells which selectively accumulate fluorescein diacetate and is capable of measuring cytotoxicity over a 4–5 log dynamic range by measuring total cellular fluorescence per well (which is proportional to viable, clonogenic cells) after eliminating background fluorescence using digital thresholding and eosin Y quenching. Cell lines were seeded into 96-well plates with 50000 cells in  $100\ \mu\text{l}$  of complete medium per well. Drugs were added in  $50\ \mu\text{l}$  volumes of complete medium per well to achieve various final concen-

trations in replicates of 12 wells per concentration. Control wells received ethanol (final concentration = 0.12–0.20%) in complete medium equivalent to the maximum final ethanol concentration of drug-treated wells. To measure cytotoxicity, plates were assayed at 4 days after initiating drug exposure to allow for maximum cell death and outgrowth of surviving cells. FDA (stock solution of 1 mg/ml in DMSO) was added in  $50\ \mu\text{l}$  of complete medium per well, to a final concentration of  $10\ \mu\text{g}/\text{ml}$ . The plates were incubated for an additional 15–45 min at  $37^{\circ}\text{C}$  and then  $30\ \mu\text{l}$  of eosin Y (0.5% in normal saline) was added per well. Total fluorescence of each well was then measured using DIMSCAN. To ensure that 4-HPR was not acting to block or inhibit proliferation in the absence of cell death, in addition to microscopic visual inspection of all DIMSCAN assay plates for the presence of non-fluorescing (dead) cell bodies, an assessment of 4-HPR-induced apoptosis in CEM and MOLT-4 cells was made by flow cytometry using propidium iodide to identify cells with a sub  $G_0/G_1$  DNA content and analyzed on a Coulter Epics ELITE flow cytometer with a 488 nm Argon laser and a 610/20 nm band pass filter.<sup>58</sup>

Results were analyzed and expressed as survival fractions by comparing the quantified fluorescence of surviving cells to that of control cells using Excel software (Microsoft, Seattle, WA, USA), and graphed using SigmaPlot 5.0 (Jandell Scientific, San Rafael, CA, USA). Limits of detection were determined by the number of cells (50000) present in control wells at the time of drug treatment, providing a detection limit for cytotoxicity of 4.7 logs. The stability of 4-HPR and PPMP for 4 days in the DIMSCAN cytotoxicity assay system is not known.

### Lipid analysis

Methods were modified from Lavie *et al.*<sup>59,60</sup> Cells were seeded into six-well plates at  $1 \times 10^6$  cells/well. At time = 0,  $^3\text{H}$ -palmitic acid ( $1\ \mu\text{Ci}/\text{ml}$  medium) and 4-HPR and/or PPMP (drug-treated) or ethanol (control) were added to wells in complete medium to achieve various final drug concentrations in a final volume of 2 ml per well. For some assays, an inhibitor of ceramide synthase (dihydroceramide synthase; sphinganine N-acyltransferase), fumonisin B<sub>1</sub>, was added to  $50\ \mu\text{M}$  at 16 h

prior to time = 0, or a serine palmitoyltransferase inhibitor, L-cycloserine, was added to 300  $\mu\text{M}$  at time = 0,<sup>61,62</sup> Cells were harvested at +24 h (for dose–response) or at the appropriate time (for time–response) and lipids were extracted. For lipid extraction, cells were transferred into glass vials and collected by centrifugation with subsequent aspiration of the medium. The effect of 4-HPR and PPMP on  $^3\text{H}$ -palmitic acid uptake was determined by assaying and comparing the remaining radiolabel in 100  $\mu\text{l}$  of medium from each well. Cells were washed with ice-cold phosphate-buffered saline (PBS), centrifuged, and the buffer aspirated. Then, to each sample vial were added 1.0 ml methanol/2% acetic acid, 1.0 ml distilled water and 1.0 ml chloroform. The vial was vigorously vortexed for 30 s, and the phases were separated by centrifugation. The lower (organic) phase was then transferred to a new vial and volatiles were evaporated under a nitrogen stream. Lipids were stored at  $-20^\circ\text{C}$ .

For analysis, 100  $\mu\text{l}$  of chloroform/methanol (2:1) was added to each vial. A 10  $\mu\text{l}$  aliquot was assayed for tritium and, from this, the tritium in the total lipid sample was calculated. Commercial lipid standards ( $\sim 15 \mu\text{g}/\text{lane}$ ) were co-spotted on to TLC plates with the cellular  $^3\text{H}$ -labeled lipids (10  $\mu\text{l}$  aliquots). Ceramide was resolved in a solvent system containing chloroform/acetic acid (90:10, v/v). Glucosylceramide was resolved using chloroform/methanol/ammonium hydroxide (70:20:4, v/v). The lipid standards were visualized by iodine vapor and the co-migrating tritiated lipid sample was assayed by scraping the TLC plate in the area of interest, adding 0.5 ml water and 4.5 ml Ecolume, and measuring tritium counts per minute (c.p.m.) by liquid scintillation counting. This value was then corrected for the amount of the original sample previously removed for other assays. Lipid changes are expressed as the mean fold-increase or decrease in three drug-treated samples as compared to that of three matched controls. The ceramide isolated by the above assay method has been verified to contain only ceramide species by mass spectroscopy.<sup>34</sup> Drugs did not affect  $^3\text{H}$ -palmitic acid uptake.

### Statistical analysis

Cytotoxicity and lipid data are presented as means  $\pm$  standard deviation. The statistical significance of differences in means was evaluated by the unpaired, two-sided Student's *t*-test using Microsoft Excel 97 software (Redmond, WA, USA). All *P* values are two-sided.

Drug-induced cytotoxic synergy was analyzed using 'CalcuSyn' software from Biosoft (Cambridge, UK) and expressed as the Combination Index (CI) at a given drug concentration. The CI is a method for quantifying drug cytotoxic synergism, based upon the mass-action law approach and the median-effect principle derived from enzyme kinetic models as developed by Chou,<sup>63</sup> which has been widely used to evaluate interactions of antineoplastic drugs. Using the CI, synergism is defined as a more than expected additive effect ( $\text{CI} < 1$ ), antagonism as a less than expected additive effect ( $\text{CI} > 1$ ), and  $\text{CI} = 1$  indicates an additive effect. The CI value can be calculated at different 'effect levels' or 'fraction effected' levels (eg  $\text{LC}_{50}$  or  $\text{LC}_{99}$ ) and may vary depending on the fractional effect level at which it is calculated.

All results were experimentally reproducible.

## Results

### 4-HPR was cytotoxic to six ALL cell lines

Cytotoxicity of 4-HPR (3–12  $\mu\text{M}$ ) to human T cell (MOLT-3, MOLT-4, CEM), pre-B cell (NALM-6, SMS-SB), and null cell (NALL-1) ALL cell lines was determined by DIMSCAN assay (Figure 2). The average 4-HPR concentration that killed 99% of cells ( $\text{LC}_{99}$ ) was 4.8  $\mu\text{M}$  (range: 1.5–8.9  $\mu\text{M}$ ). Exposure to 12  $\mu\text{M}$  for 96 h caused 4.7 (MOLT-3), 3.5 (MOLT-4), 3.9 (CEM), 2.9 (NALM-6), 4.7 (SMS-SB) and 4.5 (NALL-1) logs of cell kill. Visual microscopic inspection of DIMSCAN assay plates to rule out cytostatic effects of 4-HPR revealed an increase of non-fluorescing (dead) cell bodies proportionate to drug dose. Additionally, apoptosis, as reflected by subgenomic ( $\text{G}_0/\text{G}_1$ ) DNA content, was assessed in CEM and MOLT-4 cells by flow cytometry after a 24 h exposure to 4-HPR (10  $\mu\text{M}$ ). In MOLT-4 cells, 4-HPR exposure increased the fraction of cells with a sub- $\text{G}_0/\text{G}_1$  DNA content from 4% to 80%, and in CEM cells, 4-HPR exposure increased the fraction of cells with a sub- $\text{G}_0/\text{G}_1$  DNA content from 8% to 47%, at 24 h (data not shown). Thus, 4-HPR was highly cytotoxic for all six ALL cell lines at medium drug concentrations *in vitro* that approximate plasma levels currently achievable clinically using oral fenretinide.<sup>64,65</sup>

### 4-HPR increased ceramide in a time- and dose-dependent manner via *de novo* synthesis

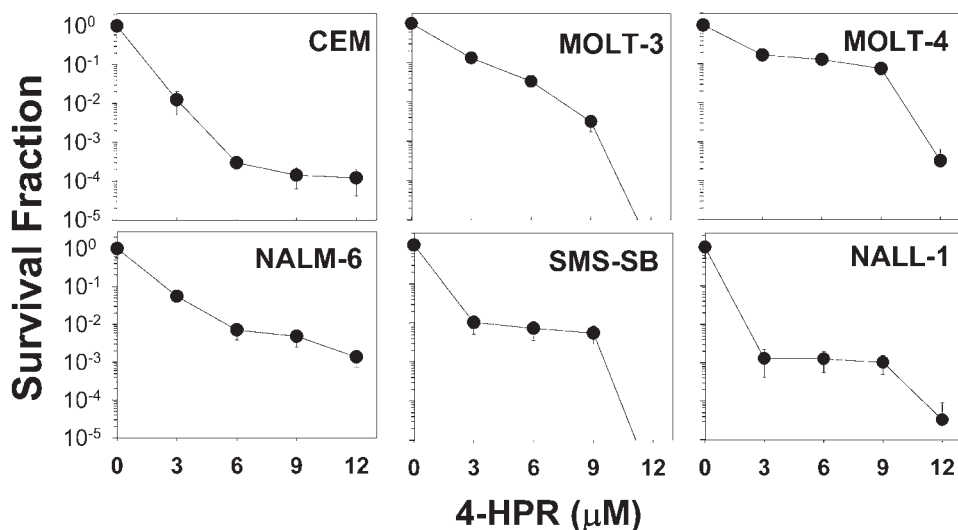
4-HPR treatment caused a dose-dependent increase of ceramide levels in all six ALL cell lines (Figure 3). Exposure to 4-HPR (9  $\mu\text{M}$ ) for 24 h resulted in an average  $8.9 \pm 1.0$ -fold (range: 4.9–15.7-fold) increase in ceramide relative to untreated controls for the six ALL lines ( $P \leq 0.01$ ) (Figure 3). The time-dependence of ceramide increase upon 4-HPR treatment was demonstrated in the CEM cell line (Figure 4). Ceramide increase was time-dependent at all 4-HPR dose levels ( $P < 0.05$ ).

Further, we demonstrated that the ceramide induced by 4-HPR treatment resulted from *de novo* synthesis, rather than sphingomyelinase activation, by employing inhibitors of serine palmitoyltransferase (SPT) and ceramide synthase (CS), key enzymes involved in *de novo* ceramide synthesis (see Figure 1).<sup>66</sup> The effect of 4-HPR treatment on ceramide increase in the presence or absence of L-cycloserine (an inhibitor of SPT), and fumonisin B<sub>1</sub> (a fungal derivative which blocks CS), is shown in MOLT-4 cells (Figure 5). Both inhibitors abrogated 4-HPR-induced ceramide increase in MOLT-4 cells, demonstrating ceramide was increased by *de novo* synthesis. These data corroborate similar findings in neuroblastoma cell lines and in HL-60 myeloid leukemia cells.<sup>34,35</sup>

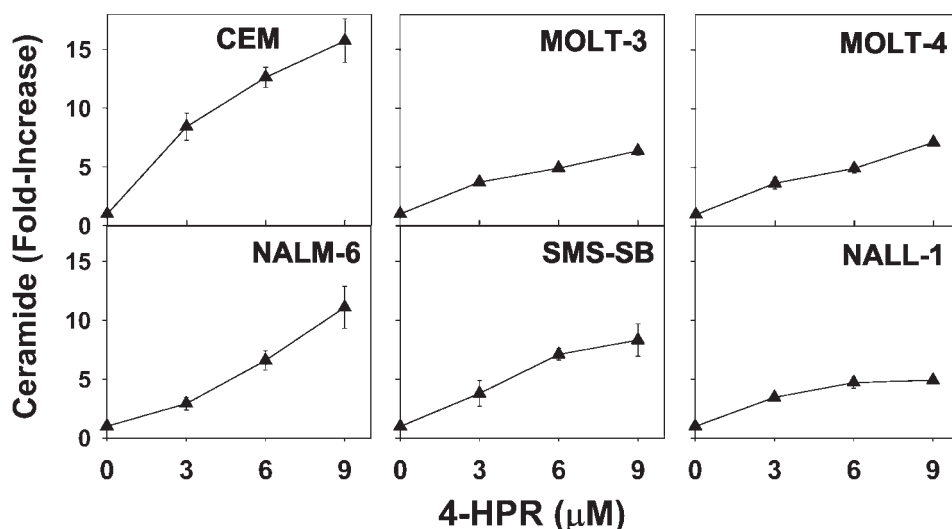
To correlate 4-HPR-induced ceramide and 4-HPR-induced cytotoxicity in ALL cells, we attempted to determine whether blocking ceramide production with these inhibitors would decrease the cytotoxicity of 4-HPR. Unfortunately, L-cycloserine and fumonisin B<sub>1</sub> were themselves sufficiently toxic to the MOLT-4 cell line as to render the results of 96 h cytotoxicity assays inconclusive (data not shown).

### PPMP increased 4-HPR-induced ceramide levels and synergized cytotoxicity

We sought to determine if decreasing the metabolic shunting of ceramide into nontoxic glucosylceramide and/or 1-*O*-acyl-



**Figure 2** Cytotoxicity of 4-HPR in ALL cell lines. Survival fraction was measured using a digital imaging fluorescence-based microscopy assay (DIMSCAN) with  $\sim 5$  log sensitivity. Assayed at +96 h. 4-HPR caused multi-log cytotoxicity in all six ALL cell lines.

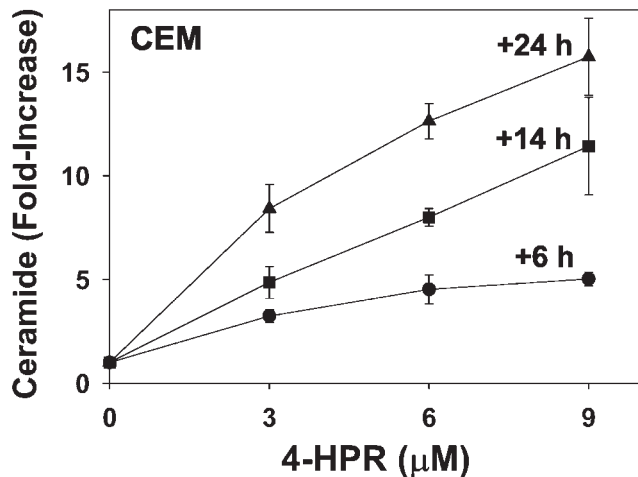


**Figure 3** Effect of 4-HPR on ceramide levels in ALL cell lines. Ceramide levels were measured by labeling with  $^3\text{H}$ -palmitic acid and thin-layer chromatography. Assays were performed at +24 h. 4-HPR significantly increased ceramide in a dose-dependent manner in all six ALL cell lines, at all doses except as noted: (CEM: 0–6  $\mu\text{M}$ ,  $P < 0.02$ ; 6–9  $\mu\text{M}$ ,  $P = 0.08$ ; MOLT-3: 0–9  $\mu\text{M}$ ,  $P < 0.02$ ; MOLT-4: 0–9  $\mu\text{M}$ ,  $P < 0.02$ ; NALM-6: 0–6  $\mu\text{M}$ ,  $P < 0.02$ ; 6–9  $\mu\text{M}$ ,  $P = 0.03$ ; SMS-SB: 0–3  $\mu\text{M}$ ,  $P = 0.05$ ; 3–6  $\mu\text{M}$ ,  $P = 0.02$ ; 6–9  $\mu\text{M}$ ,  $P = 0.25$ ; NALL-1: 0–3  $\mu\text{M}$ ,  $P = 0.005$ ; 3–6  $\mu\text{M}$ ,  $P = 0.03$ ; 6–9  $\mu\text{M}$ ,  $P = 0.58$ ).

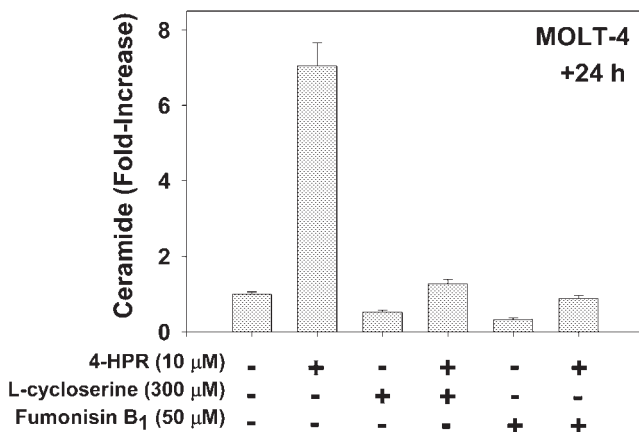
ceramide could increase 4-HPR cytotoxicity in ALL cell lines. PPMP, an inhibitor of both GCS and 1-*O*-ACS enzymatic activity (see Figure 1), was added simultaneously with 4-HPR, and the effect on ceramide levels, glucosylceramide levels, and cytotoxicity was assayed. Escalating doses of PPMP (3–12  $\mu\text{M}$ ) at a 1:1 molar ratio with 4-HPR (3–12  $\mu\text{M}$ ) synergistically increased cytotoxic activity in four of six ALL cell lines ( $\text{CI} < 1.0$ ), and was additive in two cell lines (Figure 6). Moreover, in three cell lines examined (NALM-6, MOLT-3, MOLT-4), combining PPMP with 4-HPR increased ceramide levels above those of 4-HPR alone (Figure 7). In NALM-6, the combination of PPMP (9  $\mu\text{M}$ ) and 4-HPR (9  $\mu\text{M}$ ) (4-HPR+PPMP) induced a  $14.5 \pm 0.5$ -fold induction of ceramide relative to controls, compared to  $9.1 \pm 1.1$ -fold induction with 4-HPR alone ( $P = 0.005$ ). A similar ceramide increase which tended toward statistical significance ( $P = 0.07$ ) was observed in

MOLT-3 cells ( $9.5 \pm 2.2$ -fold increase with 4-HPR+PPMP vs  $5.6 \pm 1.0$ -fold increase with 4-HPR (9  $\mu\text{M}$ ) alone). In MOLT-4 cells, a statistically significant ( $P = 0.04$ ), but less dramatic, increase in ceramide levels resulted from combining 4-HPR+PPMP ( $10.4 \pm 0.4$ -fold compared to  $8.8 \pm 0.7$ -fold with 4-HPR (9  $\mu\text{M}$ ) alone). Concordantly, whereas 4-HPR (9  $\mu\text{M}$ ) increased glucosylceramide levels by an average of  $1.9 \pm 0.2$ -fold over untreated controls in these three cell lines ( $P \leq 0.03$ ), the addition of PPMP (9  $\mu\text{M}$ ) abrogated the glucosylceramide increase in all three cell lines ( $P \leq 0.02$ ) (Figure 7).

Thus, in NALM-6 and MOLT-3 cells, the increase in ceramide resulting from combining PPMP with 4-HPR paralleled the observed increases in cytotoxicity (Figure 6); whereas in MOLT-4, the ceramide increase mediated by PPMP and 4-HPR was modest compared to the increase in cytotoxicity elicited by combining PPMP with 4-HPR.



**Figure 4** Time-dependence of 4-HPR-induced ceramide increase in the CEM cell line. Ceramide levels were measured at +6 h, +14 h and +24 h after addition of 4-HPR. Ceramide increase was time-dependent at all 4-HPR dose levels tested (3 µM: 6–14 h,  $P = 0.04$ ; 14–24 h,  $P = 0.02$ ; 6 µM: 6–24 h,  $P < 0.02$ ; 9 µM: 6–14 h,  $P = 0.04$ ; 14–24 h,  $P = 0.07$ ).



**Figure 5** Effect of inhibitors of *de novo* ceramide synthesis on ceramide increase by 4-HPR in the MOLT-4 cell line. L-cycloserine (300 µM) and fumonisins B<sub>1</sub> (50 µM) are inhibitors of serine palmitoyltransferase and ceramide synthase, respectively. L-cycloserine significantly blunted the 4-HPR-induced ceramide increase ( $P = 0.004$ ) compared to 4-HPR alone, as did fumonisins B<sub>1</sub> ( $P = 0.003$ ). Assayed at +24 h. Labeled ceramide level of drug-free controls is defined as '1-fold'.

#### Effects of 4-HPR on non-malignant lymphoid cell lines

These data demonstrate that 4-HPR caused increases of ceramide by *de novo* synthesis and suggest that cytotoxicity in ALL cell lines may correlate with ceramide increase. To address whether 4-HPR-induced cytotoxicity and ceramide increases are malignancy-specific events, we utilized monocyte-depleted normal peripheral blood mononuclear cells (PBMC) and an immortalized Epstein–Barr virus-transformed B cell lymphoblastoid line (SMS-SAL) to serve as staged, non-malignant counterparts to ALL cell lines. In PBMC, 4-HPR was not cytotoxic and minimally increased ceramide (Figure 8). Cell survival upon exposure to 4-HPR at three concentrations (3 µM, 6 µM, 12 µM) was not statistically different to survival of untreated controls ( $P = 0.49$ ;  $P = 0.25$ ;  $P = 0.82$ ,

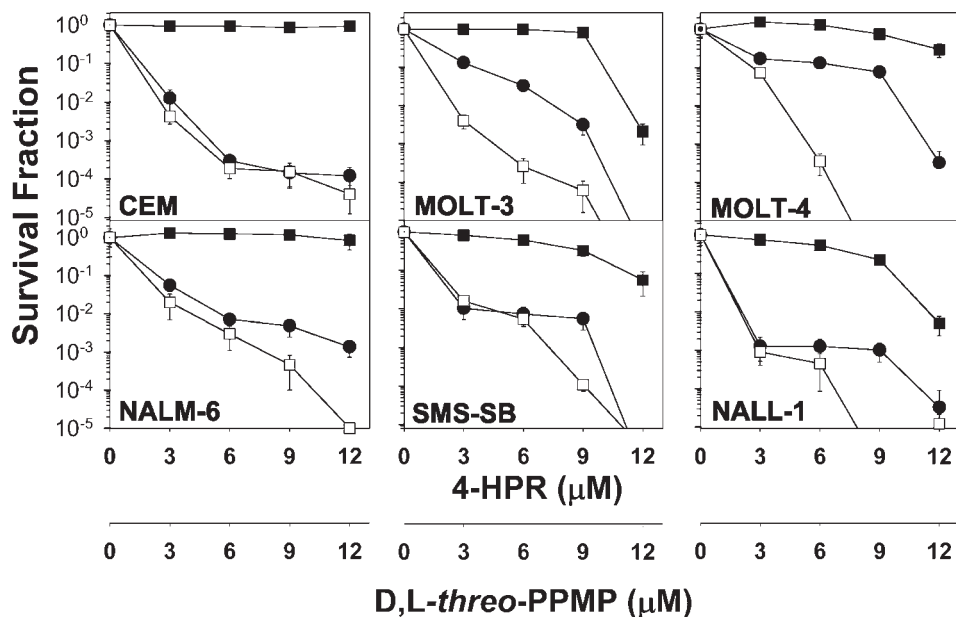
respectively) after 96 h (Figure 8a). At 9 µM 4-HPR, cell survival was minimally increased compared to controls ( $P = 0.001$ ). Concurrently, exposure of PBMC to 4-HPR (9 µM) increased ceramide only ~80% at 24 h compared to controls (Figure 8b), significantly lower than the average  $8.9 \pm 1.0$ -fold increase observed in ALL cells at 4-HPR (9 µM) ( $P \leq 0.02$ ) (Figure 3).

In the SMS-SAL lymphoblastoid cell line, 4-HPR, alone or in combination with PPMP, was minimally cytotoxic and minimally increased ceramide (Figure 8). In SMS-SAL, cell survival upon exposure to 4-HPR at lower concentrations (3–9 µM) was not statistically different to survival of untreated controls (3 µM:  $P = 0.12$ ; 6 µM:  $P = 0.72$ ; 9 µM:  $P = 0.80$ ) at 96 h (Figure 8c), while 12 µM 4-HPR caused minimal cytotoxicity (<0.15 logs cell kill). Combining PPMP (3–12 µM) with 4-HPR (3–12 µM) at a 1:1 molar ratio slightly increased cytotoxicity at higher doses (Figure 8c), although cytotoxicity at the highest drug doses was still low (<0.4 logs cell kill) compared to the logs of cell kill observed in ALL cells. Ceramide was increased <50% by 4-HPR (9 µM) at 24 h in SMS-SAL cells (Figure 8d). This fold-increase in ceramide was significantly lower than the 4.9–15.7-fold increases observed in identically treated ALL cells ( $P \leq 0.01$ ) (Figure 3). Exposing SMS-SAL cells to 4-HPR+PPMP increased ceramide more than 4-HPR (9 µM) alone ( $P = 0.03$ ) (Figure 8d), but the ~100% increase compared to controls was still significantly lower than the increase observed in ALL cells treated with 4-HPR (9 µM) alone ( $P \leq 0.02$ ) (Figure 3). Glucosylceramide levels upon treatment with 4-HPR (9 µM) were not different from controls ( $P = 0.41$ ) (Figure 8d). PPMP decreased glucosylceramide levels ( $P < 0.02$ ) as expected.

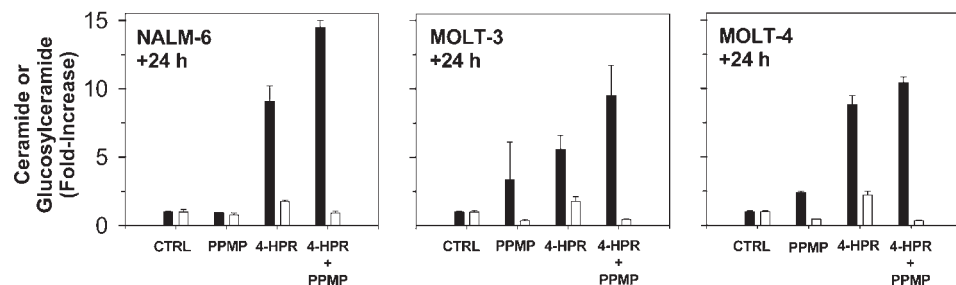
#### Discussion

We have demonstrated that 4-HPR was cytotoxic to, and caused a time- and dose-dependent increase in ceramide by *de novo* synthesis, in six ALL cell lines, but not in non-malignant lymphoid cell counterparts. There was variation in the absolute cytotoxicity associated with a given level of ceramide increase, possibly reflecting intrinsic differences in sensitivity to *de novo* ceramide in the cell lines. However, the correlation between ceramide increase and cytotoxicity was suggestive that ceramide, a molecule with ascribed pro-death functions, may be responsible, at least in part, for 4-HPR-induced cell death in ALL cell lines. To further investigate this correlation, we employed a modulator of ceramide metabolism, PPMP, which inhibits the glycosylation and acylation of ceramide into non-toxic metabolites. Overall, PPMP synergistically increased 4-HPR cytotoxicity in five of six ALL cell lines. In the single cell line (CEM) in which PPMP added no appreciable cytotoxic benefit, 4-HPR itself was highly cytotoxic, even at moderate concentrations (nearly 4 logs of cytotoxicity at 6 µM 4-HPR), and synthesized high levels of ceramide in response to 4-HPR as a single agent. Thus, the cytotoxic effects of further increasing ceramide with PPMP may have been masked.

We also directly measured the effect of combining PPMP and 4-HPR on ceramide levels in ALL cell lines. 4-HPR+PPMP increased ceramide levels more than 4-HPR alone. In the NALM-6 and MOLT-3 cell lines, the increase in ceramide by 4-HPR+PPMP paralleled the synergistic increase in cytotoxicity of the combination. In MOLT-4 cells, the increase in ceramide upon addition of 4-HPR+PPMP was statistically significant, but modest compared to the effect on cytotoxicity. It is



**Figure 6** Effect of PPMP on 4-HPR-induced cytotoxicity. PPMP, an inhibitor of glucosylceramide synthase (GCS) and 1-*O*-acylceramide synthase (1-*O*-ACS), synergized 4-HPR cytotoxicity in five of six ALL cell lines. Assayed at +96 h. (●), 4-HPR; (■), PPMP; (□), 4-HPR + PPMP (1:1 molar ratio). Measure of cytotoxic synergy, Combination Index (CI): synergy, CI < 1; additive, CI = 1; antagonism, CI > 1. CEM, CI = 1; MOLT-3, CI < 1; MOLT-4, CI < 1; NALM-6, CI < 1; SMS-SB, 3 μM (CI > 1), 6 μM (CI > 1), 9 μM (CI = 1); NALL-1, 3 μM (CI > 1), 6 μM (CI = 1), 9 μM (CI < 1).



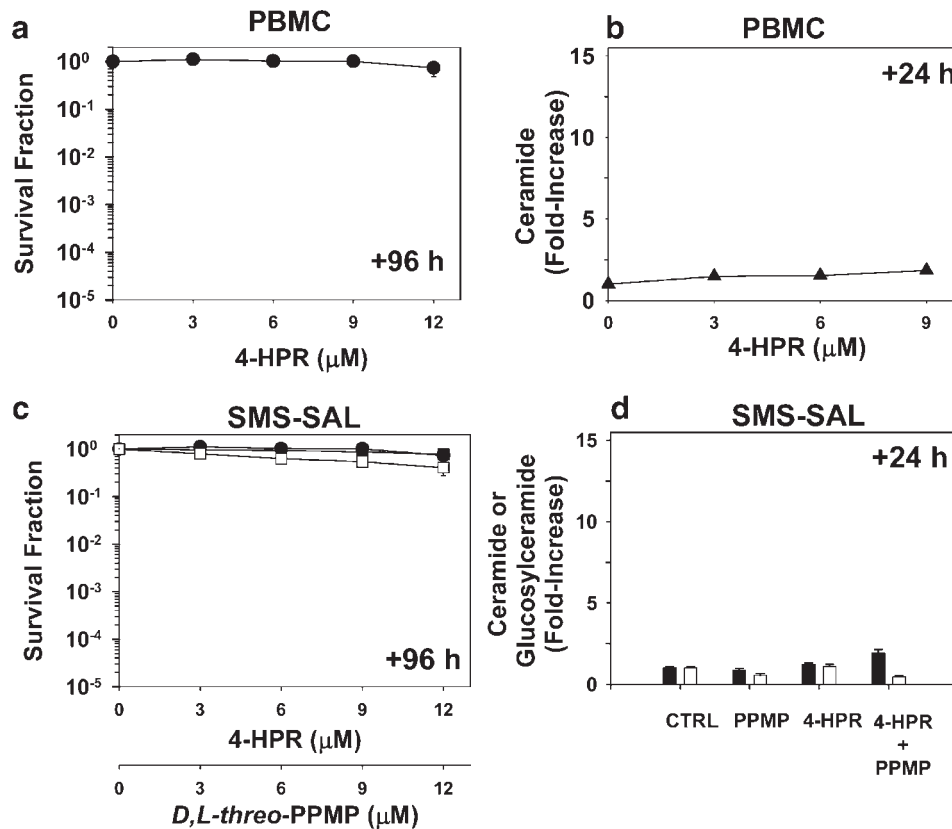
**Figure 7** Effect of PPMP on 4-HPR-induced ceramide and glucosylceramide increases. PPMP (9 μM) + 4-HPR (9 μM) increased ceramide levels (closed bars) above those of 4-HPR (9 μM) alone in NALM-6 cells ( $P = 0.005$ ), MOLT-3 cells ( $P = 0.07$ ), and MOLT-4 cells ( $P = 0.04$ ). 4-HPR (9 μM) also increased glucosylceramide levels (open bars) by approximately 2-fold over controls in all three cell lines ( $P < 0.02$ ). PPMP (9 μM) abrogated 4-HPR-induced glucosylceramide increase in all three cell lines ( $P < 0.02$ ). CTRL, vehicle-treated control. Labeled ceramide and glucosylceramide levels of drug-free controls are defined as '1-fold'.

possible that in MOLT-4 cells, although PPMP does effectively block the shunting of ceramide into glucosylceramide, the corresponding increase in total ceramide is modest because the additional ceramide is shunted through alternative metabolic pathways. Some ceramide metabolites, such as sphingosine, which might accumulate upon inhibition of GCS and 1-*O*-ACS (Figure 1), can be toxic to cells and may also synergistically augment ceramide-mediated cytotoxicity.<sup>67-71</sup> Alternatively, PPMP may have synergistic cytotoxicity in MOLT-4 cells that is unrelated to cytotoxic sphingolipids. However, taken together, these data suggest a correlation of increased ceramide levels with the synergistic cytotoxic effects of PPMP and 4-HPR in the ALL cell lines tested.

Because the inhibitors of *de novo* ceramide synthesis employed in these studies themselves proved cytotoxic in the ALL cell lines examined, alternative strategies are necessary to definitively demonstrate the dependence of 4-HPR and 4-HPR plus PPMP cytotoxicity on *de novo* ceramide increases. One approach is to overexpress GCS, thereby shunting *de*

*novo* ceramide into nontoxic glucosylceramide, in a 4-HPR-sensitive, ceramide-increasing ALL cell line, and observe the effect on cytotoxicity.<sup>72</sup> CEM cells may be an ideal candidate in which to do this. Overexpression of GCS can reduce ceramide levels produced by *de novo* synthesis, but not ceramide resulting from neutral sphingomyelinase activation, in GCS-transduced Jurkat cells.<sup>73</sup> Thus, overexpression of GCS in CEM cells should blunt 4-HPR-induced increase of *de novo* ceramide and directly demonstrate its role in the cytotoxicity of 4-HPR, and of 4-HPR combined with modulators of ceramide metabolism.<sup>34</sup>

Finally, we have also demonstrated that the ceramide-inducing and cytotoxic effects of 4-HPR appear to be malignancy-specific by comparing ALL cell lines to normal peripheral blood mononuclear cells (PBMC), and an EBV-transformed, immortalized, but non-malignant, B lymphoblastoid cell line counterpart. In these cell types, 4-HPR no more than doubled ceramide levels, and was minimally cytotoxic at the concentrations tested, consistent with the lack of hematopo-



**Figure 8** Effect of 4-HPR and PPMP on cytotoxicity and ceramide levels in non-malignant cells. (a) 4-HPR cytotoxicity to normal peripheral blood mononuclear cells (PBMC). 4-HPR was not cytotoxic and minimally increased ceramide in PBMC. Cell survival upon exposure to 4-HPR at three different concentrations (3  $\mu\text{M}$ , 6  $\mu\text{M}$ , 12  $\mu\text{M}$ ) was not statistically different to survival of untreated controls ( $P = 0.49$ ;  $P = 0.25$ ;  $P = 0.82$ , respectively). At 4-HPR (9  $\mu\text{M}$ ), cell survival was minimally increased compared to controls ( $P < 0.02$ ). (b) 4-HPR effect on ceramide in PBMC. 4-HPR (9  $\mu\text{M}$ ) increased ceramide by ~80% in PBMC compared to controls ( $P = 0.008$ ), but this increase was significantly lower than in ALL cells at 4-HPR (9  $\mu\text{M}$ ) ( $P \leq 0.02$ ). (c) Effect of 4-HPR and PPMP on cytotoxicity and ceramide levels in non-malignant, EBV-immortalized, lymphoblastoid cell line, SMS-SAL. 4-HPR, and 4-HPR plus PPMP, were minimally cytotoxic in SMS-SAL. Cell survival upon exposure to 4-HPR at lower concentrations (3  $\mu\text{M}$ , 6  $\mu\text{M}$ , 9  $\mu\text{M}$ ) was not statistically different to survival of untreated controls ( $P = 0.12$ ;  $P = 0.72$ ;  $P = 0.80$ , respectively). Combining 4-HPR and PPMP at a 1:1 molar ratio slightly increased cytotoxicity at higher doses, although cytotoxicity (<0.4 logs cell kill) was minimal compared to effects in ALL cells. CTRL, vehicle-treated control; (●), 4-HPR; (■), PPMP; (□), 4-HPR + PPMP. (d) Effect of 4-HPR and 4-HPR + PPMP on ceramide and glucosylceramide levels in SMS-SAL cells. 4-HPR (9  $\mu\text{M}$ ) increased ceramide <50% in SMS-SAL cells compared to controls ( $P = 0.03$ ). This increase was significantly lower than in ALL cells at this dose ( $P \leq 0.02$ ). Glucosylceramide levels upon treatment with 4-HPR (9  $\mu\text{M}$ ) were not different significantly lower than in similarly treated ALL cells ( $P \leq 0.02$ ). Combining PPMP (9  $\mu\text{M}$ ) + 4-HPR (9  $\mu\text{M}$ ) increased ceramide further compared to 4-HPR (9  $\mu\text{M}$ ) alone ( $P = 0.03$ ), yet the ~100% increase compared to untreated controls remained significantly lower than in similarly treated ALL cells ( $P \leq 0.02$ ). Labeled ceramide and glucosylceramide levels of drug-free controls are defined as '1-fold'.

ietic toxicity reported in animal studies and patients receiving oral 4-HPR.<sup>32,64</sup> These data further suggest the possibility that altered regulation of the *de novo* ceramide synthetic pathway exists in leukemia (malignant) cell lines, which allows 4-HPR to stimulate the enzymatic activity and/or enzyme synthesis of the *de novo* ceramide pathway, especially of SPT, the rate-limiting enzyme.<sup>62</sup> In support of this, we have recently demonstrated that 4-HPR directly stimulates the enzymatic activity of SPT and CS in a neuroblastoma cell line.<sup>74</sup> Alternatively, non-malignant lymphoid cells may be more efficient than ALL cells at shunting ceramide into nontoxic metabolites other than glucosylceramide and 1-*O*-acylceramide. We speculate that comparing ALL cell lines to non-malignant lymphoid cells will be a useful model for the elucidation of malignancy-specific differences in the regulation of *de novo* ceramide synthesis.

Thus, should they be tolerated clinically, combinations of 4-HPR and modulators of ceramide metabolism, such as GCS and 1-*O*-ACS inhibitors, among others, may form the basis for

a novel chemotherapy effective in acute lymphoblastic leukemias.

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