Synergistic cytotoxicity between tamoxifen and the plant toxin persin in human breast cancer cells is dependent on Bim expression and mediated by modulation of ceramide metabolism

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Abstract

Phytochemicals have provided an abundant source of novel therapeutics for the treatment of human cancers. We have previously described a novel plant toxin, persin, derived from avocado leaves, which has unique *in vivo* actions in the mammary epithelium and Bim-dependent, cytotoxic effects in human breast cancer cells *in vitro*. Compounds structurally similar to persin, such as the polyunsaturated fatty acid, conjugated linoleic acid, can attenuate steroid hormone receptor signaling and modulate the response of breast cancer cells to antiestrogens. Here, we provide evidence that persin may have similar effects by showing its potent proapoptotic synergy with the antiestrogen 4-hydroxytamoxifen. However, although persin transcriptionally down-regulates estrogen receptor (ER) expression, unlike conjugated linoleic acid, it also shows efficacy in ER-negative breast cancer cells, both alone and in combination with 4-hydroxytamoxifen, whereas normal breast epithelial cells are unaffected, suggesting it may act via a distinct, ER-independent mechanism. These proapoptotic synergistic interactions are associated with increased *de novo* ceramide synthesis and are dependent on expression of the proapoptotic protein Bim. These data show that persin should be further investigated as a potential novel cancer therapeutic agent because it significantly enhances the sensitivity of breast cancer cells to the cytotoxic effects of tamoxifen, regardless of their ER status, while displaying apparent specificity for the malignant phenotype. [Mol Cancer Ther 2007;6(10):2777–85]

Introduction

Breast cancer is a leading cause of cancer death among women in developed countries. A key goal in the development of novel cancer therapeutics is to achieve tissue specificity and minimize side effects in other tissues while maintaining activity against local and metastatic disease. In breast cancer, this has been successfully achieved for the subsets of tumors that overexpress receptors for endocrine or antibody-targeted therapeutics [estrogen receptor (ER) or erbB2/neu/HER2, respectively; refs. 1, 2]. However, despite the widespread clinical efficacy of these treatments, they are ineffective as therapy for receptor-negative disease, and even among those patients that do initially respond, intrinsic or acquired therapeutic resistance remains a major obstacle to an effective cure. This emphasizes the need for the development of novel therapeutics with increased selectivity and efficacy (3) and has provided the rationale for exploring the interactions between endocrine and nonendocrine therapies as a means of enhancing responsiveness and overcoming resistance.

In the search for novel and effective treatment modalities for human breast cancer, much attention has focused on exploiting the cytostatic and cytotoxic effects of phytochemicals (4). We have previously described a novel plant toxin, persin, which has unique *in vivo* actions in the mammary epithelium and potent cytotoxic effects in human breast cancer cells *in vitro* (5). Persin is the active compound in avocado leaves that induces specific necrosis and/or apoptosis of the secretory epithelium of the mammary gland in lactating livestock and mouse models (6). Our previous studies have shown that persin elicits an arrest in the G_2-M phase of the cell cycle and induces a caspase-dependent apoptotic program associated with release of mitochondrial cytochrome *c* and Smac/DIABLO. The latter is dependent on expression of the BH3-only protein Bim. Bim is a sensor of cytoskeletal integrity (7) and a key mediator of apoptosis induced by microtubule-inhibitory agents (8), and there is evidence that persin acts as a microtubule-stabilizing agent in responsive cells (5).

Persin is an avocado acetogenin, derived from the biosynthesis of long chain fatty acids with close structural homology to the essential n-6 polyunsaturated fatty acid,

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linoleic acid. Interestingly, conjugated linoleic acid has anticarcinogenic properties with particular efficacy against breast cancer (9, 10). Furthermore, polyunsaturated fatty acids structurally similar to persin, including conjugated linoleic acid and γ-linolenic acid, can attenuate steroid hormone receptor sensitivity to circulating estrogens by direct modulation of ER levels (11–14). These data have formed the rationale for a promising phase II clinical trial of γ-linolenic acid and the antiestrogen tamoxifen as primary therapy for ER-positive breast cancer, which showed significant beneficial effects on both clinical response and biological variables of the tumors (15). Due to its close structural homology to these compounds, these studies led us to hypothesize that persin may act via a similar mechanism. Thus, we examined the effects of persin and 4-hydroxytamoxifen (4-OHT) in human breast cancer cells and defined a mechanistic basis for their synergistic interaction.

Materials and Methods

Purification and Isolation of Persin

The persin compound used in these studies was isolated from avocado leaves and purified as previously described (6).

Cell Lines and Reagents

The human breast cancer cell lines MCF-7, T-47D, and SK-Br3 were routinely maintained in RPMI 1640 supplemented with 5% FCS, 10 μg/mL insulin, and 2.92 mg/mL glutamine under standard conditions. The phenotypically normal human mammary epithelial cell line (HMEC) MCF-10A was maintained in DMEM supplemented with 5% horse serum, 20 ng/mL human recombinant epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL bovine insulin, 50 units/mL penicillin G, and 50 μg/mL streptomycin sulfate under standard conditions, and the finite life span 184 HMECs were maintained in serum-free MCDB 170 medium supplemented with bovine pituitary extract. 4-OHT and L-cycloserine were purchased from Sigma-Aldrich Pty. Ltd., and 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) was purchased from BIOMOL International LP.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega Corp.) following the manufacturer's instructions. Cells were plated in 96-well plates and treated in triplicate for 24 h with persin or 4-OHT alone, and the IC_{50} of each agent was calculated. The IC_{50} was then used in the combination studies. Cell viability was assessed by the percentage of absorbance of treated cells relative to that of solvent controls using 490-nm wavelength on a spectrophotometer.

Clonogenic Assays

The long-term survival of breast cancer cells following 2 h of treatment with persin and/or 4-OHT was assessed by clonogenic assay essentially as previously described (16). Colonies were visualized using Diff-Quik stain (Lab Aids Pty. Ltd.).

Immunoblot Analysis

Proteins from whole-cell lysates were resolved under reducing conditions on 12% SDS-polyacrylamide gels using standard methods. Resolved proteins were transferred to polyvinylidene difluoride membranes and probed with antibodies against ERα (Lab Vision) overnight at 4°C or for 2 h at room temperature. Immunoreactive protein bands were detected by the relevant anti-IgG antibodies conjugated with horseradish peroxidase followed by enhanced chemiluminescence (Pierce). Blots were checked for equal loading by reprobing with anti-actin antibody (Sigma).

Measurement of Apoptosis by Flow Cytometry

For M30 analysis, floating and attached cell populations were combined, fixed, and permeabilized in ice-cold 70% ethanol and then resuspended in PBS/0.5% bovine serum albumin with FITC-conjugated M30 CytoDEATH monoclonal antibody (1:100; Alexis Biochemicals) before the M30-positive (apoptotic) population was determined by flow cytometry.

Real-Time Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen Pty. Ltd.) and 1 μg was reverse transcribed using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time PCR was done with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using inventoried (premade) Taqman probes for *ER* or *Bim* (Applied Biosystems). Data analyses were done using the ΔCt method with *RPLPO* (Applied Biosystems) as an internal loading control. Fold changes in gene expression were calculated relative to untreated controls.

RNA Interference

Small interfering RNAs (siRNA) specific for Bim (ON-TARGET plus SMART pool, human BCL2L11) and non-targeting controls (ON-TARGET plus siCONTROL) were purchased from Dharmacon, Inc. Twenty-four hours after seeding, cells were transfected with LipofectAMINE 2000 (Invitrogen Life Technologies, Inc.) in the presence of siRNAs according to the manufacturer's protocol. Following overnight incubation, cells were treated with persin and/or 4-OHT for a further 24 h.

Cell Radiolabeling and Analysis of Lipids

MCF-7 cells were treated with concentrations of persin (13.8 or 27.6 μmol/L) for 16 h, and then cell lipids were radiolabeled by adding [^{3}H]serine (2.0 μCi/mL) to the culture medium for 6 h. After labeling, cell monolayers were lysed on ice with 0.1% SDS. Cells were detached by scraping and lipids were extracted as previously described (17). After centrifugation, the organic lower phase was withdrawn and evaporated under nitrogen. Lipids were resuspended in chloroform/methanol/acetic acid (190:9:1, v/v) and aliquots were analyzed by TLC.

Statistical Analysis

Statistical analysis was carried out using StatView 4.02 (Abacus Concepts, Inc.). Differences between groups were evaluated by Fisher's protected least significant difference test after ANOVA or factorial analysis where appropriate.

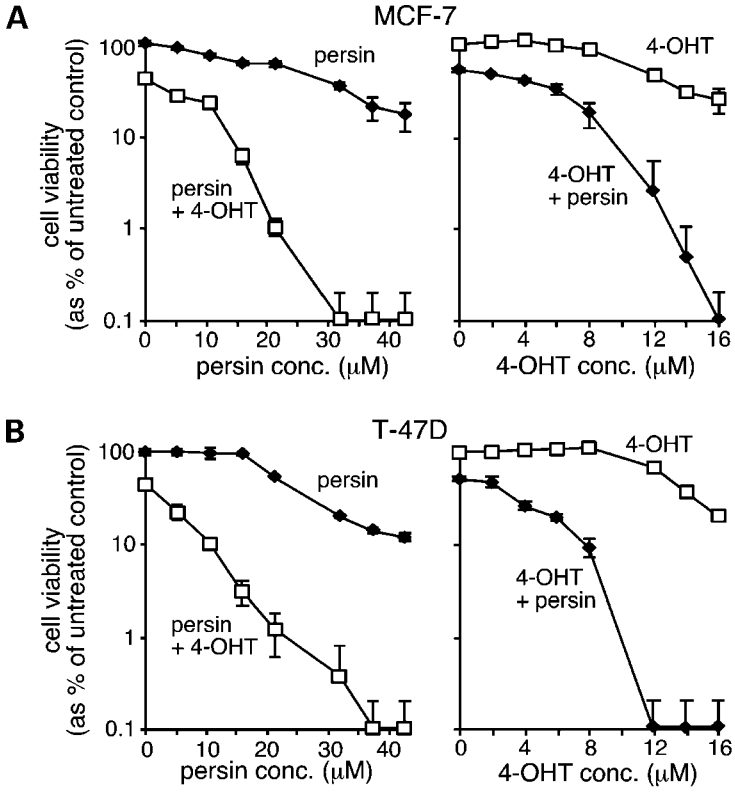
Results

Persin Enhances the Responsiveness of ER-Positive Breast Cancer Cells to 4-OHT

The effects of 24 h of treatment with persin and 4-OHT, alone or in combination, were examined in ER-positive MCF-7 and T-47D human breast cancer cells. Cell viability was determined by MTT assay and expressed as a percentage of vehicle-treated controls. Dose-response curves for persin and 4-OHT treatment alone were determined in MCF-7 (Fig. 1A) and T-47D (Fig. 1B) cells, and the IC_{50} for each agent was calculated. This concentration was then used for the combination studies. Figure 1A and B shows that, although both cell lines exhibit concentration-dependent sensitivity to persin and 4-OHT alone, this reduced survival was enhanced when the two agents were combined. Figure 1C further illustrates the interaction between persin and 4-OHT. The IC_{50} concentration of 4-OHT alone was ~13.5 μmol/L in MCF-7 and T-47D cells, but this was significantly reduced in the presence of persin (to 6.82 ± 0.41 μmol/L and 9.81 ± 0.63 μmol/L, respectively, when combined with 13.8 μmol/L persin; $P < 0.05$). The potentiation of 4-OHT cytotoxicity in the presence of persin was expressed as a sensitization factor (18) by dividing the IC_{50} value of 4-OHT alone by that obtained following combination treatment (Fig. 1C). At the highest concentration of persin used (26.7 μmol/L), the degree of potentiation was approximately 7-fold and 4.5-fold in MCF-7 and T-47D cells, respectively.

Persin Treatment Enhances the Apoptotic Response to 4-OHT

To investigate the mechanism of the interaction between persin and 4-OHT, we examined the effects of combined doses on the long-term survival of human breast cancer cells using clonogenic assays. Figure 2A shows the survival of T-47D cells treated with single and combined doses of the agents compared with untreated controls. Treatment



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	MCF-7		T-47D	
Persin conc. (μM)	4-OHT IC50 (μM)	SF[a]	4-OHT IC50 (μM)	SF[a]
0	13.13 ± 0.67	-	13.84 ± 0.77	-
13.8	6.82 ± 0.41**	**1.9**	9.81 ± 0.63*	**1.4**
20.7	3.68 ± 0.75**	**3.6**	6.94 ± 0.52**	**2.0**
26.7	1.82 ± 0.60**	**7.2**	3.09 ± 0.78**	**4.5**

[a]Sensitization Factor: obtained by dividing IC50 of 4-OHT alone by the combination

Figure 1. Interaction between 4-OHT and persin in human breast cancer cells. Representative MTT assays showing the response of MCF-7 (**A**) and T-47D (**B**) cells to 24-h treatment with persin or 4-OHT alone or in combination with an IC_{50} concentration of the other agent. *Points,* mean of duplicate wells from at least three independent experiments; *bars,* SE. Where error bars are not shown, they did not exceed the size of the respective symbols. **C,** data from MTT assays were used to calculate the IC_{50} values of 4-OHT alone and in combination with persin. The potentiation of 4-OHT cytotoxicity in the presence of persin was expressed as a sensitization factor (*SF*) by dividing the IC_{50} value of 4-OHT alone by that obtained following combination treatment. Values are calculated from at least three independent experiments ± SE. *, $P < 0.05$; **, $P < 0.001$, for combination treatment versus single treatment.

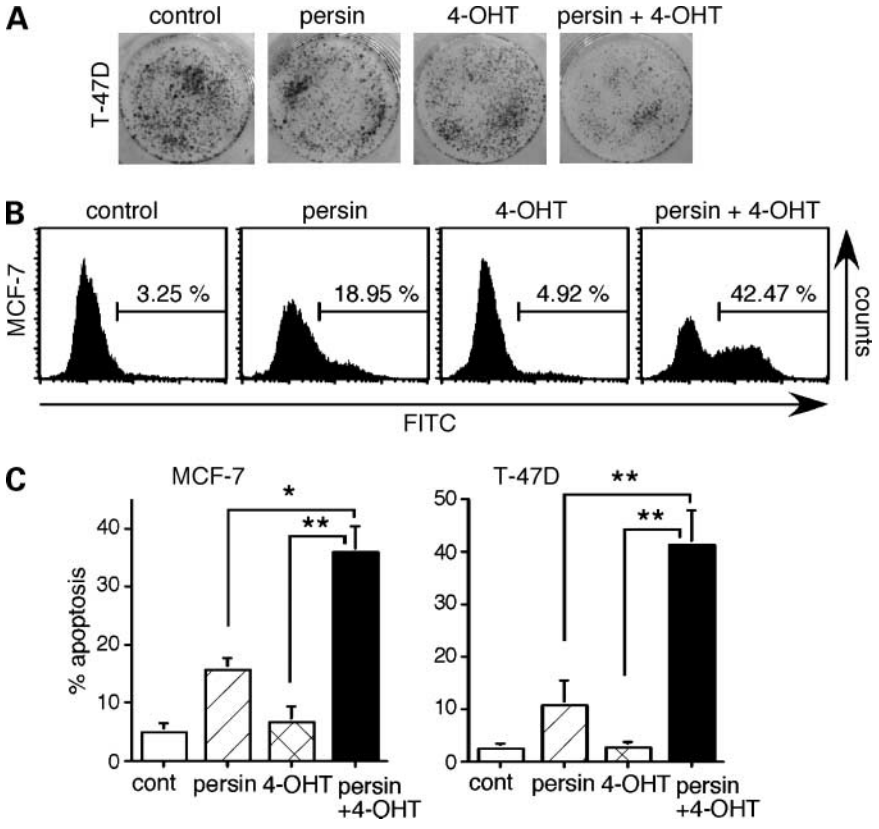

Figure 2. Enhanced responsiveness to 4-OHT is due to an induction of apoptosis. **A,** T-47D cells were seeded at low density and then treated with persin (2.76 μmol/L) or 4-OHT (10 nmol/L) alone or in combination for 2 h. Survival was measured 14 d after treatment by clonogenic assay. Data are representative of experiments done at least thrice. **B,** cells were treated with persin (2.76 μmol/L) or 4-OHT (7.5 μmol/L) alone or in combination for 24 h, and then attached and floating populations were analyzed for M30-FITC positivity by flow cytometry. Representative histograms showing the M30-FITC–positive (apoptotic) fraction. **C,** *columns,* mean of M30-positive fractions from three independent experiments; *bars,* SE. *, $P < 0.01$; **, $P < 0.001$, for combination treatment versus single treatment.

with low nontoxic concentrations of persin (2.76 μmol/L) or 4-OHT (10 nmol/L) alone resulted in no significant changes in survival compared with controls (Fig. 2A). However, there was a significant decrease in survival when the agents were combined.

To confirm that this reduced survival was due to an induction of apoptosis, we used the M30 CytoDEATH antibody that detects caspase-cleaved cytokeratin 18 (19). Treated cells were incubated with M30 CytoDEATH monoclonal antibody before flow cytometric analysis. Figure 2B illustrates representative histograms from MCF-7 cells treated for 24 h with persin (2.76 μmol/L) and 4-OHT (7.5 μmol/L) alone and in combination. The levels of apoptosis (determined by the M30-positive population) were enhanced when persin and 4-OHT were used in combination (~42.5%) compared with persin (~19.0%) or 4-OHT (~5.0%) alone. The graphs in Fig. 3C show data combined from three independent experiments and show that in both MCF-7 and T-47D cells there was a significantly enhanced apoptotic response when persin was used in combination with 4-OHT compared with either single agent alone, suggesting a synergistic interaction between the two compounds.

Effects of Persin on ERα Expression

Previous studies have shown that polyunsaturated fatty acids structurally similar to persin, such as conjugated linoleic acid and γ-linolenic acid, can mediate responsiveness to antiestrogens by down-regulating ERα expression (11–14). To determine if persin acts via a similar mechanism, we examined its effects on the expression of ERα. Cells were treated with cytotoxic concentrations of persin (27.6 μmol/L) for up to 24 h, and ERα mRNA and protein expression was determined at various time points by quantitative PCR and immunoblot, respectively. There was an approximately 25% to 60% decrease in ERα protein expression in T-47D and MCF-7 cells, respectively, by 24 h after treatment (Fig. 3A), and a significant decrease in ERα mRNA expression was also observed (data not shown). To further examine the role of ERα in the observed synergy with 4-OHT, mRNA levels were determined by quantitative PCR following 24 h of treatment with nontoxic concentrations of persin (2.76 μmol/L) or 4-OHT (7.5 μmol/L) alone and in combination. Figure 3B shows that, although there was little effect of each agent alone on the levels of ERα mRNA expression, there was a significant decrease in expression when both agents were combined. Similar effects were seen in both MCF-7 and T-47D cells (Fig. 3B).

To determine if the synergistic interactions between 4-OHT and persin were dependent on modulation of ERα expression, we examined the effects of persin and 4-OHT treatment in the ER-negative cell line SK-Br3 that we have previously shown to be sensitive to the apoptotic effects of persin (5). Cells were treated with 4-OHT alone (up to 16 μmol/L) or in combination with an IC_{50} concentration of persin (27.6 μmol/L), and cell viability was determined by M30 positivity. Figure 3C shows that, although SK-Br3 cells

were relatively resistant to the cytotoxic effects of 4-OHT alone, there was a significant induction of apoptosis when treatment was combined with persin compared with either single-agent treatment. Similar results were observed with another ER-negative, persin-responsive cell line, MDA-MB-157 (data not shown).

Phenotypically Normal Mammary Epithelial Cells Do Not Respond to Persin

To further characterize the specificity of the actions of persin, we examined its effects on the phenotypically normal, ER-negative mammary epithelial cell lines MCF-10A and HMEC 184. Figure 3D shows that both cell lines were completely resistant to the cytotoxic effects of persin (up to 40 μmol/L) alone and in combination with 4-OHT (10 μmol/L), and no synergistic interaction between the two agents was observed.

Enhanced Responsiveness to 4-OHT Is Dependent on Bim Expression

Persin induces an apoptotic pathway in sensitive breast cancer cell lines that is dependent on expression of the BH3-only protein Bim (5). We further examined the role of this proapoptotic protein in the synergistic response to persin and 4-OHT. The effect of cytotoxic concentrations of persin (27.6 μmol/L) on Bim mRNA expression was quantitated using quantitative PCR over a time course of treatment up to 24 h. Figure 4A shows that persin treatment resulted in a significant induction of Bim mRNA by 24 h in both MCF-7 and T-47D cells. To determine what role the transcriptional regulation of Bim plays in the synergistic actions of persin, cells were treated with low concentrations of persin (2.76 μmol/L) or 4-OHT (7.5 μmol/L) alone or combined for 24 h, and Bim mRNA expression was determined by quantitative PCR. Figure 4B shows that combined treatment with persin and 4-OHT resulted in a significant induction of Bim mRNA expression compared with single-agent treatment.

To determine whether the observed proapoptotic synergy was dependent on Bim expression, we examined the proapoptotic effects of the two agents when Bim expression was attenuated. MCF-7 cells were treated with persin and/or 4-OHT for 24 h in the presence of Bim-specific siRNAs or nontargeting controls, and then the induction of apoptosis was determined and quantitated. Bim protein expression is reduced ~70% in the presence of Bim siRNAs (Fig. 4C; ref. 5). Figure 4D shows that the proapoptotic synergy between persin and 4-OHT was significantly attenuated in the presence of Bim siRNAs compared with nontargeting controls, suggesting that it may be mediated, in part, by Bim.

Involvement of Ceramide Production

We then continued our investigations aimed at defining a mechanistic basis for the observed synergy between these two compounds. It is known that tamoxifen increases levels of the intracellular apoptotic second messenger, ceramide, by preventing its conversion to glucosylceramide by glucosylceramide synthase (20), and this may mediate the ability of tamoxifen to circumvent multidrug resistance in breast cancer cells (21). This led us to investigate whether the observed synergy between 4-OHT and persin was

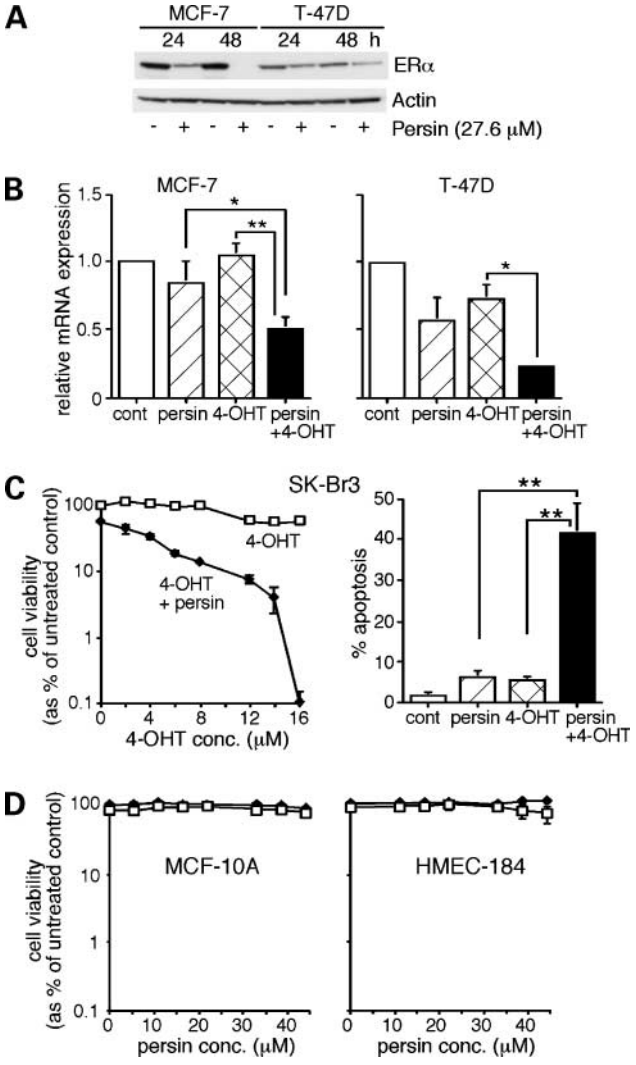

Figure 3. Effects of persin are independent of ERα expression. **A,** MCF-7 and T-47D cells were treated with persin (27.6 μmol/L) for 24 or 48 h, and then levels of ERα protein were determined by immunoblot. Actin was used as a loading control. Representative blots from three independent experiments. **B,** levels of ERα mRNA following treatment with persin (2.76 μmol/L) or 4-OHT (7.5 μmol/L) alone or in combination for 24 h. Expression levels were determined by quantitative PCR and quantitated relative to the loading control. *Columns,* mean of triplicate samples from three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, for combination treatment versus single treatment. **C,** *left,* MTT assay showing the effect of 24-h treatment with 4-OHT alone and in combination with IC_{50} dose of persin in ER-negative SK-Br3 cells. *Points,* mean of triplicate wells; *bars,* SE. Where error bars are not shown, they did not exceed the size of the respective symbols. *Right,* effect of persin and 4-OHT treatment on the induction of apoptosis in SK-Br3 cells. Cells were treated alone or in combination for 24 h and then analyzed for M30-FITC positivity by flow cytometry. *Columns,* mean of M30-positive fractions from three independent experiments; *bars,* SE. **, $P < 0.001$, for combination treatment versus single treatment. **D,** effect of persin and 4-OHT in the phenotypically normal, ER-negative mammary epithelial cell lines MCF-10A and HMEC 184. Cells were treated for 24 h with concentrations of persin alone or in combination with 4-OHT as indicated, and cell viability was determined by MTT assay. *Points,* mean of triplicate wells from three independent experiments; *bars,* SE. Where error bars are not shown, they did not exceed the size of the respective symbols.

mediated via modulations in intracellular ceramide production. We quantitated *de novo* ceramide production following exposure to cytotoxic concentrations of persin (13.8 and 27.6 μmol/L) in the presence of [^{3}H]serine tracer and observed a significant increase in ceramide production compared with untreated controls, which was attenuated in the presence of an inhibitor of *de novo* ceramide synthesis, L-cycloserine (Fig. 5A). We then further investigated whether ceramide was causal in the apoptotic response to combined treatment with persin and 4-OHT. First, we determined whether blocking ceramide glycosylation could mimic the effects of 4-OHT and enhance persin-induced apoptosis. MCF-7 cells were treated with a relatively nontoxic concentration of persin (2.76 μmol/L) and the selective glucosylceramide synthase inhibitor PPMP (10 μmol/L) for 24 h, and then apoptosis induction was analyzed by M30 positivity. Figure 5B shows that combined treatment with PPMP and persin resulted in a synergistic induction of apoptosis compared with single-agent treatment. We then examined the effects of the ceramide inhibitor L-cycloserine (10 mmol/L) on persin-induced and 4-OHT–induced apoptosis. Treatment with the ceramide inhibitor significantly abrogated both persin-induced apoptosis (Fig. 5C) and persin/4-OHT proapoptotic synergy (Fig. 5D), indicating that *de novo* generation of ceramide is causal, at least in part, in the cytotoxic response to persin alone and the synergistic response to combined treatment with 4-OHT.

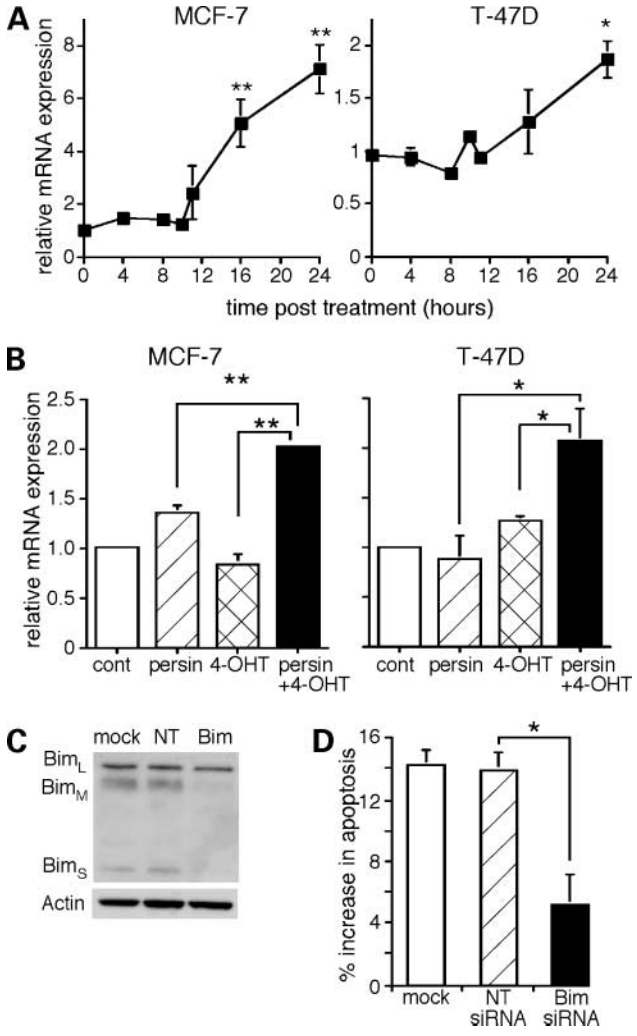

Figure 4. Synergy between persin and 4-OHT is dependent on Bim expression. **A,** effect of persin on Bim transcription was determined by real-time quantitative PCR. Cells were treated with 27.6 μmol/L persin, and then levels of Bim mRNA were quantitated relative to the loading control RPLPO at various time points after treatment. *Points,* mean of triplicate samples from three independent experiments; *bars,* SE. *, $P < 0.01$; **, $P < 0.0001$, for persin treatment versus untreated control. **B,** MCF-7 and T-47D cells were treated with persin (2.76 μmol/L) or 4-OHT (7.5 μmol/L) alone or in combination for 24 h, and then levels of Bim mRNA were determined by quantitative PCR and quantitated relative to the loading control. *Columns,* mean of triplicate samples from three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.0001$, for combination treatment versus single treatment. **C,** MCF-7 cells were treated for 16 h with Bim-specific siRNAs and nontargeting (*NT*) controls or mock transfected, and then Bim expression was determined by immunoblotting. Actin was used as a loading control. **D,** MCF-7 cells were treated for 16 h with Bim-specific siRNAs and nontargeting controls or mock transfected and a combination of persin (4.14 μmol/L) or 4-OHT (7.5 μmol/L), alone or in combination. Cells were then incubated with M30-FITC and analyzed by flow cytometry. *Columns,* data from three independent experiments and expressed as percentage increase in apoptosis of combined treatment over single agent alone; *bars,* SE. *, $P < 0.01$, Bim siRNA versus nontargeting control.

Discussion

Since its first clinical use in the early 1970s (1), antiestrogen therapy has had a significant effect on survival for breast cancer patients with endocrine-responsive disease (22). However, its efficacy is primarily limited to a subset of patients whose tumors express ER and is often compromised by the development of resistance (1, 23, 24). This has led to a need for the development of novel therapeutics to improve and broaden efficacy against refractory disease.

We have previously shown the cytostatic and cytotoxic effects of the plant toxin persin in human breast cancer cell lines *in vitro* (5). Persin-induced apoptosis is dependent on the expression of the BH3-only protein Bim and is associated with changes to the microtubular architecture characteristic of increased tubulin polymerization (5). Compounds structurally related to persin, such as the polyunsaturated fatty acids γ-linolenic acid and conjugated linoleic acid, have demonstrable anticancer activity (25) and can modulate the response of ER-positive human breast cancer xenografts to the antiproliferative and/or cytotoxic effects of tamoxifen through down-regulation of ERα expression (13). Here, we investigated whether persin elicits a similar effect in human breast cancer cell lines, examining the interplay between persin and tamoxifen and further defining the mechanistic basis for its actions.

Our results have shown that the human breast cancer cell lines MCF-7 and T-47D have a markedly enhanced response to the cytotoxic effects of persin and 4-OHT when the two agents are combined as opposed to single-agent treatment. The mitogenic effects of estrogen are mediated through nuclear ligand-activated transcription factors, the ERs, predominantly ERα. Selective ER modulators, such as

4-OHT, can mediate their actions in human breast cancer cells primarily by modulation of ERα expression and function (1, 23). Thus, although endocrine therapy has become an integral component of breast cancer treatment in both the metastatic and adjuvant setting, its efficacy is limited to a subset of tumors expressing ER, with little, if any, benefit in ER-negative cancers (22, 26). Although we have previously shown that the response to persin is independent of ER status (5), both MCF-7 and T-47D cells are ER positive, and the synergistic effects of combined treatment with persin and 4-OHT were associated with a significantly enhanced decrease in ERα mRNA compared with single-agent treatment. However, the ER-negative cell line SK-Br3 displayed a similar response to 4-OHT in the presence of persin, suggesting that the ability of persin to modulate the response to antiestrogens may be mediated by a parallel, ER-independent pathway. These data also suggest that persin may have a wider efficacy and, potentially, distinct mechanism of action compared with other polyunsaturated fatty acids, as the latter have no efficacy against ER-negative breast cancer cells *in vitro* (12). From a clinical perspective, this is of particular importance, as it implies that persin has efficacy against a subset of breast cancers with poor prognosis that are nonresponsive to antiestrogen therapy and could also, potentially, circumvent endocrine resistance. Of further potential clinical relevance is the data showing that the viability of normal mammary epithelial cells is unaffected by persin treatment or synergy with 4-OHT, suggesting that the actions of persin are selective for a malignant phenotype.

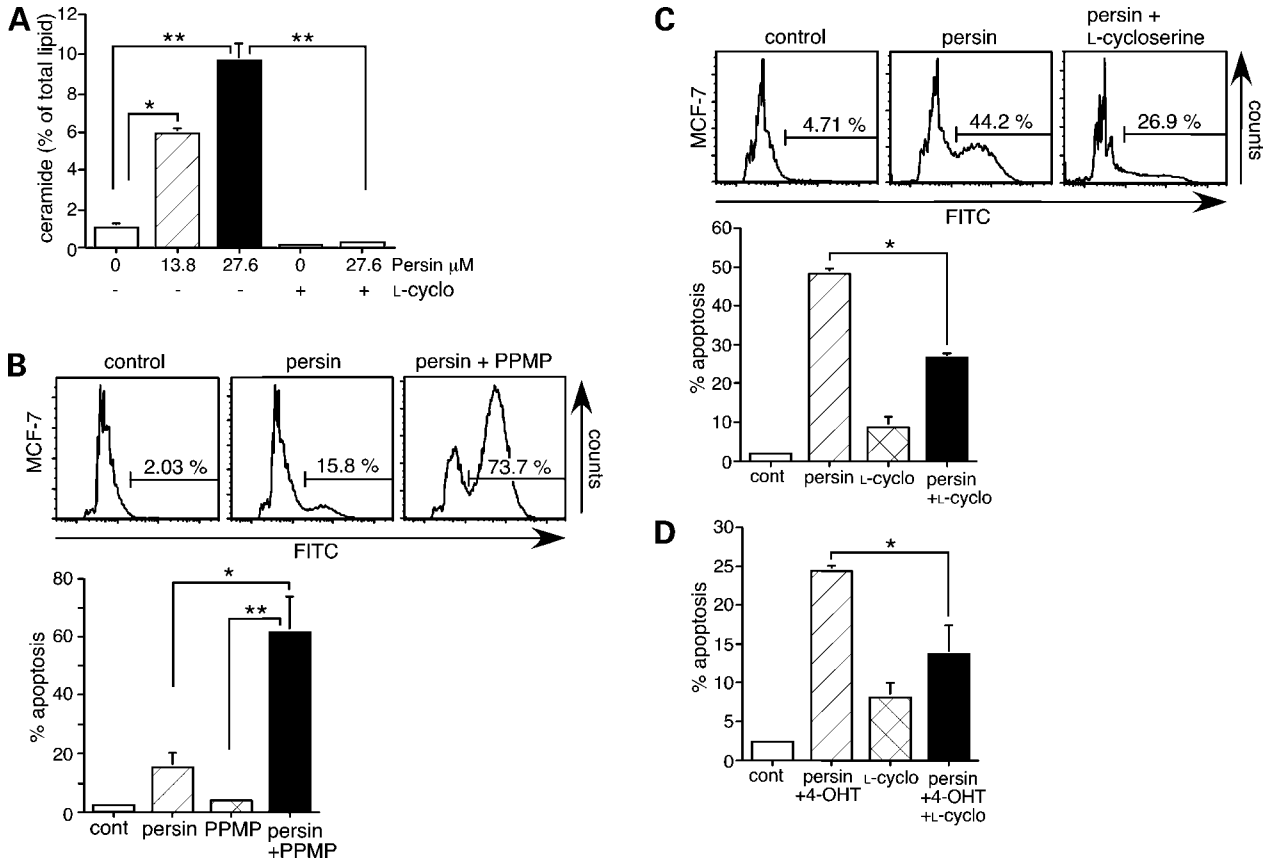

Figure 5. Modulation of ceramide metabolism influences persin and 4-OHT synergy. **A,** MCF-7 cells were treated with concentrations of persin alone or in combination with L-cycloserine (10 mmol/L) as indicated in the presence of [^{3}H]serine, and then lipids were extracted and ceramide species were analyzed by TLC and autoradiography. Levels of ceramide are expressed as a percentage of total lipids. *Columns,* mean of two independent experiments; *bars,* SE. *, $P < 0.01$; **, $P < 0.0001$, for treated cells versus control. **B,** MCF-7 cells were treated with low concentrations of persin (2.76 μmol/L) alone or following a 2-h pretreatment with PPMP (10 μmol/L) for 24 h, and then apoptosis induction was determined by M30 positivity. Representative histograms showing the M30-FITC – positive (apoptotic) fraction. *Columns,* mean of M30-positive fractions from three independent experiments; *bars,* SE. *, $P < 0.01$; **, $P < 0.001$, for combination treatment versus single treatment. **C,** MCF-7 cells were treated with a cytotoxic concentration of persin (13.8 μmol/L) alone or following a 2-h pretreatment with L-cycloserine (10 mmol/L) for 24 h, and then apoptosis induction was determined by M30 positivity. Representative histograms showing the M30-FITC – positive (apoptotic) fraction. *Columns,* mean of M30-positive fractions from three independent experiments; *bars,* SE. *, $P < 0.01$, for combination treatment versus single treatment. **D,** MCF-7 cells were treated with combined persin (2.76 μmol/L) and 4-OHT (7.5 μmol/L) for 24 h with or without a 2-h pretreatment with L-cycloserine (10 mmol/L), and then apoptosis induction was determined by M30 positivity. *Columns,* mean of M30-positive fractions from two independent experiments; *bars,* SE. *, $P < 0.01$, combination treatment versus single treatment.

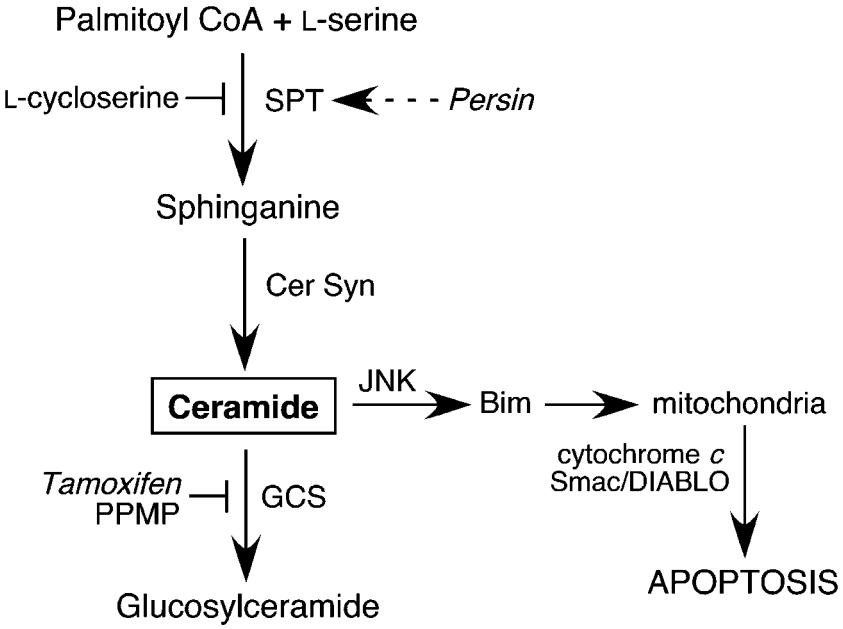

Figure 6. A schematic representation of the putative interactions of persin and 4-OHT with ceramide metabolism. The first step of *de novo* sphingolipid biosynthesis is the condensation of L-serine with palmitoyl-CoA, which is catalyzed by serine palmitoyltransferase (*SPT*) and blocked by the inhibitors myriocin and L-cycloserine. Persin-induced apoptosis is attenuated in the presence of serine palmitoyltransferase inhibitors, suggesting it may modulate ceramide production at this step. Glucosylceramide synthase (*GCS*) catalyses the conversion of ceramide to glucosylceramide. Tamoxifen and PPMP increase intracellular ceramide by preventing the conversion of ceramide glucosylceramide by glucosylceramide synthase. *Cer Syn,* ceramide synthase. Intracellular ceramide can activate the stress kinase c-Jun NH_2-terminal kinase (*JNK*), which acts on the mitochondria either directly or through the BH3-only protein Bim to trigger the release of apoptotic intermediates and initiate the downstream apoptotic cascade.

This requires further investigation but seems to be in contrast to early work on this compound that showed its apparent *in vivo* specificity for the mammary gland in lactating livestock (6); however, lactating mammary epithelium is known to exhibit changes in lipid composition, which may result in increased uptake of persin metabolites and a consequential elevated responsiveness to its cytotoxic effects in this tissue.

Ceramide is a membrane sphingolipid that functions as an important second messenger during cellular stress-induced apoptosis (27). Although the precise mechanism by which intracellular ceramide accumulation mediates the apoptotic process is unclear, it is thought to center on mitochondrial changes leading to the release of apoptotic intermediates (28). Experimental manipulation of ceramide metabolism has shown the therapeutic potential of targeting sphingolipid biosynthesis to enhance the efficacy of cancer therapy (29). Addition of exogenous ceramide or inhibitors of ceramide metabolism enhances the cytotoxic effects of many chemotherapeutic agents, such as paclitaxel (30). Conversely, high levels of glucosylceramide, the inactive metabolite of ceramide generated by glucosylceramide synthase, are associated with multidrug-resistant cancers, and very low levels, with drug-sensitive cells (31). Tamoxifen increases the levels of intracellular ceramide by blocking ceramide glycosylation (20, 21) and, thus, modulating cellular sensitivity to cytotoxic agents (32). Treatment with the selective glucosylceramide synthase inhibitor PPMP mimicked the effects of 4-OHT in our system and enhanced persin-induced apoptosis, suggesting that the cytotoxic synergy between persin and 4-OHT occurs via a modulation of ceramide metabolism. This concept was supported by the observed increase in intracellular ceramide following persin treatment and the attenuation of persin-induced apoptosis by the serine palmitoyltransferase inhibitor L-cycloserine. Thus, we hypothesize that the synergistic proapoptotic effects elicited by persin and 4-OHT are mediated, at least in part, by the *de novo* generation of ceramide by persin and the inhibition of its glycosylation by 4-OHT (Fig. 6).

Our previous studies have identified Bim and the stress kinase c-Jun NH_2-terminal kinase as upstream mediators of persin-induced apoptosis (5), and here, we show that Bim plays a role in the synergistic interactions between persin and 4-OHT. Ceramide is known to activate c-Jun NH_2-terminal kinase (33), which in turn can induce the phosphorylation of Bim and its translocation from the microtubules to the mitochondria (34). Thus, we propose that the effects of persin and 4-OHT on ceramide production trigger a downstream signaling cascade, leading to the activation of Bim and the induction of an intrinsic apoptotic program. Persin may also activate Bim directly through its effects on the microtubular architecture and tubulin polymerization (5).

In summary, our data suggest that persin mediates its cytotoxic effects in human breast cancer cells via the *de novo* generation of ceramide, and proapoptotic synergy is achieved by addition of 4-OHT to inhibit ceramide glycosylation. The resulting increase in intracellular ceramide triggers apoptosis via a Bim-dependent signaling cascade (Fig. 6). Thus, persin shows considerable potential as a clinically relevant anticancer agent due to its intrinsic cytotoxicity and potent proapoptotic synergy with tamoxifen, independent of ER status. Our results provide the rationale for the further preclinical evaluation of this compound and the development of more effective synthetic analogues, with the potential to act as a novel strategy to enhance and broaden therapeutic efficacy and counteract endocrine resistance in breast cancer.

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