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Intrinsic cytotoxicity and chemomodulatory actions of novel phenethylisothiocyanate sphingoid base derivatives in HL-60 human promyelocytic leukemia cells

Charlene R. Johnson[1,2], Jiong Chun[3], Robert Bittman[3], and W. David Jarvis[1,3,4]§

Department of Integrative Biology & Pharmacology[1] and the Institute for Molecular Medicine[4]
University of Texas Health Sciences Center – Houston
Houston, TX 77030

Research & Development Division[2]
Dominion Diagnostics, Inc.
North Kingstown, RI 02852

Department of Chemistry and Biochemistry[3]
Queens College of the City University of New York
Flushing, NY 11367

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Correspondence (§):

Dr. W. David Jarvis
Dominion Diagnostics, Inc.
Research & Development
211 Circuit Drive
North Kingstown, RI 02852
telephone: 401.667.0800; *telefax:* 401.667.0330

david.jarvis@dominiondiagnostics.com

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Abbreviations:

araC	1-[β-D-arabinofuranosyl]cytosine (cytarabine)
PEITC-Sa	phenethylisothiocyanate-substituted sphinganine
PEITC-So	phenethylisothiocyanate-substituted sphingosine
CLP	calphostin C (UCN-1028c)
SPC-100270	safingol

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ABSTRACT

The protein kinase C (PKC) isoenzyme superfamily represents a popular target in pharmacological interventions designed to elicit apoptosis directly in tumor cells or to potentiate the lethal effects of antineoplastic agents. Numerous observations support the clinical utility of PKC inhibition by experimental sphingolipid derivatives such as safingol. The present studies document the cytotoxicity and chemomodulatory capacity of phenethylisothiocyanate derivatives of sphinganine and sphingosine (PEITC-Sa; PEITC-So) in the human myeloid leukemia cell line HL-60. The biological actions of these novel derivatives were compared directly with those of the parent compounds sphinganine and sphingosine. Exposure to natural and modified sphingoid bases promoted extensive apoptotic cell death. The PEITC-sphingoid base derivatives exhibited higher cytotoxicity than their natural counterparts and were also distinctly superior to the clinically relevant sphingoid base analog safingol. In each instance, lethality was shown to correlate with inhibition of cPKC/nPKC and downstream loss of ERK1/ERK2. The involvement of these signaling systems in potentiating the lethal actions of 1-[β-D-arabinofuranosyl]cytosine (araC) was also examined with regard to the differential actions of PEITC-Sa and PEITC-So to that of the parent compounds as well as safingol. Exposure to araC alone rapidly increased PKC activity. In the presence of PEITC-Sa or PEITC-So, the therapeutic efficacy of araC increased markedly; moreover, potentiation was directly related to the loss of araC-stimulated PKC activity. These findings demonstrate that PEITC-substituted sphingoid base analogs exert potent antineoplastic effects in human leukemia cells. We suggest that these synthetic lipids represent potentially useful agents in the development of cPKC/nPKC-directed chemotherapeutic strategies.

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INTRODUCTION

It is widely documented that acute signaling through the *conventional* and *novel* isoforms of protein kinase C (cPKC, nPKC) mediates a potent cytoprotective influence capable of averting apoptosis in a variety of settings [reviewed in Jarvis and Grant, 1999]. Accordingly, the selective targeting of the *conventional* and *novel* isoforms of protein kinase C (cPKC, nPKC) has become a mainstream approach to pharmacological interventions designed to confer chemosensitizing or radiosensitizing advantages in therapeutic settings.

Pharmacological manipulations that impinge selectively upon cPKC/nPKC signaling are particularly effective in many hematopoietic malignancies. Previous efforts in this laboratory have focused on chemosensitization of myeloid leukemia cells to the deoxycytidine analog cytarabine (1-[β-D-arabinofuranosyl]cytosine; ara-C). The cytocidal properties of this highly effective antileukemic agent are well characterized (reviewed in Grant, 1998). The cytotoxicity of araC emerges through repeated phosphorylation to form the lethal derivative ara-CTP. Template-specific incorporation of this anabolite into elongating DNA strands interferes with normal DNA synthesis, and subsequently elicits the extensive endonucleolytic DNA degradation associated with apoptosis.

In addition to derangement of DNA biosynthesis, araC engages an array of intracellular signals in myeloid cells including, paradoxically, a cytoprotective response pathway that favors cell survival [reviewed by Jarvis and Grant, 2000]. Among various metabolic disturbances caused by araC is the inappropriate accumulation of the lipid messenger diglyceride [Kucera and Capizzi, 1992; Strum *et al.*, 1994], which profoundly attenuates the apoptotic process in many settings [Jarvis *et al.*, 1994, 1996, 1998]. The increased availability of free diglyceride results in rapid stimulation of cPKC/nPKC activity [Kharbanda *et al.*, 1991; Jarvis *et al.*, 1998] which, further downstream, promotes recruitment of the cytoprotective MAPK-ERK cascade [Kharbanda *et al.*, 1994] and up-

regulation of the pro-survival transregulatory factor NFκB [Kharbanda *et al.*, 1990; Brach *et al.*, 1992a, 1992b].

The involvement of multiple lipid messengers in the physiological regulation of leukemic cell survival is well established. Although cytotoxic sphingolipid messengers such as ceramide and sphingosine exert their effects through a complex of distinct subcellular targets, it is significant to the present studies that inhibition of one or more cPKC or nPKC isoforms is associated with sphingolipid-induced cell death, the inhibitory influence being either direct or indirect. In the case of structurally simple lysosphingolipids, this interaction is mediated through direct binding with the conserved regulatory domain of cPKC and nPKC subfamilies. As such, sphingoid bases have been found to exhibit anti-tumor activity by limiting PKC-dependent survival signaling. This is reflected in studies demonstrating the attenuation of tumor cell proliferation *in vitro* [Merrill *et al.*, 1990] and tumor growth and metastasis *in vivo* [Sadahira *et al.*, 1992]. In addition, the lethal response to sphingoid bases is closely reproduced by pharmacological agents that inhibit cPKC/nPKC through direct targeting of the enzymes' regulatory domain, such as the photo-activated alkaloid, calphostin C (UCN-1028c). Based on the ability of these agents to limit cPKC/nPKC activity, other studies have subsequently shown that araC action is substantially augmented by pharmacological reductions in PKC activity through inhibition upon acute exposure to sphingosine and the nonphysiological sphingoid base analog L-*threo*-dihydrosphingosine (SPC-100270; safingol) [Kedderis *et al.*, 1995; Jarvis *et al.*, 1998], as well as in response to pharmacological inhibitors such as calphostin.

Both naturally occurring and synthetic isothiocyanates function as highly effective chemopreventive agents, inhibiting cell growth both *in vivo* and *in vitro* [Conaway, *et al.*, 2002]. Moreover, it has been shown that sphingoid base adducts of phenethylisothiocyanate (PEITC) inhibit cell growth in HL-60 cells [Xu and Thornalley, 2000]. The cur-

rent studies examined the anti-proliferative effects of two such derivatives, PEITC-sphinganine (PEITC-Sa) and PEITC-sphingosine (PEITC-So) with respect to the parent compounds, sphingosine and sphinganine. To address this question directly, we monitored apoptotic commitment as well as selective cPKC/nPKC and MAPK-ERK inhibition in HL-60 human promyelocytic leukemia cells upon acute exposure to all four compounds and the nonphysiological sphingoid base analog safingol. In addition, we compared the ability of sphingosine and sphinganine, as well as their phenethylisothiocyanate derivatives, to potentiate the cytotoxic actions of araC. Our results demonstrate the intrinsic lethality of PEITC-Sa and PEITC-So in HL-60 cells, the potencies and efficacies of which are superior to those of safingol and the parent lipid compounds. Furthermore, we show a dramatic amplification in araC action in the presence of both phenethylisothiocyanate derivatives. This increase in cytotoxicity is shown to correlate with inhibition of cPKC/nPKC activity and subsequent downstream loss of ERK1/ERK2.

These findings demonstrate that PEITC-substituted sphingoid base analogs exert potent antineoplastic effects in human myeloid leukemia cells. We suggest that these synthetic lipids represent useful agents in the development of cPKC/nPKC-directed chemotherapeutic strategies.

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MATERIALS & METHODS

Drugs and reagents. Crystalline preparations of araC (Sigma Chemical, St. Louis, MO) were stored desiccated at 4° and dissolved in sterile PBS immediately before use. Synthetic preparations of sphingosine and dihydrosphingosine (sphinganine) were obtained from Biomol; corresponding *N*-(*N*'-phenethylthiocarbamoyl) sphingoid base derivatives were synthesized essentially as described [Xu and Thornalley, 2000]; lipids were purified by silane thin-layer chromatography using Merck 60F254 silica gel plates developed in a solvent system of methanol-chloroform (1:9, v/v). Safingol was obtained either from Calbiochem or from Sphinx Pharmaceuticals. All sphingolipids were initially dissolved in 100% ethanol and stored at -70°C; for experimental use, ethanol stocks of these agents were complexed with delipidated albumin. Lipid preparations were prewarmed to 37°C and delivered directly into complete medium as indicated below. Lyophilized preparations of calphostin C were dissolved in dimethylsulfoxide and delivered as a pre-warmed concentrated organic stock. All test reagents were presented at final concentrations in complete medium at 37°C; appropriate controls confirmed that each of the vehicles used was without effect in HL-60 cells.

Cell culture. The human promyelocytic leukemia cell line HL-60 was grown in complete RPMI-1640 medium (phenol red-free formulation, supplemented with 1% sodium pyruvate, nonessential amino acids, L-glutamine, penicillin, and streptomycin; all from Life Technologies) and 10% heat-inactivated fetal bovine serum. Cultures were passed twice weekly and exhibited a doubling time of ~24 hr. Cultures were maintained under a humidified atmosphere of 95% room air, 5% CO_2, at 37°C. Cell density was determined by liquid suspension particle counter, and basal cell viability was routinely assessed by vital dye exclusion.

Test exposures. All experimental incubations were performed as described previously [Jarvis *et al.*, 1994a]. Cells in log-phase growth were pelleted, rinsed twice in complete medium, resuspended at a density of 4×10^5 cells/ml, and maintained as indicated above. Cells were exposed to test agents for appropriate intervals in complete medium; loss of cells under these conditions caused by either washing or cell adherence was negligible (≤5%). Test incubations were terminated with gentle pelleting of the cells by centrifugation at 400 × *g* for 10 min at 4°; in some instances, aliquots of the medium were retained for direct assay of released DNA. After the determination of cell density, the cells were pelleted and prepared as outlined below for morphology inspection by conventional light or fluorescent microscopy, assay of clonogenicity, or *in vitro* assays of cPKC/nPKC and ERK1/ERK2 activities.

Measurement of clonogenicity. Pelleted cells were rinsed extensively and prepared for soft-agar cloning as described previously [Jarvis *et al.*, 1994c]. Cells were resuspended in cold PBS and seeded onto 35-mm culture plates at a fixed density (400 cells/ml/well) in complete RPMI-1640 medium containing 20% fetal calf serum, 10% 5637-CM, and 0.3% Bacto agar. Cultures were maintained for 10-12 days before the formation of colonies (defined as groups of ≥50 cells) was scored.

Cytological characterization of apoptosis. Pelleted cells were resuspended in PBS and fixed in cytocentrifuge preparations according to established procedures [Jarvis *et al.*, 1997]. For visualization of apoptotic morphological alterations, fixed cells were stained with 20% May-Grünwald-Wright-Giemsa stain. At least five 100-cell fields were scored for each treatment by conventional light microscopy by assessing the expression of cytoarchitectural characteristics of apoptosis (*i.e.*, condensed nucleoplasm and cytoplasm, formation of membrane blebs, karyolytic degeneration of the nucleus into apoptotic bodies, and overall cell shrinkage). For visualization of apoptotic DNA damage,

fixed cells were sequentially (*a*) treated with ethanol-acetic acid (2:1, v/v) at 20° for 5 min, (*b*) stained for broken DNA by treatment with terminal deoxynucleotidyl transferase in the presence of fluorescein isothiocyanate-dUTP; Molecular Probes) at 37°C for 60 min, and (*c*) counterstained for intact DNA with 0.01% propidium iodide in sodium citrate at 20°C for 10 min. At least three 100-cell fields were scored for each treatment by fluorescent microscopy by assessing increased direct fluorescence of end-labeled double-stranded DNA.

*Assessment of ara-CTP metabolism***.** Pelleted cells were rinsed in cold PBS, repelleted, and then lysed in 0.6 N trichloroacetic acid. Pyrimidine nucleotide extracts were then prepared as previously explained in detail [Jarvis *et al.*, 1994c]. Levels of ara-CTP were separated by high pressure liquid chromatography; values are expressed as pmol of ara-CTP present in 1.5×10^6 cells.

Determination of cPKC/nPKC activity. Pelleted cells were rinsed in PBS, repelleted, and homogenized in 20 mM Tris·HCl, 500 µM EDTA, and 500 µM EGTA, pH 7.5, containing protease inhibitors (40 µg/ml aprotinin, 15 µg/ml leupeptin). After partial purification of homogenates over DEAE-cellulose, particulate *(i.e.*, membrane-associated) and soluble (*i.e.*, cytosolic) enzyme fractions were separated by ultracentrifugation at 100,000 × *g* at 4° for 2 hr. Subcellular fractions of membrane and cytosol were added to reaction mixtures containing lysis buffer and mixed micelles of synthetic phosphatidylserine and dioleoylglycerol (10 µM). Particulate activity was assayed using synthetic acetylated myelin basic protein *N*-terminal peptide $AcMBP_{4-14}$ as described previously [Jarvis *et al.*, 1998]. Reactions were initiated by the addition of 25 µCi of [γ-^{32}P]ATP and 20 µM nonisotopic ATP and allowed to proceed for 5 min at 30°C. Reactions were terminated by transfer to nitrocellulose filters and immersion in cold *ortho*phosphoric acid (1% v/v). Filters were rinsed sequentially in *ortho*phosphoric

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acid and PBS, and radioactivity was determined by non-aqueous liquid scintillometry. Because this *in vitro* assay monitors only diglyceride-driven PKC activity, only the activity conventional and novel isoforms are directly measured; the contribution of diglyceride-*in*sensitive (*i.e.*, atypical) species is subtracted upon correction for nonspecific and background activity; accordingly, data derived from this assay system are reported as cPKC/nPKC activity. In a limited number of trials, the actions of synthetic sphingolipids were examined in basal and stimulated recombinant human cPKCα purified (enzyme was derived from a baculovirus expression system, then reconstituted in a mixed micelle assay format of phosphatidylserine/phosphatidylcholine/Triton X-100 as described previously [Sando and Beals, 2003].

Determination of MAPK activity. Pelleted cells were rinsed in PBS, repelleted, and flash-frozen. Cell pellets were lysed in 25 mM HEPES, pH 7.4, containing 5 mM EGTA, and 5 mM EDTA, supplemented with protease inhibitors (5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml soybean trypsin inhibitor, 40 µg/ml pepstatin, 40 µg/ml chymotrypsinogen, 40 µg/ml E64, 40 µg/ml aprotinin, 1 µM microcystin LR), phosphatase inhibitors (0.5 mM trisodium orthovanadate, 0.5 mM tetrasodium pyrophosphate), and containing 0.05% sodium deoxycholate (w/v), 1% Triton X-100 (v/v), and 0.1% 2-mercaptoethanol (v/v). Lysates were clarified by centrifugation at 5000 × *g* at 4°C for 5 min. ERK1/ERK2 was immunoprecipitated from clarified lysates with protein A-agarose-conjugated antibody/antisera, and activities were determined as described previously [Jarvis *et al.*, 1997]. MAPK activity was assayed after immunoprecipitation of p42-ERK1/p44-ERK2 using MBP as substrate. Preimmune controls were also run to ensure selectivity of substrate phosphorylation. Reaction mixtures consisted of immunoprecipitated enzyme, substrate, and [γ-^{32}P]ATP (5000 Ci/pmol) in 25 mM HEPES, pH 7.4, containing 15 mM $MgCl_2$, 100 mM trisodium *ortho*vanadate, 0.01% (v/v) 2-mercapto-

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ethanol, and 1 μM microcystin LR. Reactions were initiated by the addition of substrate. MAPK-ERK reactions were terminated by transfer to p81 filter paper; filters were rinsed repeatedly in 185 mM *ortho*phosphoric acid and then dehydrated in acetone. Total radioactivity resident in filters was determined by non-aqueous liquid scintillometry.

Statistical analyses. In all cases, quantitative determinations are expressed as the mean ± SEM, derived from replicate (n) values, with adequate replicative trials, as indicated. Conventional analysis of variance, in conjunction with ythe Bonferroni test for multiple comparisons (Wallenstein, 1980; Grove 1982) were for statistical evaluation of all data sets in order to restrict the overall *P* of type-I error within acceptable ranges, as Fscited in the text.

RESULTS

Inhibition of phenethylisothiocyanate-substituted sphingoid base analogs.

The *N*-(*N*'-phenethylthiocarbamoyl) adducts of sphinganine and sphingosine were prepared and purified as outlined in previous reports [Xu & Thornalley, 2000]. Conventional structural projections of the resulting derivatives, PEITC-Sa and PEITC-So, are presented in *Figure 1*; it should noted that, apart form the isothiocyanate substituents, these synthetic lipid are otherwise identical with their naturally occurring counterparts.

Initial studies assessed the capacity of PEITC-substituted sphingoid bases to modify cPKC/nPKC activity. HL-60 cells were exposed to a synthetic preparation of the naturally lipid sphinganine, an isothiocyanate-substituted form of sphinganine (PEITC-Sa), or the clinically relevant sphinganine analog safingol for 30 min in serum-free medium; cPKC/nPKC was then recovered from particulate and soluble subcellular fractions and subjected to *in vitro* assay in the presence of synthetic diglyceride. As shown in *Figure 2*, only ~30% ($p \leq 0.001$) of the total diglyceride-sensitive cPKC/nPKC activity was available to the particulate (*i.e.*, membrane) fraction under basal conditions. A brief treatment with sphinganine, PEITC-Sa, or safingol resulted in a substantial redistribution of essentially all assayable PKC activity back into the soluble cytosolic fraction ($p \leq 0.001$), however, consistent with the patterns of acute cPKC/nPKC inhibition that we previously obtained in HL-60 cells. Related test exposures compared alterations in cPKC/nPKC activity in response to synthetic sphinganine, sphingosine, and the corresponding derivatives. Each of these lipids effectively depleted cellular membrane fractions of diglyceride-sensitive PKC activity by ≥95%(*Table 1A*); of equal interest, while free sphinganine suppressed cPKC/nPKC activity almost as effectively as PEITC-Sa, free phenethylisothiocyanate alone over the same range of concentrations failed to modify activity discernibly (*Table 1B*).

Intrinsic cytotoxicity of PEITC-substituted sphingoid bases

Other studies compared the lethal effects of PEITC-derivatized sphingoid bases with respect to cytotoxic potential. Acute (6-hr) exposure of HL-60 cells to the physiological lipids sphingosine and sphinganine and the corresponding analogs PEITC-So and PEITC-Sa potently elicited apoptotic DNA degradation and cell death (to 85%-95% of the total treated fraction; $p \leq 0.001$; *Tables 2A & 2B*).

Direct comparisons of the HL-60 cell response to sphinganine, PEITC-Sa, and the therapeutically relevant synthetic sphinganine analog safingol (also designated *SPC-100270*) are presented in *Figures 3 & 4*. The relative apoptotic potential of all three lipids was compared. Exposure of cells to PEITC-Sa (10 μM) or sphinganine (10 μM) for 6 hr resulted in extensive induction of apoptotic cell death (81% and 94%, respectively) (*Figure 3A*). The appearance of cytoarchitectural alterations consistent with apoptosis was demonstrated by light microscopy and confirmed by analysis of apoptotic DNA degradation by fluorescence microscopy. These studies were further validated by the demonstrated loss of clonogenic potential (a decline of ≥97%; $p \leq 0.001$) within the population of treated cells (*Figure 3B*). Safingol, on the other hand, proved substantially less effective in inducing apoptosis, initiating cell death in 36% of the cell population but effectively inhibiting ≥90% of clonogenic growth. On the other hand, PEITC-Sa lethality was equivalently matched by calphostin C (also designated UCN-1028c), a photo-activated inhibitor that interacts directly with the (*Table 3*). It was next necessary to verify that modified sphingoid base derivatives were directly capable of inhibiting lipid-sensitive PKC activity; accordingly, the suppressive effects of So and PEITC-So on re-combinant human cPKCα, the predominant isoform expressed in HL-60 cells) were next assessed. Consistent with the *in* vivo cPKC/nPKC responses described above, recom-

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binant cPKCα reconstituted into an optimized phospholipid-based micellar assay system was potently inhibited by sphinganine and PERITC-sphinganine. Thus, while basal activity in this preparation was minimal (≤7% of the total enzyme present), synthetic dioctanoylglycerol (10 μM) elicited a robust stimulation, activating ~90% of the total measurable activity in the reaction mixture within 15 min; in contrast, inclusion of synthetic sphingolipids dramatically reversed this response, such that diglyceride-driven cPKCα activity was reduced ~72% and ~91% by sphinganine and PEITC-sphinganine respectively, whereas safingol was markedly less effective ($p \leq 0.001$ for each; *Table 4*).

Consistent with their observed toxicities, both sphinganine and PEITC-Sa produced marked parallel inhibitions of the basal activities of both cPKC/nPKC (*Figure 4A*) and ERK1/ERK2 (*Figure 4B*). Following acute 6-hr exposures to either lipid, activities of both enzymes were dramatically suppressed, with Sa eliciting an 88% decrease in cPKC/nPKC activity and PEITC-Sa an 83% decrease below that of vehicle-treated cells. ERK1/ERK2 activity was similarly altered with Sa affecting an 89% inhibition and PEITC-Sa a slightly higher 94% inhibition. In line with its decreased apoptotic capacity, safingol produced a substantial (~90%) suppression of cPKC/nPKC, yet inhibited the activity of ERK1/ERK2 only partially (52%).

To assess the time frame in which cPKC/nPKC and ERK1/ERK2 inhibition was occurring, *in vitro* kinase assays were performed. Exposure of HL-60 cells to PEITC-Sa (10 μM) for 90 min elicited an extremely rapid inhibition of both enzymes, with maximal suppression occurring within the first 15 min (*Figure 5*); the response was slightly more pronounced with respect to cPKC/nPKC activity. An essentially identical profile was obtained with PEITC-So (*data not shown*).

Potentiation of drug-induced apoptosis by PEITC-Sa and PEITC-So.

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Other studies characterized the potential of PEITC-substituted sphingoid base analogs to enhance or amplify the apoptotic capacity of araC. Brief (30-min) exposure of HL-60 cells to araC (10 μM) in combination with increasing concentrations (0-500 nM) of PEITC-Sa sharply decreased the activities of cPKC/nPKC and ERK1/ERK2, with maximal inhibition observed at higher lipid concentrations (*Figure 6A*). Furthermore, the suppression of araC-stimulated cPKC/nPKC and ERK1/ERK2 activity correlated directly with the induction of apoptosis (*Figure 6B*). A 48% increase in the onset of apoptotic commitment (at 6 hr; $p \leq 0.05$) was demonstrated in response to a combination of PEITC-Sa (500 nM) and araC (10 μM) over that seen with respect to araC (10 μM) treatment alone.

A concentration-response profile of apoptotic commitment in response to increasing amounts of araC alone or in combination with a fixed concentration of PEITC-Sa was also determined. Cells were exposed to 500 nM PEITC-Sa in combination with varying amounts of araC and cell death assessed at 6 hr. As presented in *Figure 7*, treatment with araC alone resulted in a detectable increase in apoptosis at concentrations of 1 μM. Combination treatments resulted in a leftward shift in the concentration-response profile, with apoptotic cell death occurring at araC concentrations of 100 nM. Furthermore, treatment with PEITC-Sa and araC together dramatically increased the population of nonviable cells, with apoptotic percentages as high as 77% compared to only 31% in response to treatment with araC alone, effectively a doubling of the drug's lethal capacity ($p \leq 0.05$). Throughout these trials, there was no discernible increase in conversion of ara-C to the lethal metabolite ara-CTP by natural sphingolipids of sphingolipid analogs (*data not shown*), demonstrating that the observed augmentation of drug-induced cytotoxicity was unrelated to accelerated ara-CTP formation or extended ara-CTP stability.

Preceding results indicated that both PEITC-Sa and Sa performed more effectively in inducing apoptotic DNA degradation and cell death than did safingol. Likewise,

clonogenicity was shown to correlate inversely with the demonstrated cytotoxic potential of the lipid. Accordingly, all three lipids were further examined for their ability to potentiate araC-induced apoptosis and inhibit clonogenic growth (*Figure 8*). Both Sa (500 nM) and PEITC-Sa (500 nM), in the presence of araC (10 μM) potently induced both apoptotic DNA degradation and apoptotic cell death, with increases of 3.1-fold and 2.7-fold, respectively (significant to a level of $p \leq 0.001$ for both lipids), of the responses observed for araC alone. Parallel treatment with safingol resulted in a modest increase in the lethality of araC. Inhibition of clonogenic growth was directly associated with increased cytotoxic capacity. Surprisingly, while safingol failed to obliterate clonogenicity in araC-treated cultures, its effectiveness was more considerably pronounced at the clonogenic level than may have been anticipated based on its ability to induce apoptosis (*i.e.*, ≥95% suppression for the combination).

DISCUSSION

The present studies assessed the antineoplastic properties of two novel phenethyl-isothiocyanate sphingoid base derivatives. The results demonstrate that PEITC-Sa and PEITC-So exert a substantial antileukemic influence in HL-60 cells. Like sphingosine and sphinganine, PEITC-derived analogs potently inhibit basal and stimulated activity of cPKC/nPKC; the inhibitory properties of these derivatives exhibit comparable efficacy (but somewhat higher potency) than do the effects of their naturally occurring counter-parts. We find that the cytotoxicity of these derivatives is mediated through abrogation of one or more cPKC/nPKC-driven survival signals, as in the case of physiologically rel-evant sphingoid bases or other highly selective inhibitors such as calphostin-C. Together, these findings suggest a useful application for these experimental compounds in the development of PKC-directed chemotherapeutic strategies.

Endogenous sphingoid bases subserve an important physiological function as inhibi-tory effectors of the lipid-sensitive isoforms of PKC [reviewed in Hannun & Bell, 1989]. Similarly, the antiproliferative and often cytotoxic properties of free sphingoid bases is well established [Stevens *et al.*, 1990]. Both sphingosine and sphinganine have been shown to exert lethal influences in a variety of cellular settings, and can potently initiate apoptosis in HL-60 and other cells of hematopoietic origin [Amin *et al.*, 2000; Auzenne *et al.*, 1998; Jarvis *et al.*, 1996, 1997; Klostergaard *et al.*, 1998; Ohta *et al.*, 1994, 1995; Pyne, 2002; Smith *et al.*, 2000; Shirahama *et al.*, 1997; Sweeny *et al.*, 1996].

Current appreciation of an implicit relationship between the lipid-/phorboid-sensitive cPKC/nPKC signaling and cell survival [reviewed by Desai *et al.*, 2002] supports anti-neoplastic applications for agents such as the non-physiological sphingoid base analog safingol (formerly designated SPC-100270) [Rajewski *et al.*, 1995]. Structurally safingol is defined as L-*threo*-sphinganine (*2R,3S*), an enantiomer of the natural lipid D-*erythro*-

sphinganine (*2S,3R*) [Rajewski *et al.*, 1995]. Safingol evokes apoptosis in a variety of human neoplastic cell types *in vitro* [Kedderis *et al.*, 1995]. This cytotoxicity corresponds directly with the inhibition of basal cPKC/nPKC activity [Rajewsk *et al.*, 1995; Kedderis *et al.*, 1995; Jarvis *et al.*, 1998], and consequent alterations in cellular processes further downstream [reviewed in Hoffman, 2001]. Exposure to safingol produces a rapid collapse of the MDR-mediated drug efflux system that is normally sustained via PKC-dependent signals [Sachs *et al.*, 1995]. Beyond retardation of MDR drug efflux rates, however, safingol effectively arrests PKC-driven expression of p-glycoprotein drug transporters at the transcriptional and translational levels, ultimately reducing steady-state levels of *mdr1*/MDR1 and *mdr2*/MDR2 [Castro *et al.*, 1999; Klostergaard *et al.*, 1998]. Other preclinical findings support the use of safingol in chemosensitization. *In vitro* studies have demonstrated potent amplification of the tumoricidal actions of mainstream antineoplastic agents in human tumors, including mitomycin C [Schwartz *et al.*, 1995, Hsueh *et al.*, 2000], irinotecan [Litvak *et al.*, 2003], and vinblastin [Sachs *et al.*, 1995]. Again, these interactions appeared to be in register with direct inhibition of basal and/or drug-stimulated cPKC/ nPKC activity. Substantial potentiation of doxorubicin toxicity has been documented by defined *in vitro* assessments in human tumor cells [Sachs *et al.*, 1995], by parallel *in vivo* evaluations in canine and rodent animal models [Kedderis *et al.*, 1995] and by pilot clinical trials in humans [Schwartz *et al.*, 1997]. Interestingly, while safingol does augment araC lethality in HL-60 cells [Jarvis *et al.*, 1998], we have noted that the effects of this analog on cPKC/nPKC inhibition and apoptotic chemopotentiation are discernibly less than that afforded by naturally occurring sphingoid bases or cPKC/ nPKC-selective agents such as calphostin C. This discrepancy is not presently understood.

Despite widespread interest in the pharmaceutical development of PKC inhibitors as therapeutic reagents, virtually all of the PKC-selective compounds presently under clinical development (*e.g.*, 7-hydroxystaurorine) preferentially target sites within the carboxy-terminal catalytic domain conserved throughout the entire isoenzyme superfamily. Conversely, there are no clinically directed agents that target the aminoterminal regulatory domain of the conventional and novel subfamilies apart from calphostin and safingol. It is also significant that safingol is the only sphingolipid analog developed for selective pharmacological modulation of cPKC and nPKC activity in therapeutic settings.

Other studies from this laboratory in both myeloid (*e.g.*, HL-60) and lymphoid (*e.g.*, REH) leukemias demonstrated that using pro-apoptotic cPKC/nPKC-directed agents such as the *bis*indolylmaleimides (W.D. Jarvis, unpublished observations). The generally high potency and intrinsic cytotoxicity of these compounds, noted in our test exposures, rendered them somewhat unpredictable as chemosensitizing agents. This factor, in part, redirected our interest toward the application of potential modulatory compounds such as sphingoid base analogs.

Recently, Xu and Thornalley [2000] described the synthesis and biological activity of novel phenethylisothiocyanate derivatives of sphinganine and sphingosine. The anticarcinogenic properties associated with a variety of free isothiocyanates — most notably methyl-, benzyl-, phenyl, and allyl-substituted — have been recognized in a variety of settings (Conway *et al.*, 2002), convincingly supporting the use of this class of compounds in chemopreventive applications. Whether such anticarcinogenic properties are retained in more complex isothiocyanate adducts is uncertain. In any case, derivatives such as PEITC-Sa and PEITC-So represent novel compounds for use as antineoplastic agents. Results from initial *in vitro* screening demonstrated that these analogs produce potent suppressions of proliferative capacity in HL-60 cells at high nanomolar concen-

trations; to date, however, the relative apoptotic capacities of PEITC sphingoid bases have not been determined, nor have their effects on cPKC/nPKC activity been characterized.

In the present studies, we have documented both the cytotoxic potential and chemomodulatory capacity of PEITC-Sa and PEITC-So. Specifically, we demonstrated a pronounced and rapid decrease in cPKC/nPKC and MAPK-ERK activity following treatment of HL-60 cells with either sphingolipid analog. Moreover, at high concentrations this reduction in enzyme activity was accompanied by the appearance of morphological and biochemical alterations consistent with apoptosis, while at low (*i.e.*, sublethal) concentrations both PEITC-Sa and PEITC-So produced substantial increases in araC lethality. These findings support those obtained earlier using the cPKC/nPKC modulator, bryostatin and the MAPK inhibitor, PD98059 (Jarvis *et al.*, Mol. Pharm, 1998) and further strengthen the proposed involvement of cPKC/nPKC and downstream MAPK signaling in attenuating the cytotoxic influence of araC. In araC-treated HL-60 cells, drug-induced apoptosis is dramatically amplified upon acute disruption of the MEK-ERK module with flavonoid MEK inhibitors, including aminomethoxyflavone (PD-098059) or trihydroxyflavone (apigenin), but not by simpler hydroxylated flavones or free flavonic acid [Jarvis *et al.*, 1998]. In parallel studies, we similarly have found that the response to araC is effectively exacerbated by MEK-directed bisbutadiene agents; specifically, araC lethality is sharply amplified by the full inhibitor U0126, moderately enhanced by the partial inhibitor U0125, and unaffected by the structurally simplified inactive control U0124. Concordant with these findings, we note that araC action in HL-60 cells is also moderately augmented by the weak ERK inhibitor iodotubercidin (W.D. Jarvis, unpublished observations). In repeated experimental trials, however, we have never discerned that any of the compounds effective in the exacerbation of araC lethality possess intrinsic

cytoxicity in human myeloid malignancies; it is nonetheless noteworthy that at least one report has described such effects of flavonoid compounds in primary cultures of leukemic blasts [Milella, 2001].

Finally, it should be noted that, whereas free isothiocyanates may exert substantial chemopreventive (antiproliferative) influences in some tumors, we found no evidence that free PEITC possesses any intrinsic biological activity in HL-60 cells, even at very high (*i.e.*, mid-millimolar) concentrations. Thus, we conclude that the chemotherapeutic potential of PEITC-Sa and PEITC-So is exclusively related to the biology of the adduct structure itself, rather than to the individual properties of the constituents.

Isothiocyanates have been applied in conjunction with directly toxic agents for combination approaches to cancer treatment. Previous studies have reported similar findings with respect to the cytotoxic properties of the individual compounds when presented independently, as well as those observed in treatments employing the co-exposure of a known toxic reagent in combination with a free isothiocyanate analog; as implied above, the bioactivity of isothiocyanates may depend to a great extent upon both cell type and kinetic circumstances. The current strategy makes use of a biologically active isothiocyanate chemically coupled to a biologically active cytotoxic lipid. In HL-60 cells, the antiproliferative capacity of the resulting compounds, PEITC-Sa and PEITC-So, markedly surpasses the pharmacological effects of either of their constituents when applied singly or together as paired agents. This enhanced therapeutic efficacy may simply reflect enhanced bioavailability of the PEITC adducts compared with natural sphingoid bases, but could also derive from a new essential biochemical property of these compounds. In any case, the cytotoxic effects observed upon treatment of myeloid leukemia cells with either PEITC-Sa and PEITC-So — both alone and in concert with an estab-

lished antileukemic such as araC — suggests a unique opportunity for the development of novel chemosensitizing strategies that utilize PEITC derivatives of sphingoid bases.

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FIGURE LEGENDS

FIGURE 1: Structural projections of phenethylisothiocyanate-substituted sphingoid bases. Stereochemical representations of phenethylisothiocyanate-D-*erythro*-sphinganine (PEITC-Sa) and phenethylisothiocyanate-D-*threo*-sphingosine (PEITC-So).

FIGURE 2: Acute PKC response to sphinganine and PEITC-Sa. HL-60 cells were exposed to synthetic preparations of sphinganine, PEITC-Sa, or safingol for 30 min at equimolar concentrations (10 μM). Relative cPKC/nPKC activity in particulate and soluble fractions was determined by *in vitro* assays as described in *Materials and Methods*. Values reflect the mean ± standard error of three determinations and are expressed as a percentage of basal activity detected in untreated controls. Data shown are from a representative study performed four times with comparable results.

FIGURE 3: Induction of apoptosis by sphinganine and PEITC-Sa. HL-60 cells were exposed to equimolar concentrations of sphinganine, PEITC-Sa, or safingol (10 μM) for 6 hr. ***A.*** Cytocentrifuge preparations of fixed cells were examined for DNA degradation (dark gray bars) using fluorescence microscopy following manual staining with FITC-dUTP in the presence of Tdt. Apoptotic cell death (light gray bars) was evaluated by light microscopy as described in *Materials and Methods*. ***B.*** Suppression of clonogenicity (black bars). All values represent the mean ± standard error of three determinations. Results shown are from a representative experiment performed four times with comparable outcomes.

FIGURE 4: Acute inhibition of PKC and MAPK by sphinganine and PEITC-Sa. HL-60 cells were exposed to equimolar concentrations (10 μM) of sphinganine or PEITC-Sa for 6 hr. ***A.*** cPKC/nPKC (particulate) and ***B.*** MAPK (p42-ERK1/p44-ERK2) activities were determined by *in vitro* kinase assays as described in *Materials & Methods*. Values reflect the mean ± standard error of three determinations and are expressed as a percentage of basal activity detected in untreated controls. Data shown are from a representative study performed four times with comparable results.

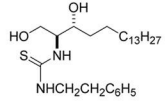
FIGURE 5: Time course of PKC and MAPK inhibition by PEITC-Sa. HL-60 cells were exposed to PEITC-Sa (10 μM) for 90 min. Relative cPKC/nPKC (▼) and MAPK (p42-ERK1/p44-ERK2) (▲) activities were determined by *in vitro* kinase assays as described in *Materials and Methods*. Values reflect the mean ± standard error of three determinations and are expressed as a percentage of basal activity detected in untreated controls. Data shown are from a representative study performed four times with comparable results.

FIGURE 6: Suppression of cPKC/nPKC and MAPK activities and potentiation of araC action by PEITC-Sa. HL-60 cells were exposed to araC (10 μM) with increasing concentrations (10-500 nM) of PEITC-Sa. ***A.*** cPKC/nPKC (▼) and MAPK (p42-ERK1/p44-ERK2) (▲) activities were determined by *in vitro* assay after 30 min. ***B.*** Apoptotic cell death (◆) was assessed after 6 hr. Values reflect the mean ± standard error of

three determinations. Data shown are from a representative study performed four times with comparable outcomes.

FIGURE 7: Amplification of araC cytotoxicity by PEITC-Sa. HL-60 cells were exposed to increasing concentrations of araC (10^{-9}-10^{-4} M) alone (solid line) or in combination (dashed line) with a fixed concentration of PEITC-Sa (500nM) for 6 hr. Cytocentrifuge preparations of fixed cells were examined by light microscopy following manual staining with conventional May-Grünwald-Wright-Giemsa reagents. Values reflect the mean ± standard error of three determinations. Results shown are from a representative experiment performed four times with comparable results.

FIGURE 8: Potentiation of araC related apoptosis. HL-60 cells were exposed to araC (10 μM) in the absence or presence of equimolar concentrations of sphinganine, PEITC-Sa, or safingol (SPC) (500nM) for 6 hr. ***A.*** Apoptotic DNA degradation (single-hatched) was assessed by fluorescence microscopy following manual staining with FITC-dUTP in the presence of Tdt. Apoptotic cell death (cross-hatched) was examined by light microscopy as described in *Materials and Methods*. ***B.*** Suppression of clonogenicity (black bars). Values reflect the mean ± standard error of three determinations. Data shown are from a representative study performed four times with comparable outcomes.



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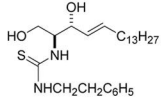

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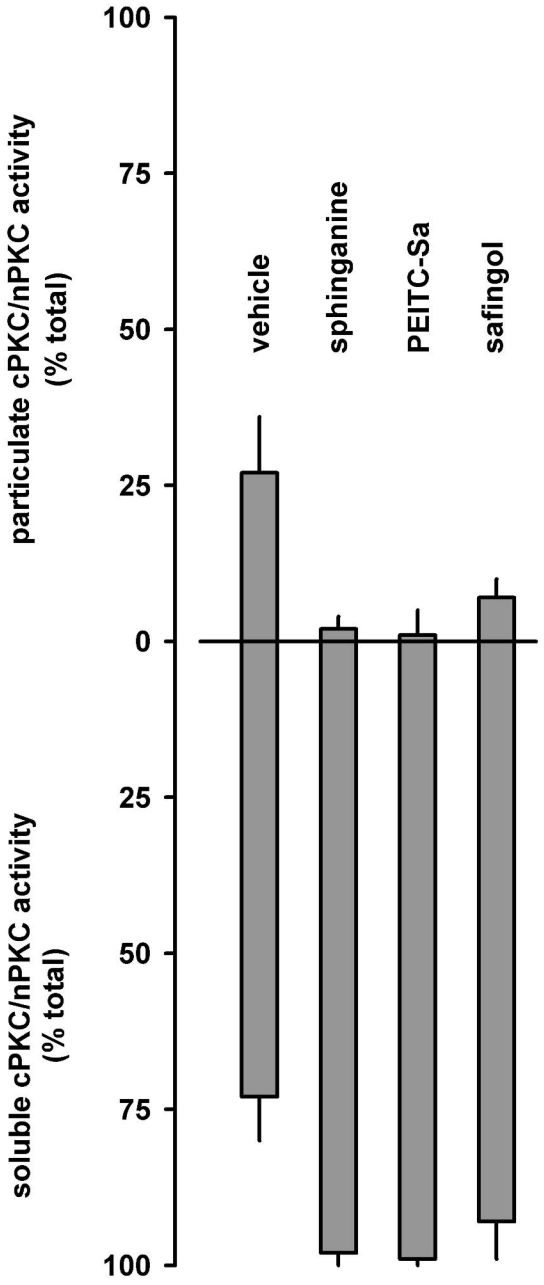

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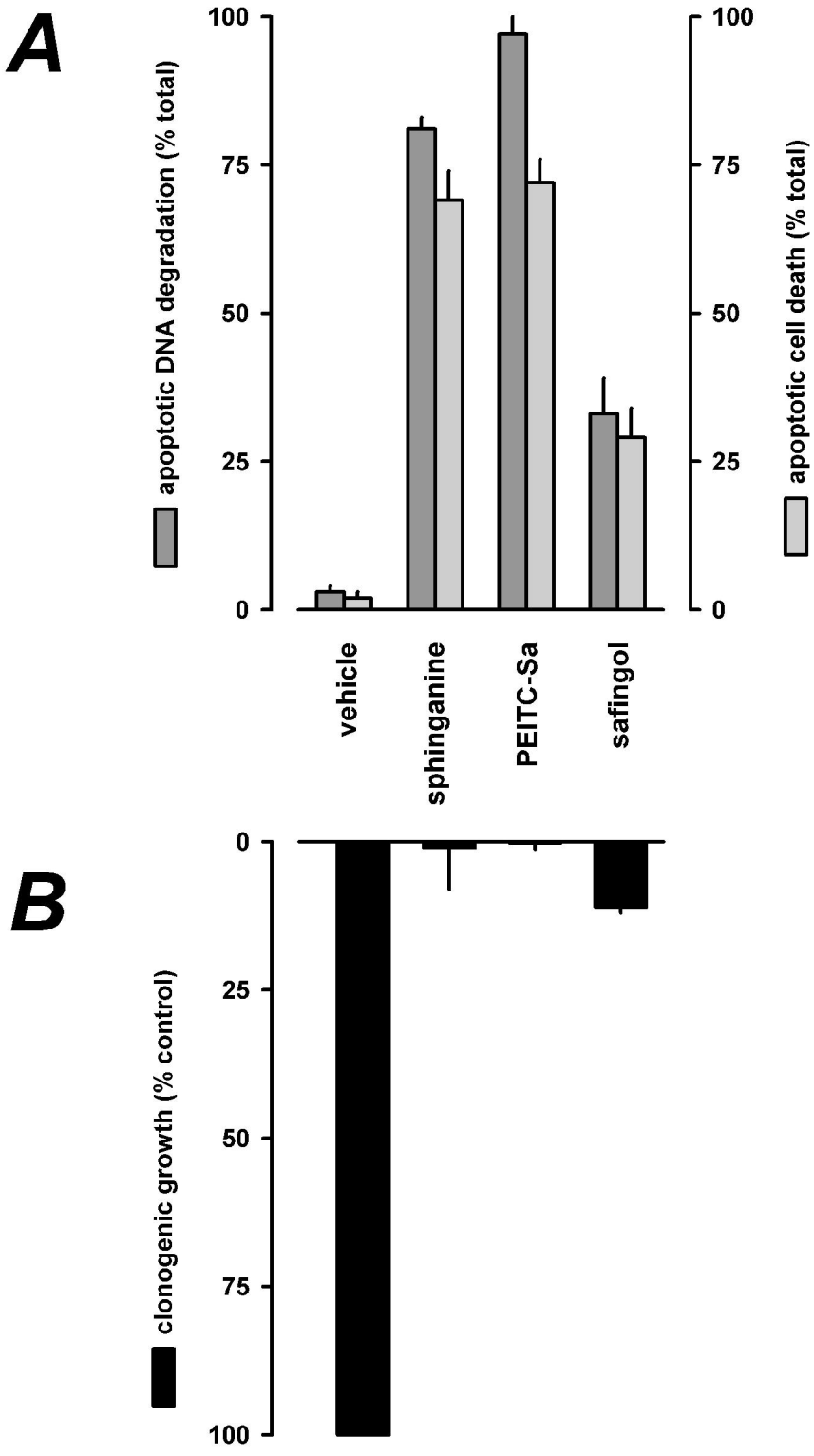

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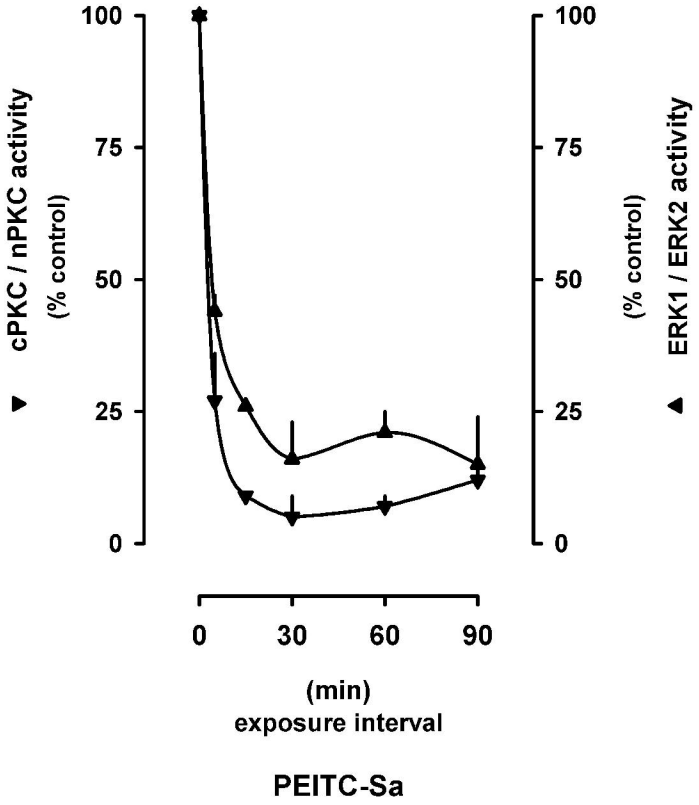
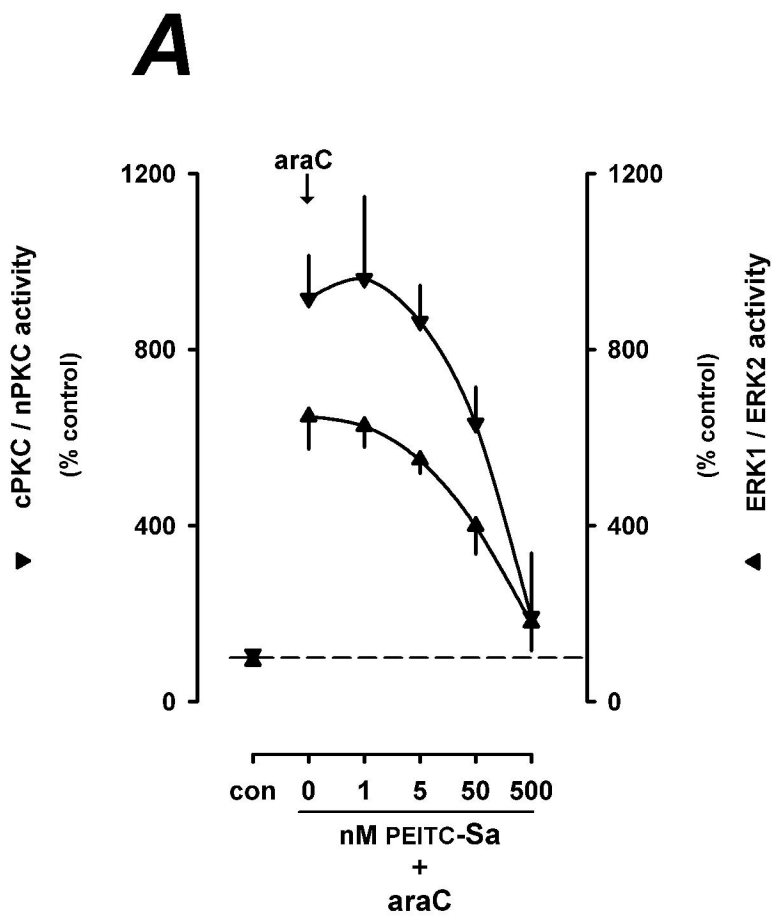
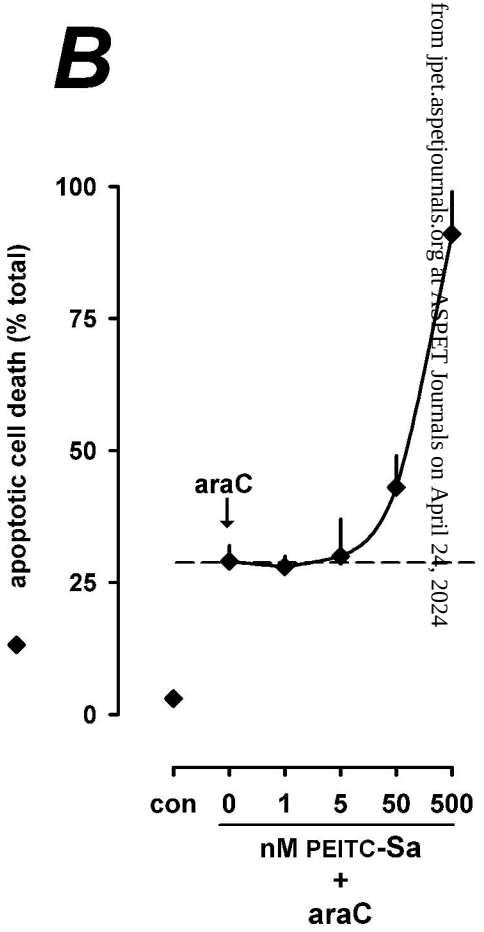
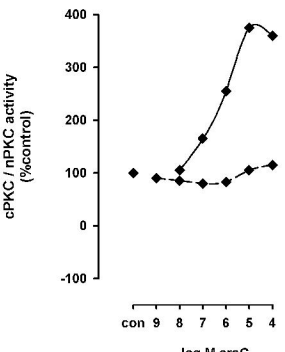
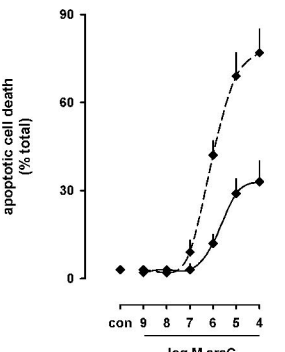







cPKC / nPKC activity
(%control)
400
300
200
100
0
-100
con 9 8 7 6 5 4
-log M araC


apoptotic cell death
(% total)
90
60
30
0
con 9 8 7 6 5 4
-log M araC

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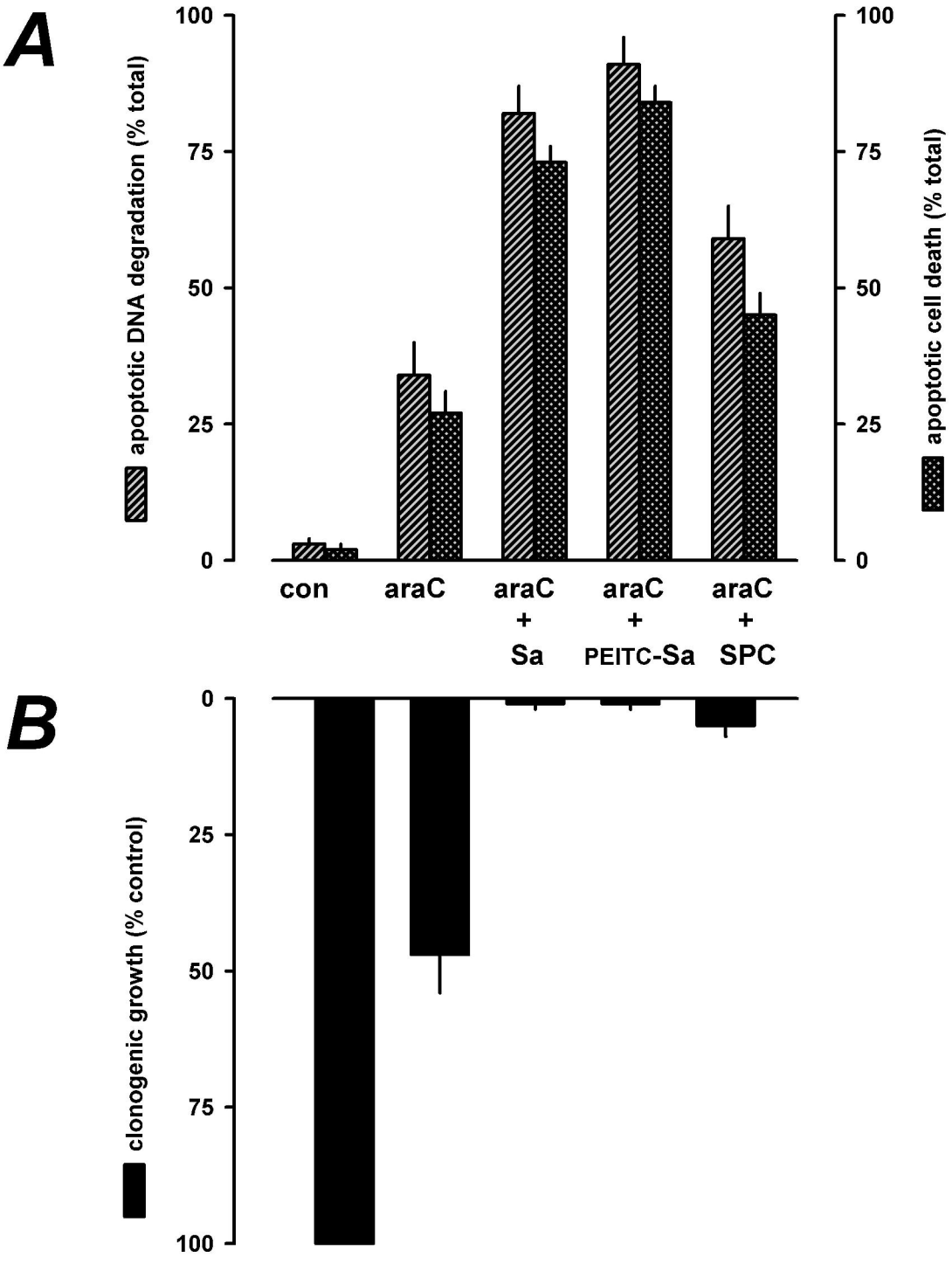

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Table 1a

treatment	*cPKC/nPKC activity (% untreated control)*
vehicle	100 ± 1
So	7 ± 1
Sa	4 ± 4
PEITC-So	3 ± 2
PEITC-Sa	5 ± 1

Table 1b

treatment	*cPKC/nPKC activity (% untreated control)*
vehicle	100 ± 3
PEITC-Sa	6 ± 2
Sa	6 ± 3
PEITC	97 ± 5

TABLE 1: Acute actions of lysosphingolipid analogs on lipid-driven PKC activity.

A. HL-60 cells were exposed to sphingosine (So), sphinganine (Sa), the corresponding PEITC-substituted analogs (PEITC-So, PEITC-Sa), or vehicle for 30 min. **B.** HL-60 cells were exposed to PEITC-sphinganine (PEITC-Sa), free sphinganine (Sa), free (PEITC), or vehicle for 30 min. Membranal cPKC/nPKC activity was then isolated and assayed *in vitro* as described in *Materials & Methods*. Values reflect the mean ± standard error for quadruplicate determinations. Data shown are from a representative study performed at least three times with comparable outcomes.

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Table 2a

treatment	*apoptotic DNA damage (% total)*	*apoptotic cell death (% total)*
vehicle	4 ± 1	2 ± 1
So	87 ± 6	74 ± 7
Sa	84 ± 4	75 ± 3
PEITC-So	93 ± 4	88 ± 6
PEITC-Sa	95 ± 7	84 ± 8

Table 2b

treatment	*apoptotic DNA damage (% total)*	*apoptotic cell death (% total)*	*clonogenicity (% control)*
vehicle	3 ± 0	2 ± 1	100 ± 2
Sa	82 ± 3	74 ± 3	2 ± 1
PEITC-Sa	91 ± 6	80 ± 4	1 ± 0
PEITC	5 ± 2	3 ± 2	92 ± 7

TABLE 2: Effects of lysosphingolipid analogs on leukemic cell survival.

A. HL-60 cells were exposed to sphingosine (So), sphinganine (Sa), the corresponding PEITC-substituted analogs (PEITC-So, PEITC-Sa), or vehicle for 6 hr. **B.** HL-60 cells were exposed to PEITC-sphinganine (PEITC-Sa), free sphinganine (Sa), free (PEITC), or vehicle for 6 hr. Cell survival was evaluated along multiple indices as outlined in *Materials & Methods*. Apoptotic DNA degradation was assessed by fluoroscent microscopy, and apoptotic cytoarchitecture was assessed by light microscopy; clonogenic growth was monitored by soft agar assay for colony formation. All values reflect the mean ± standard error for triplicate determinations. Data shown are from a representative study performed at least three times with comparable outcomes.

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Table 3

treatment	*apoptotic DNA damage (% total)*	*apoptotic cell death (% total)*
vehicle	3 ± 0	4 ± 1
PEITC-Sa	93 ± 4	91 ± 3
safingol	37 ± 3	24 ± 4
calphostin	89 ± 7	77 ± 8

TABLE 3: Relative toxicities of PEITC-sphinganine.

HL-60 cells were exposed to PEITC-Sa or the clinically relevant cPKC/nPKC-directed chemotherapeutic agents safingol and calphostin for 6 hr. The intrinsic cytotoxicity of these agents, defined by microscopic visualization of apoptotic chromatinolysis and cytology were assessed as before. All values reflect the mean ± standard error for triplicate determinations. Data shown are from a representative study performed three times with comparable outcomes.

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Table 4

cotreatment	*% inhibition of dioctanoylglycerol-stimulated cPKCα activity*
vehicle	1 ± 1
sphinganine	72 ± 4
PEITC-sphinganine	91 ± 3
safingol	54 ± 8

TABLE 4: Relative effects of cytotoxic sphingolipids on recombinant cPKCα.

Relative *in vitro* effects of sphinganine (Sa; 5.0 μM), PEITC-sphinganine (PEITC-Sa; 5.0 μM), ad safingol (5.0 μM) on diglyceride-stimulated recombinant human cPKCα purified (enzyme was derived from a baculovirus expression system, then reconstituted in a mixed micelle assay format of phosphatidylserine – phosphatidylcholine – Triton X-100. Results are expressed in terms of relative inhibition of the extent of acute dioctanoyl-glycerol-stimulated cPKCα activity. All values reflect the mean ± standard error for quadruplicate determinations. Data shown are from a representative study performed six times with comparable results.