Natural Sphingadienes Inhibit Akt-Dependent Signaling and Prevent Intestinal Tumorigenesis

Henrik Fyrst, Babak Oskouian, Padmavathi Bandhuvula, Yaqiong Gong, Hoe Sup Byun, Robert Bittman, Andrew R. Lee, and Julie D. Saba

Introduction
Sphingolipid metabolites regulate cell proliferation, migration, and stress responses. Alterations in sphingolipid metabolism have been proposed to contribute to carcinogenesis, cancer progression, and drug resistance. We identified a family of natural sphingolipids called sphingadienes and investigated their effects in colon cancer. We find that sphingadienes induce colon cancer cell death in vitro and prevent intestinal tumorigenesis in vivo. Sphingadienes exert their influence by blocking Akt translocation from the cytosol to the membrane, thereby inhibiting protein translation and promoting apoptosis and autophagy. Sphingadienes are orally available, are slowly metabolized through the sphingolipid degradative pathway, and show limited short-term toxicity. Thus, sphingadienes represent a new class of therapeutic and/or chemopreventive agents that blocks Akt signaling in neoplastic and preneoplastic cells. [Cancer Res 2009;69(24):9457–64]

Abstract
Sphingolipid metabolites regulate cell proliferation, migration, and stress responses. Alterations in sphingolipid metabolism have been proposed to contribute to carcinogenesis, cancer progression, and drug resistance. We identified a family of natural sphingolipids called sphingadienes and investigated their effects in colon cancer. We find that sphingadienes induce colon cancer cell death in vitro and prevent intestinal tumorigenesis in vivo. Sphingadienes exert their influence by blocking Akt translocation from the cytosol to the membrane, thereby inhibiting protein translation and promoting apoptosis and autophagy. Sphingadienes are orally available, are slowly metabolized through the sphingolipid degradative pathway, and show limited short-term toxicity. Thus, sphingadienes represent a new class of therapeutic and/or chemopreventive agents that blocks Akt signaling in neoplastic and preneoplastic cells. [Cancer Res 2009;69(24):9457–64]

Materials and Methods
Antibodies and reagents. Anti-Akt, anti–4EBP-1, anti–phospho-4EBP-1, anti–poly(ADP-ribose) polymerase (PARP), and horseradish peroxidase–linked anti-rabbit IgG antibodies were from Cell Signaling Technology. Anti-actin antibody, monodansylcadaverine (MDC), 3-methyladenine (3-MA), and insulin-like growth factor 1 (IGF-1) were from Sigma-Aldrich. n-Erythro-C14:So and N-acetyl-v-erythro-C14:So (C2-Cer) were from Matreya. n-Erythro-C17:So, v-erythro-C17:1-SIP, GluCer (soy), and 1-C16d-2-lysoPC were from Avanti Polar Lipids. AC-DEVD-pNA was from BioMol. DMEM with high glucose (DME H-21), fetal bovine serum (FBS), and antibiotics were from University of California, San Francisco, Cell Culture Facility. Cell lines were transfected with FuGENE HD transfection reagent (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen Corporation). Beclin small interfering RNA (siRNA) was from Dharmacon, Inc.

Human cell culture and transfections. HT29, HCT116, SW480, and DLD1 cell lines were from American Type Culture Collection and grown in DME H-21 containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. SW480 cells were transiently transfected with recombinant human-Akt1 containing a hemagglutinin tag and the Src myristoylation (myr) sequence cloned into pcDNA3.0 (myr-Akt1; ref 20) from Addgene. SW480 cells were transiently transfected with light chain 3–green fluorescent protein (LC3-GFP), a construct encoding a microtubule-associated protein 1 LC3-GFP fusion or pDest-mCherry-EGFP-LC3B plasmid DNA (21). In some experiments, cells were cotransfected with pcDNA3-myr-Akt1. HeLa cells were transiently transfected with a plasmid expressing a GFP-Akt1 pleckstrin homology domain (PHD) fusion or with the corresponding mutant GFP-Akt1-PHD (E17K; a gift from James Thomas, Lilly Research Laboratories, Indianapolis, IN; ref. 22).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Cell viability and caspase-3 activity. Lipids and inhibitors were solubilized in ethanol or DMSO from 2.5 to 5 mmol/L and diluted into fresh medium. Cells were grown in medium containing 10% FBS until 70% to 80% confluent and then treated with lipids. Viability of cells was determined by measuring their ability to hydrolyze the tetrazolium compound MTS into formazan using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). After the addition of MTS to the medium, cells were incubated at 37°C and absorbance at 490 nm was determined.

Proliferation Assay (Promega). After the addition of MTS to the medium, cells were incubated for 18 h, followed by visualization using fluorescence microscopy. Some cells transiently transfected with Akt1-PHD GFP fusion constructs were treated with lipids. Viability of cells was determined by quantifying caspase-3 activation in the supernatants as described (23).

Western blotting. Western blotting was performed as described (23). Signals were visualized using the SuperSignal West-Pico kit (Pierce) and quantified by densitometry using ImageJ software (NIH).

Autophagy. SW480 cells transfected with LC3-GFP or pDest-mCherry-GFP-LC3B were treated with SD. Punctate formation was monitored by fluorescence microscopy. Punctate formation was monitored by fluorescence microscopy. Pictures were taken at 3, 6, and 18 h after SD treatment. In some experiments, cells were cotransfected with pcDNA3-myr-Akt1, leading to expression of myristoylated Akt. For beclin downregulation, HeLa cells transiently transfected with Akt1-PHD GFP fusion constructs were treated with lipids. Viability of cells was determined at indicated time points (D). Human colon cancer cell lines were treated with 10 μmol/L C14(4,6)SD. Viability was determined after 18 h (E). Columns, mean of at least three independent experiments; bars, SD.

Figure 1. SDs are cytotoxic to human colon cancer cells. Drosophila SDs have a 14-carbon backbone containing conjugated double bonds at positions 4,5 and 6,7 [C14(4,6)SD]; soy SDs have an 18-carbon backbone with double bonds at positions 4,5 and 8,9 [C18(4,8)SD] (A). HT29 cells were incubated with C14(4,6)SD (white columns), C18(4,8)SD (gray columns), or soy GluCer (black columns). Viability was determined after 18 h by the MTT assay (B). HT29 cells were incubated with C14(4,6)SD. Viability was determined after 18 h by the MTT assay (C). HT29 cells were treated with 10 μmol/L C14(4,6)SD. Viability was determined at indicated time points (D). Human colon cancer cell lines were treated with 10 μmol/L C14(4,6)SD. Viability was determined after 18 h (E). Columns, mean of at least three independent experiments; bars, SD.

Statistical analysis. Statistical analysis was performed using Student’s t test; P values ≤0.05 were considered significant.

Results

SDs inhibit the growth of colon cancer cells. The natural isoforms of fly SD [(2S,3R,4E,6E)-2-amino-tetracdecadiene-1,3-diol] and soy SD [(2S,3R,4E,8E)-2-amino-octadecadiene-1,3-diol; Fig. 1A], hereafter called C14 and C18 SDs, were compared with soy glycosylceramides (GluCer) containing a SD backbone for their effects on the viability of HT29 colon cancer cells. SDs derived from either source inhibited cell viability with similar potency, whereas soy GluCer was less effective (Fig. 1B). Subsequent experiments were performed with C14 SD unless stated otherwise, due to greater availability of this compound. As shown in Fig. 1C and D, SDs inhibited cell viability in a dose- and time-dependent manner. The cytotoxic effect of SDs was also observed in other colon cancer cell lines (Fig. 1E). In contrast, nonmalignant human colon mucosal epithelial cells were less sensitive to the cytotoxic effects of SDs (data not shown).

SDs induce conserved cell death pathways. To determine the mechanism of SD-mediated cytotoxicity, we incubated HT29 cells

Animals. For toxicity study, ten 10- to 12-wk-old male C57BL/6 mice were given, by gavage, 25 mg/kg SD (n = 7) or 0.5% methycellulose vehicle control (n = 3) for 10 d. Subjects were euthanized and phlebotomized, and organs were harvested and fixed. For tumorigenicity study, 12-wk-old Apc(+/−) male mice were given, by gavage, either 25 mg/kg SD (n = 10) or vehicle (n = 10) for 10 d. Subjects were euthanized by CO2 asphyxiation. Grossly visible polyps were counted, measured (diameter), removed at the base, and frozen in LN2. The remaining intestinal tissues were prepared for histologic examination.

Statistical analysis. Statistical analysis was performed using Student’s t test; P values ≤0.05 were considered significant.

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SDs induce conserved cell death pathways. To determine the mechanism of SD-mediated cytotoxicity, we incubated HT29 cells...
for 24 hours with SDs and evaluated the effect of treatment on apoptosis. As shown in Fig. 2A, SDs elicited a 3.5-fold increase in caspase-3 activity compared with vehicle-treated control cells. Apoptosis was confirmed by the presence of the cleaved form of PARP, as shown in Fig. 2B. The presence of the second double bond conferred a 2-fold increase in apoptosis when compared with a 36 405 416 mol/L C14(4,6)SD for 24 h. Caspase-3 activity was determined on whole cell extracts. For control versus myr-Akt-transfected cells, P = 0.0001 (C). Columns, mean of at least three independent experiments; bars, SD.

SDs induce cell death through an Akt-dependent mechanism. Apoptosis and autophagy are both under repressive control by the PI3K/Akt pathway, which is constitutively activated in many cancer cells. To explore the role of the PI3K/Akt pathway in SD-mediated cytotoxicity, the effect of SDs was tested on cells expressing a myristoylated, constitutively active form of Akt (myr-Akt) compared with cells transfected with empty plasmid. As shown in Supplementary Fig. S3A and B, expression of myr-Akt in SW480 cells attenuated SD-mediated conversion of endogenous LC3-I to LC3-II, indicating that autophagy was inhibited. However, at high doses of SD, some conversion was evident. Therefore, to confirm the role of Akt in SD-mediated autophagy, SW480 cells coexpressing LC3-GFP plus myr-Akt or LC3-GFP plus a critical component of autophagic pathways. Downregulation of beclin-1 was accomplished by siRNA, as shown in Supplementary Fig. S4. Whereas 10 μmol/L C18 SD treatment for 24 hours reduced the viability of mock-transfected SW480 cells to 66.7 ± 13% of vehicle-treated controls, SW480 cells lacking beclin-1 maintained 95 ± 9% viability after SD treatment. In addition, autophagosome formation was reduced in SW480 cells downregulated for beclin-1, using pDest-mCherry-EGFP-LC3B as a marker of autophagy (Fig. 3D). These results confirm that SD treatment induces beclin-dependent autophagy.

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Figure 2. SDs induce apoptosis in colon cancer cells. HT29 cells were treated for 24 h with 10 μmol/L C14-So, C14(4,6)SD, or C2-Cer containing a C14-sphingoid base. Apoptosis was evaluated by caspase-3 activity (A) and PARP cleavage (B). HT29 cells transfected with myr-Akt were incubated with 10 μmol/L C14(4,6)SD for 24 h. Caspase-3 activity was determined on whole cell extracts. For control versus myr-Akt–transfected cells, P = 0.0001 (C). Columns, mean of at least three independent experiments; bars, SD.

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plasmid control were analyzed after SD treatment. Figure 3C shows that the induction of autophagy by SDs was almost completely inhibited by constitutive Akt activation, as shown by lack of autophagosome formation. Constitutive activation of Akt also abrogated SD-mediated apoptosis in HT29 cells (Fig. 2C). Thus, SDs induce apoptotic and autophagic cell death programs through an Akt-dependent mechanism.

**SDs inhibit Akt translocation, activation, and signaling in colon cancer cells.** To investigate the effect of SDs on Akt signaling, we treated HT29 cells with SDs for various periods and analyzed expression of total and phosphorylated Akt by immunoblotting of whole cell extracts. Figure 4A and B show that SDs inhibit Akt activation. The effect was apparent from 30 minutes to 3 hours of incubation.

One of the major functions of Akt is the regulation of protein synthesis (26). Akt activation results in mammalian target of rapamycin–catalyzed phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4EBP-1), an inhibitor of protein translation, promoting its release from translational initiation factor eIF4E and thereby stimulating cap-dependent mRNA translation (26). Treatment of HT29 cells with SDs reduced cellular phosphorylated 4EBP-1 compared with total 4EBP-1 (Fig. 4A and B). The inhibition of 4EBP-1 phosphorylation was evident as early as 1 hour after treatment, and by 3 hours, 4EBP-1 phosphorylation was reduced to <60% of control cells. In SW480 cells treated with C14 SD, 4EBP-1 phosphorylation was reduced to 50% after 1 hour and to 20% in 3 hours (Fig. 4C). Taken together, our results show that SDs attenuate Akt activation and signaling, SD-mediated inhibition of the PI3K/Akt pathway and downstream effects on 4EBP-1 would be predicted to inhibit protein translation. To verify this possibility, a cell-free translation assay was devised in which HT29 cells treated with SDs or vehicle were disrupted, and whole cell extracts were evaluated for their ability to translate an mRNA template encoding firefly luciferase into active enzyme. To avoid effects that could potentially be caused by inhibition of amino acid uptake, amino acids were provided in excess in the translation assay. Figure 4D shows that pretreatment of HT29 cells with SDs inhibits protein translation compared with cells treated with lysophosphatidylcholine or vehicle. In contrast to the observed effects on apoptosis, autophagy, and protein translation, SD treatment did not alter the cell cycle profile of HT29 or SW480 cells (Supplementary Fig. S5).

The PHD of Akt proteins is lipophilic and capable of interacting with phosphatidylinositol-tris-3,4,5-phosphate (PIP3), thereby recruiting Akt to the membrane in response to PI3K activation (27). Subsequent to membrane localization, Akt is activated by phosphorylation. We examined the effect of SDs on subcellular localization in HeLa cells of a fusion protein consisting of GFP and the PHD of Akt1 (Akt1-PHD; ref. 22). Similar experiments were conducted in cells expressing a PHD mutant containing a single amino acid substitution in the PHD (Akt1-E17K-PHD) that facilitates interaction with phosphoinositide ligand, leading to
pathologic membrane localization, constitutive Akt activation, and cellular transformation (22). As shown in Fig. 5, Akt1-PHD localized primarily to the cytoplasm of vehicle-treated control cells. As expected, treatment with insulin induced the translocation of Akt1-PHD to the membrane. However, pretreatment with SDs prevented Akt1-PHD translocation in response to insulin treatment. These findings indicate that SDs inhibit Akt translocation by preventing interactions between the PHD and PIP3 that are required for its recruitment to the membrane. In contrast, the subcellular localization of a constitutively membrane-bound mutant Akt1-E17K-PHD was unaffected by SDs, consistent with our finding that cells with constitutively active Akt are able to resist SD-mediated cytotoxicity. These results were further substantiated by subcellular fractionation studies demonstrating that SD treatment of HT29 cells reduces membrane-bound, activated Akt but has no effect on constitutively membrane-bound Akt (Supplementary Fig. S6).

SDs could inhibit Akt translocation by reducing the amount of PIP3 lipid formed in response to insulin stimulation. To address this possibility, phosphatidylinositols in SW480 cells were labeled by incorporation of radioactive phosphate. Cells were then treated with SDs and evaluated for effects of IGF-I administration on the formation of PIP3. In control cells, treatment with IGF-I resulted in a 2-fold increase in the ratio of PIP3/PIP (Supplementary Fig. S7). A similar effect of IGF-I administration was observed in SD-treated cells. These findings indicate that under the experimental conditions used, SD treatment does not affect PIP3 formation.

Figure 4. Akt and 4EBP-1 phosphorylation are inhibited by SDs in colon cancer cells. HT29 cells were incubated with 10 μmol/L SDs for the indicated times. Cells were harvested, and protein levels were evaluated by Western blotting (A). Actin is the loading control. The ratios of phosphorylated Akt to total Akt and phosphorylated 4EBP-1 to total 4EBP-1 were quantified by densitometry (B). SW480 cells were incubated with 10 μmol/L C14(4,6)SD for the indicated times. Actin and phosphorylated 4EBP-1 were evaluated by Western blotting and quantified by densitometry (C). HT29 cells were incubated for 6 h with 10 μmol/L C14(4,6)SD, harvested, and homogenized in lysis buffer, and subjected to centrifugation at 30,000 × g. Supernatants were recovered and their translational activity was measured with the Dual Design Luciferase Reporter Assay System (D). Columns, mean of at least three independent experiments; bars, SD.
SDs suppress intestinal tumorigenicity in vivo. Based on our in vitro findings, we hypothesized that SDs would reduce intestinal tumorigenicity. To test this hypothesis, we assessed the influence in vitro of various compounds, including sphingolipids (12). Further, ApcMin/+ mice have been used to show the chemopreventive effects of many natural compounds, including sphingolipids (12). These results are consistent with the in vitro finding that SDs are effectively metabolized by enzymes of the sphingolipid-degradation pathway but at a slower rate than So (Supplementary Fig. S8A and B).

ApcMin/+ male mice were given food and water ad libitum, and beginning at 80 days of life, either 25 mg/kg body weight of C14 or C18 SDs in 0.5% methylcellulose by gavage for 10 days. Other than a grossly evident reduction in hematocrit, SDs produced no appreciable effects on mouse health parameters, including body weight, hydration status, and activity. Blood chemistries, liver functions, and hematologic parameters were not different in the treated and untreated groups (Supplementary Table S1). Further, no gross or microscopic pathology was noted in major solid organs of SD-treated animals. These results are consistent with the in vitro findings that SDs are effectively metabolized by enzymes of the sphingolipid-degradation pathway but at a slower rate than So (Supplementary Fig. S8A and B).

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Discussion

We have observed that the SD family of natural sphingolipids is cytotoxic to colon cancer. In contrast, GluCer compounds derived from soy were less efficient in affecting the growth of colon cancer cells in vitro, which suggests that they must be metabolized to SDs by brush border enzymes of the gut before acquiring cytotoxic and tumor-preventive properties.

Sphingolipids influence the activity of various cellular targets involved in the regulation of cell survival, proliferation, and apoptosis. Our findings suggest that SDs act by inhibiting Akt signaling. It was shown previously that the unnatural Cer analogue C2-Cer regulates insulin action in 3T3-L1 preadipocytes by interfering with the translocation of Akt to the membrane (31). SDs seem to act in a similar fashion in colon cancer cells but with greater potency than C2-Cer. The immediate downstream consequences of SD-mediated Akt inhibition include a block in protein translation and induction of autophagy. If the SD treatment is sustained, the colon cancer cells may undergo cell death by autophagy or concurrently with apoptosis. Which of these outcomes predominates in a particular cell type is presumably dictated by cellular context.

Mutations in the PI3K/Akt pathway, including activating mutations in PI3K and loss-of-function mutations in the phosphatase PTEN (a negative regulator of PI3K signaling that is produced by a tumor suppressor gene), are commonly found in human malignancies (32). More rarely, activating mutations in Akt have been detected in cancer (22). Activation of this pathway enhances the biosynthesis of macromolecules needed to sustain the growth of rapidly proliferating cells and also
that increasing the oral intake of natural SDs from soy products may provide protection against colon cancer in humans and that direct delivery of SDs may be useful in colon cancer treatment. However, studies that specifically address the efficacy of SDs in preventing colonic tumors are needed to confirm this. Studies conducted over longer periods will be required to fully characterize the toxicity profiles of natural sphingolipids. Since we have shown previously that SIP lyase and other genes in the sphingolipid degradative pathway are downregulated in intestinal polyps of the ApcMin/+ mouse and in human colon cancers, we suspect that intestinal neoplastic tissue may be more sensitive to SDs than surrounding uninvolved tissues (23). This is supported by in vitro studies demonstrating that SIP lyase down-regulation confers greater sensitivity to SDs (Supplementary Fig. S9).

Sphingolipids found in meat and milk are metabolized to So, which is also cytotoxic to colon cancer cells. However, So is less potent than SD, consistent with previous reports demonstrating the importance of the second double bond in Cer potency (35). In addition, So is rapidly metabolized to SIP, and this conversion contributes to the enlargement and progression of intestinal polyps (36). SIP acts through a family of G protein–coupled receptors to promote angiogenesis and block apoptotic pathways (37). SIP activation of its receptors leads to stimulation of the PI3K/Akt pathway (38–40). The major So kinase gene SPHK1 functions as an oncogene and is upregulated in colon cancer; administration of SIP-specific antibodies slows tumor progression and angiogenesis in murine models of cancer (41, 42). Thus, one major concern in the development of strategies to utilize sphingolipids for chemotherapeutic purposes is the potential conversion of endogenous So or sphingolipid analogues to the corresponding phosphorylated sphingoid base. This conversion would create a self-limiting effect and could even have counterproductive effects on cell growth and tumor progression. In this regard, SDs show a distinct advantage over other sphingolipids, as they seem to be poorly converted to SD-phosphates, have a long half-life in intestinal epithelial cells, and the toxicity profile associated with short-term oral delivery of SDs is favorable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/7/09; revised 9/16/09; accepted 10/8/09; published OnlineFirst 11/24/09.

Grant support: NIH grants R01GM085366, CA129438, CA77528 and GM66954 (J.D. Saba), and HL-083187 (R. Bittman).

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doi:10.1158/0008-5472.CAN-09-2341

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