The marine lipopeptide somocystinamide A triggers apoptosis via caspase 8

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Screening for novel anticancer drugs in chemical libraries isolated from marine organisms, we identified the lipopeptide somocystinamide A (ScA) as a pluripotent inhibitor of angiogenesis and tumor cell proliferation. The antiproliferative activity was largely attributable to induction of programmed cell death. Sensitivity to ScA was significantly increased among cells expressing caspase 8, whereas siRNA knockdown of caspase 8 increased survival after exposure to ScA. ScA rapidly and efficiently partitioned into liposomes while retaining full antiproliferative activity. Consistent with the induction of apoptosis via the lipid compartment, we noted accumulation and aggregation of ceramide in treated cells and subsequent co-localization with caspase 8. Angiogenic endothelial cells were extremely sensitive to ScA. Picomolar concentrations of ScA disrupted proliferation and endothelial tubule formation in vitro. Systemic treatment of zebrafish or local treatment of the chick chorioallantoic membrane with ScA resulted in dose-dependent inhibition of angiogenesis, whereas topical treatment blocked tumor growth among caspase-8-expressing tumors. Together, the results reveal an unexpected mechanism of action for this unique lipopeptide and suggest future development of this and similar agents as antiangiogenesis and anticancer drugs.

Results

ScA Induces Apoptosis Selectively via Caspase 8. In initial studies, we reported that ScA isolated from mixed assemblage L. majuscula/ Schizothrix species yielded modest cytotoxic effects against a murine neuroblastoma cell line (11). Initial studies were somewhat limited by the relatively low abundance of this compound. In continuing investigations, we now document that freshly isolated ScA shows potent antiproliferative activity against a number of human tumor cells (Table 1). Microscopic examination reveals that the loss of proliferation is associated with a “blebbing” morphology (Fig. 1A and B), whereas biochemical analysis revealed proteolytic processing of cellular proteins, such as caspase 8 and poly(ADP-ribose) polymerase (PARP), that are hallmark indicators of apoptosis (Fig. 1C). To determine whether caspase 8 expression could account for increased ScA activity, we examined the ability of ScA to induce apoptosis in wild-type Jurkat cells or those deficient in caspase 8. Compared with wild-type cells, apoptosis was dramatically decreased among caspase-8-deficient Jurkat cells exposed to ScA (Fig. 1D). Extending these studies, we knocked down expression of caspase 8 (~80%) by using a short-hairpin RNA (shRNA) approach in the A549 cell line and found a 5-fold loss in sensitivity to ScA (Fig. 1E). Finally, we also tested NB7 neuroblastoma tumor cells, which are deficient for caspase 8 expression, and the matched sister line, NB7C8, which is reconstituted for physiological levels of caspase 8 expression (12). In this case, the expression of caspase 8 increased the potency (IC50) of ScA by 50-fold (Fig. 1F). The results implicate caspase 8 as an effector of apoptosis after ScA treatment.

Caspase 8 is an effector of death receptor (DR)-mediated apoptosis after DR ligation by an appropriate agonist, which triggers the recruitment of the adaptor protein FADD and subsequent formation of the death-inducing signaling complex.

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(13). Accordingly, Jurkat cells lacking FADD were protected from ScA-mediated killing (Fig. 1D), implicating DR-mediated killing in this process. However, NB7C8 cells are resistant to DR-mediated killing (12), and similarly A549 cells do not undergo Fas-mediated apoptosis (ref. 14; our unpublished results), although both cell lines were sensitive to ScA-induced killing. In agreement with these results, the addition of the Fas agonist CH11 did not cooperate with ScA-induced killing. In agreement with these results, the addition of the Fas agonist CH11 did not cooperate with ScA-induced killing. 

**ScA Partitions into Phospholipids and Alters Membrane Structure.**

Because ScA is a lipophilic compound (logP = 10.3), we assessed the capacity of ScA to partition from liquid phase into 100-nm liposomes (nanosomes). Remarkably, intercalation of ScA within the nanosomes was essentially complete within 30 min, with no residual unincorporated drug detected (data not shown). Testing whether the liposome-borne ScA maintained cytotoxic activity, we found that apoptosis induced by treatment of cells with ScA nanosomes mimicked treatments with ScA as a "free" compound (Fig. 2B). Treatment with control, unloaded nanosomes had no effect on cell viability. Together, these results support the potential for future nanosome-based delivery of ScA in vivo, whereas implicating the lipid compartment in ScA-mediated cell death.

In this respect, it is known that alterations to the cell lipid compartment can promote caspase-mediated cell killing. For example, ceramide-enriched membrane domains can promote DR clustering and activation of caspase 8 (17, 19, 20), whereas treatment with arachidonic acid can mediate caspase-3-dependent cell death (21). Therefore, we examined the A549 cells for evidence of alteration to the lipid compartment after exposure to ScA. Interestingly, we observed an accumulation, and aggregation, of cell surface ceramide (Fig. 2B, red channel) that was absent among cells treated with diluent (our unpublished results) or those treated with arachidonic acid (control) (Fig. 2B). This result supported the notion that ScA acted via alterations to the plasma membrane. To evaluate whether observed alterations to membrane lipid distribution corresponded to interactions with apoptotic effectors, in particular caspase 8, we next examined whether these aggregated "clusters" of ceramide in the membrane colocalized with this caspase (Fig. 2B, green channel; colocalization is shown in the yellow channel). The results supported the notion that ScA partitions within cell membranes, alters the lipid compartment and induces the external death pathway in susceptible cells. Although ceramide was used as a reporter of changes within the organization of the lipid compartment, it remains unclear which lipids (if any) directly contribute to apoptosis. However,
it was not simply the lipophilic nature of ScA that resulted in proapoptotic activity. ScA is a disulfide-linked lipopeptide dimer (Fig. 2C), and in parallel structure–function studies we found that individual monomers retained essentially no ability (<0.1% potency) to induce apoptosis. Similarly, chemical modification of either the lipopeptide tail of the molecule or manipulations of the disulfide bond abrogated all tumouricidal activity of the compound. Thus, the combination of lipopeptide and disulfide moieties appears critical to ScA activity.

**Antiangiogenic and Antitumoral Effects of ScA.** Angiogenic endothelial cells are susceptible to apoptosis initiated by caspase 8 (20, 22, 23). We therefore tested the effect of ScA treatment on endothelial cells in vitro and in vivo. Cultured endothelial cells were extremely sensitive to ScA (Fig. 3A), with an IC_{50} in the picomolar range. In agreement with these results, we found that ScA potently blocked endothelial cell tube formation in vitro (Fig. 3B), suggesting that ScA might act both on endothelial and on tumor cells in vivo.

To test this, we first examined whether systemic exposure to ScA blocked developmental angiogenesis in zebrafish. During development of the zebrafish, intersegmental vessels sprout and grow upwards from the dorsal aorta, and then the tips join to form a dorsal vein (Fig. 4A). In a dose-dependent manner (Fig. 4 B–F), ScA blocked blood vessel growth and angiogenesis, although all of the fish remained viable during the 24-h period of the study. Similarly, local introduction of ScA into the chick chorioallantoic membrane potently blocked growth-factor-induced angiogenesis (Fig. 4G). The results demonstrate the sensitivity of endothelial cells to ScA in vivo, in agreement with our observations in vitro, and indicate that ScA acts as an antiangiogenic agent. However, it was not clear whether ScA could act directly on tumor cells in vivo.

To tested the capacity of ScA to directly inhibit tumor growth in vivo, neuroblastoma tumors expressing (or lacking) caspase 8 were seeded into the chick chorioallantoic membrane, and a tumor mass was allowed to establish for 3 days. Tumors then were treated topically with 100 pmol of ScA and allowed to grow a further 5 days before being harvested. In this case, we observed an inhibition of tumor growth selectively in the caspase-8-expressing tumor cells. The results show that, in addition to an antiangiogenic effect, low levels of ScA also can act to inhibit the growth of caspase-8-expressing tumor cells.

**Discussion**

The genetic diversity in the world’s oceans recently has become more widely appreciated (2–4, 24, 25). In particular, the diversity of biochemical matter that arises from unique and previously uncharacterized metabolic pathways holds promise for the development of new compounds in the treatment of human malignancy. This study focuses on the activity of ScA, a lipopeptide derived from the cyanobacteria *L. majuscula* (26, 27). We show here that ScA selectively activates a caspase-8-dependent cell death pathway. Interestingly, apoptosis occurs among tumor lines that are normally resistant to treatment with DR agonists that act via caspase 8, which is of particular interest because many tumors tend to maintain caspase 8 expression, likely because of its ability also to fulfill nonapoptotic roles (10, 28), but are resistant to DR-mediated killing (29). Other natural products, including the complex heterocycle gambogic acid (30) and the related kaurene diterpene (31), also activate caspase-8-dependent killing. Although gambogic acid is structurally unrelated to ScA and acts via distinct molecular pathways (30), the shared property of caspase 8 activation is interesting, and it is conceivable that such compounds could function as defensive adaptations (9).

ScA induces alterations in the plasma membrane lipid compartment, as indicated by clustering of ceramide on the cell surface associated with activation of caspase 8 and cell death. At micromolar concentrations, ScA can induce cell death via caspase-8-independent pathways, as shown by its capacity to induce apoptosis in caspase-8-deficient cells. This finding is in agreement with other lipid agents, such as ceramide, which can induce apoptosis via several different actions (19, 32). Nonetheless, the cytotoxic actions of ScA at nanomolar and picomolar concentrations are caspase-8-dependent. Our results group the cell lines examined into two categories: those that are sensitive in both lower nanomolar and above concentrations are required to induce cell death (Table 1). All of the more sensitive lines can be killed by caspase-8-mediated pathways. However, we expect that metabolic pathways that regulate the lipid composition
of the plasma membrane will also act as independent factors that can modulate cell sensitivity to ScA. ScA contains a disulfide bond that is anticipated to be reduced upon exposure to the inner leaflet of the plasma membrane. However, it is not yet clear whether covalent modification of membrane components by ScA is necessary to activate (or inactivate) ScA-mediated killing. ScA does represent an uncommon structure among compounds isolated from marine cyanobacteria, containing a disulfide moiety and lipopeptide tails, and the cytoxic activity of ScA requires both lipopeptide and disulfide moieties to initiate caspase-8-dependent death. Truncation of the lipopeptide, or reduction or substitution of the sulfhydryl bonds, abrogated the cytocidal activity of ScA. However, ScA integrated into nanosomes maintained full activity, suggesting an alternative mechanism for delivery in which ScA would be sequestered and protected within a hydrophobic environment, which may be particularly desirable for in vivo applications, based on the ability of liposomes to stabilize and provide a targeting function for ScA. For example, intercalation within nanosomes would be expected to protect the disulfide bond moiety from metabolic degradation after administration in vivo. Thus, nanosomes are an attractive candidate for preclinical delivery of ScA.

Synthesis of ScA involves known organic chemistry reactions and can be accomplished via more than one approach. Currently, we are investigating two distinct reaction schemes to optimize yield and purity for future large-scale production. Storage of ScA in an inert dry atmosphere is anticipated to be necessary to avoid eventual hydrolytic degradation of the unsaturated amide, but the compound is otherwise quite stable. Similarly, the production of the nanosomes involves the assembly from commercially available structural and targeting components, and occurs in quantitative yields via standard liposome production techniques. Thus, ScA represents an active natural compound that appears unencumbered by issues with complex synthesis or upscaling commonly associated with natural products.

The optimization of targetable nanoplatforms that home to specific tumor or vascular beds is rapidly proceeding (33). This therapeutic approach will benefit from both pro-drug approaches and from drugs, such as ScA, that show limited solubility in aqueous media and act directly at the plasma

Fig. 3. Endothelial cells are highly sensitive to ScA. (A) Human endothelial cells were incubated with ScA at decreasing concentrations, as shown, and viability was assessed by XTT assay after 72 h. Data shown are the mean ± SE of triplicate wells from a representative experiment. (B) Human endothelial cells were plated on Matrigel-coated surfaces and allowed to form tubes for 48 h in the presence of DMSO diluent (Top). When ScA was added (Middle and Bottom) cell viability was compromised, and endothelial cell tube formation was disrupted in dose-dependent manner.

Fig. 4. Antiangiogenic and antitumor activity of ScA. (A–F) Transgenic Tg(fli1:EGFP) zebrafish embryos in which GFP is expressed in endothelial cells were incubated without (A) or with increasing concentrations of ScA: 80 nM (B), 160 nM (C), 300 nM (D) 1.6 μM (E), or 3 μM (F). Blood vessel morphology was recorded by fluorescence microscopy. (G) Filter disks impregnated with 100 ng of basic FGF were placed on the chorioallantoic membrane of 11-day-old chicks to induce angiogenesis in the absence or presence of ScA, as shown. After 72 h, disks were removed and the vascularity of the underlying chorioallantoic membrane determined by direct counting of branch points using a dissecting microscope. (H) NB7 neuroblastoma cells lacking caspase 8 (filled bar) or NB7C8 cells reconstituted for caspase 8 expression (open bars) were seeded into 10-day-old chick chorioallantoic membranes to form tumors. After 72 h, ScA was added topically to each growing tumor mass. Tumors were harvested and resected on day 8, and mass (wet weight) was determined. Data shown is the mean ± SD (n = 8–12). The mass of the NB7C8 is significantly decreased (P < 0.002).
membrane of susceptible cells. Toxic and relatively insoluble drugs, such as taxols, may find advantages conferred by nano-platform-mediated delivery. Similarly, we expect that the ability of ScA to readily partition within the nanosize lipid component is advantageous for the targeted delivery of this compound to tumor sites. The targeted nanopharmacology strategy overcomes some issues in terms of solubility and metabolism of ScA while in circulation. In turn, drugs such as ScA do not compete for interior “cargo” space designated for soluble drug payloads. We speculate that such approaches therefore may represent an efficient design for the delivery of combination therapies. Given the sensitivity of proliferating endothelial cells to ScA, it is likely that targeted delivery to cells of the vascular compartment will promote a potent and specific antiangiogenic response.

**Materials and Methods**

**Confocal Microscopy.** For confocal analysis, AS49 cells were seeded on glass coverslips, treated with ScA, DMSO, or arachidonic acid (controls) at 1 or 0.1 μM concentration for 30 min or 6 h. After treatment, the cells were fixed for 10 min with methanol, stained with 1 μg/ml propidium iodide in PBS, permeabilized for 2 min at room temperature with 0.1% Triton in PBS, and blocked for 30 min with 2% BSA in PBS. The staining was performed at room temperature for 2 h, with mouse monoclonal anti-human ceramide (Alexis) and rabbit anti-human caspase 8 (BD PharMingen) antibodies followed by secondary goat anti-mouse Alexa Fluor 588 and goat anti-rabbit Alexa Fluor 488 antibodies (Invitrogen). The blue DNA binding dye TOPRO-3 (Molecular Probes) was added together with the primary antibodies and allowed to react for 1 h. Cells were washed three times with PBS between the different incubation steps, and the incubation with the secondary fluorescently labeled antibodies was performed in the dark. All antibodies were diluted in PBS. Confocal images were recorded on a Nikon Eclipse C1 confocal microscope.

**Cell Lines.** Cells and cell lines were maintained in either DMEM or RPMI medium supplemented with 10% FCS. The caspase-deficient NB7 neuroblastoma cells have been described in ref. 3A. Caspase-8-deficient and reconstituted Jurkat cells were provided by Steve Hedrick (University of California at San Diego). Silencing of caspase 8 gene expression in the AS49 cells was performed through the use of delivering shRNA in a lentiviral format. Briefly, 293 T cells were transfected with casp 8 (Open Biosystems) or scrambled (Addgene) shRNAs in pLKO.1 lentiviral vector, together with lentiviral packaging plasmids (PMLDL, VSV-G, and RSV-REV) using Fugene6. The ratio of target shRNAs and packaging plasmids was 1:1 for infection. At 48 h after the infection, cells were harvested for 24 h and co-transfected with ScA and caspase 8 shRNA plasmids before replacing media with fresh media. Viral constructs were incubated for 24 h with AS49 cells before replacing media with selective media containing puromycin (1 μg/ml). The suppression of caspase 8 was verified by Western blotting analysis.

**Cytotoxicity Assay.** Cytotoxicity of ScA was assessed by using the XTT cell proliferation assay. Briefly, cells were plated on 96-well plates (5,000 per well) and assayed overnight at 37°C to allow for attachment and spreading. After 24 h, ScA was added from a DMSO stock, previously frozen at −80°C, as added directly from serial dilutions in DMSO at concentrations ranging from 100 μM to 100 mM. After 72 h, XTT (Aldrich Chemicals) was added to a final concentration of 250 μg per well. The plates were then incubated under standard tissue culture conditions until the control wells (DMSO) reached an OD value between 1.0 and 1.5. as measured at 450 nm with a microtiter plate reader. The cell viability–drug dilution profiles were obtained from sigma plots, and drug concentrations that inhibited growth by 50% were calculated from multiple runs (IC50). Preparation of Liposomes. Cholesterol:1,2-dioleoyl-sn-glycerol-3-phosphoanhydrolamine (DOPE):1,2-dioleoyl-sn-glycerol-3-phosphocholine (DSPC):scA:1,2-dioleoyl-sn-glycerol-3-phosphoanhydrolamine-N-[(methoxypolyethylene glycol)-
