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Letters

Short Photoswitchable Ceramides Enable Optical Control of Apoptosis

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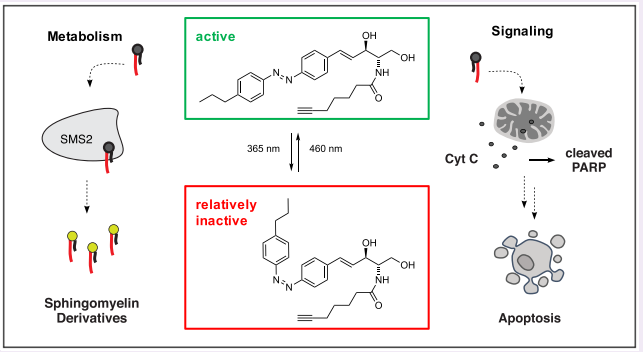
ABSTRACT: We report short ceramide analogs that can be activated with light and further functionalized using azide–alkyne click chemistry. These molecules, termed **scaCers**, exhibit increased cell permeability compared to their long-chain analogs as demonstrated using mass spectrometry and imaging. Notably, **scaCers** enable optical control of apoptosis, which is not observed with long-chain variants. Additionally, they function as photoswitchable substrates for sphingomyelin synthase 2 (SMS2), exhibiting inverted light-dependence compared to their extended analogs.



The central hub of sphingolipid metabolism is occupied by ceramides.[1] They play important roles in signal transduction, cell cycle regulation, and programmed cell death (e.g., apoptosis) and serve as biosynthetic precursors for many highly bioactive lipids.[2] The delivery of ceramides and functionalized analogs to cells has been a long-standing challenge due to the very limited cell permeability and bioactivity of exogenously added long-chain ceramides. To overcome this limitation, short-chain ceramide analogs (e.g. C2, C6, C8) have been employed. These exhibit improved cellular uptake and have markedly increased bioactivity compared to long-chain variants, which are abundant in cells.[3,4]

Photoswitchable lipids containing an azobenzene tail modification allow for the optical modulation of various aspects of lipid biology,[5] including the modulation of ion channels,[6−8] G protein-coupled receptors,[9−11] nuclear hormone receptors,[12−14] immune receptors,[15,16] quorum sensing receptors,[17] and enzymes.[18,19] They include photoswitchable derivatives of sphingosine and sphingosine-1-phosphate[10,20] and photoswitchable ceramides, which have afforded optical control over lipid rafts[21] and sphingolipid metabolism.[19] So far, the photoswitchable ceramides could not be used successfully to control ceramide-mediated signaling pathways in cells, including apoptosis. We reasoned that this limitation could potentially be overcome through the design of short-chain photoswitchable ceramide analogs.

We here describe the synthesis and application of two novel short-chain, clickable, and photoswitchable ceramides (**scaCer1** and **2**, Figure 1). These show improved cellular uptake, bioactivity, and cellular imaging compared to their long-chain congeners (**caCer3** and **4**, Figure 1). These new molecular tools enable optical control of apoptosis in HeLa cells, while the long-chain analogs are completely inactive under comparable conditions. Similar to **caCer3** and **4**, they exhibit optical control of their metabolic conversion, as shown through light-dependent metabolism by sphingomyelin synthase 2 (SMS2).

Design, Synthesis, and Photophysical Characterization of Short-Chain caCer Analogs. The clickable and azobenzene-containing ceramide analogs **caCer3** and **caCer4** contain an azobenzene moiety in their sphingoid base and an alkyne handle for click-derivatization in their *N*-acyl chain.[19] We envisioned designing short clickable and azobenzene-containing ceramide analogues **scaCer1** and **scaCer2**, which contain identical sphingoid bases and a short *N*-acyl chain (Figure 1). This design strategy allows for direct comparison to the established **caCers** and assessment of chain length variations on cellular uptake and bioactivity. **scaCer1** and **scaCer2** were synthesized through an amide coupling reaction from **aSph1** and **aSph2** in good yields (Figure 1B). The synthesis of **aSph1** and **aSph2** has been previously described.[19]

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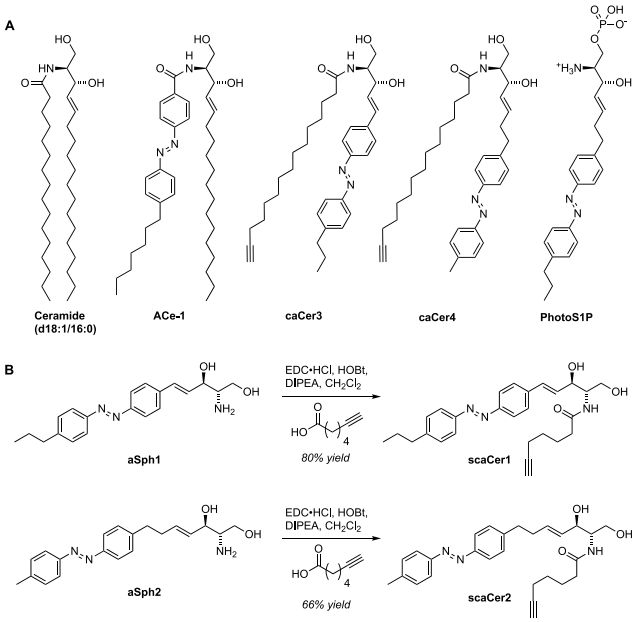

Figure 1. Design and synthesis of **scaCer1** and **scaCer2**. (A) Ceramide (d18:1/16:0) and photoswitchable sphingolipids, including the photoswitchable ceramides ACe-1, **caCer3**, **caCer4**, and a photoswitchable analog of S1P, PhotoS1P. (B) Synthesis of **scaCer1** and **scaCer2**.

Using UV−vis spectroscopy, we then evaluated the photophysical properties of **scaCer1** and **scaCer2**, in direct comparison with **caCer3** and **caCer4** (SI Figure 1). The photolipids were either kept in the dark (*trans*) or treated with blue (λ = 460 nm) or with UV-A (λ = 365 nm) light. The resulting UV−vis spectra demonstrated wavelength-dependent switching as expected for dialkyl-substituted azobenzenes (SI Figure 1A−D). Photoswitching could be repeated over multiple cycles, and all photolipids exhibit considerable thermal stability in the *cis* state in the absence of light (SI Figure 1E−H).

scaCers Exhibit Enhanced Cell Permeability. To quantify the relative cellular uptake of **caCer 3**, **caCer4**, **scaCer1**, and **scaCer2**, we incubated HeLa cells with the respective functionalized ceramide analog (dark-adapted), conducted lipid extraction, and used mass spectrometry (Figure 2B). We found that both **scaCer** variants show markedly increased cellular uptake (>10-fold) compared to the previously reported **caCers**. We further envisioned that **scaCers** could exhibit enhanced utility for the visualization using a clickable fluorophore (diSulfo-Cy5 azide). After labeling and fixation, the **scaCers** were clicked onto diSulfo-Cy5 azide via a copper(I)-catalyzed alkyne−azide cycloaddition (CuAAC). **scaCer1** and **scaCer2** showed higher fluorescence compared to their long-chain structural counterparts **caCer3** and **caCer4** indicative of higher cell loading after incubation (Figure 2C,D). We observed that an increased number of washing steps led to a decrease of the fluorescence especially for **scaCer2**. This result and the more diffuse subcellular localization of labeled **scaCer1** and **scaCer2** suggest that the click product exhibits decreased cellular retention compared to the click product of long-chain analogs.

scaCers Enable Optical Control of Apoptosis. Ceramides are pro-apoptotic lipids, and increased ceramide levels can induce apoptotic cell death (Figure 3A).[22] It has been shown that ceramide translocation to the outer mitochondrial membrane is sufficient to induce apoptosis in cells.[23] Increased ceramide production at the outer mitochondrial membrane and exogenous addition of ceramides also induce cell death.[24,25]

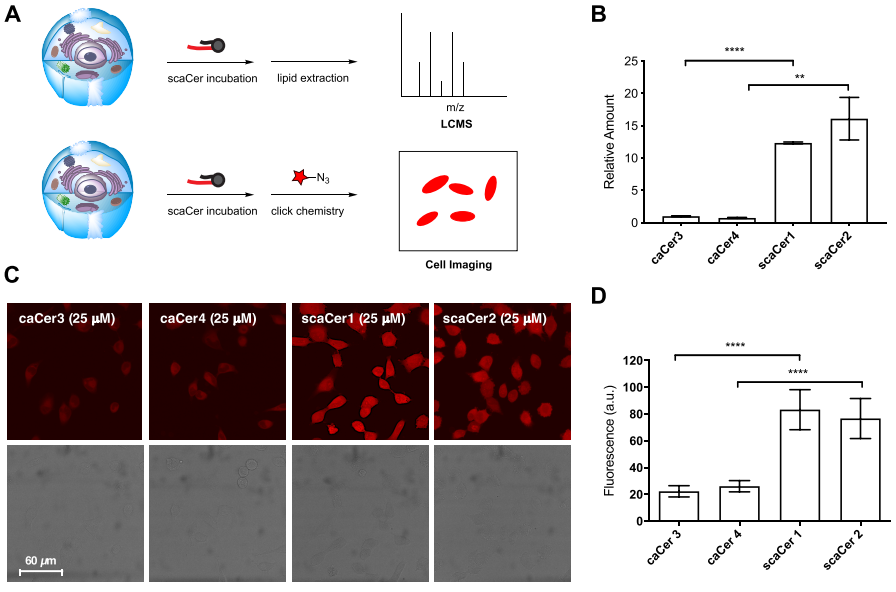

Figure 2. (A) Quantification of **caCer** and **scaCer** (dark-adapted) uptake by mass spectrometry and confocal laser scanning microscopy images of HeLa cells with **caCers** and azido-functionalized fluorophore (diSulfo-Cy5 azide). (B) Quantification of relative cell uptake by mass spectrometry (**caCer4** set to 1). Bars show the average relative uptake including the SD. Unpaired Student's *t* test; $^{**}p < 0.01$; $^{****}p < 0.0001$. (C) HeLa cells were incubated with the indicated **caCer** or **scaCer** variant, fixed with para-formaldehyde, and then labeled with sulfo-cyanin-5-azide by means of CuAAC. Images were obtained with a confocal microscope (λ_{Ex} = 646 nm). Depicted are representative fluorescence and brightfield images. (D) Bars show the average fluorescence intensity including the SD. Two biologically independent experiments were performed, and three images were acquired and analyzed for every condition. A total number of 89 (**caCer3**), 73 (**caCer4**), 92 (**scaCer1**), and 69 (**scaCer2**) cells were analyzed. Unpaired Student's *t* test; $^{****}p < 0.0001$.

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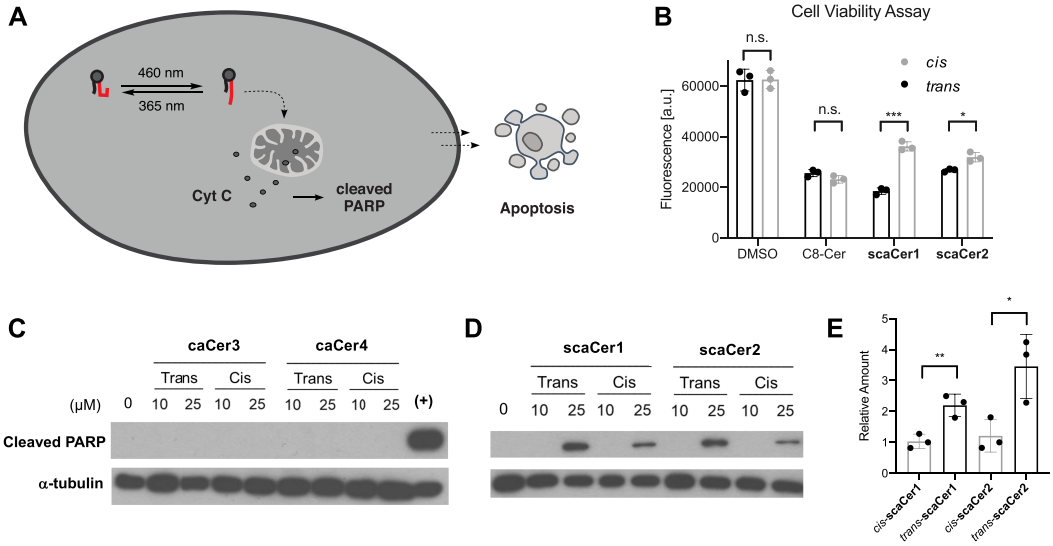

Figure 3. Optical control of apoptosis. (A) Scheme of light-dependent **scaCer**-induced apoptosis. (B) Cell viability assay with HeLa cells with 10 μM C8-ceramide, **scaCer1**, or **scaCer2** after 24 h of incubation with or without 370 nm Cell DISCO[20,27] at 100 ms irradiation every 10 s. The experiment was conducted in technical triplicates and repeated two times with similar results. Unpaired Student's *t* test; *$p < 0.1$; ***$p < 0.001$. (C, D) Immunoblot analysis after treatment of HeLa cells with **caCer3**, **caCer4**, **scaCer1**, and **scaCer2** for 8 h at different concentrations. For treatment with *cis*-photolipids, compounds were preirradiated for 2 min before addition to cells, and cells were irradiated for 2 min after 4 h to keep the photoswitch in *cis*. For treatment with *trans*-photolipid, compounds and cells were kept in the dark. Lane marked with "+" shows the cleaved PARP in an apoptotic sample as positive control. α-Tubulin is the loading control (see SI for details). (E) Quantification of cleaved PARP relative to α-tubulin (*cis*-isomer was normalized to 1 for each blot). Unpaired Student's *t* test; *$p < 0.1$; **$p < 0.01$ (see SI for details).

However, due to the limited cellular uptake of long-chain ceramides, short-chain analogs remain important tools for investigating the biological function of these lipids.[26] In order to test if **scaCers** exhibit potency for apoptosis induction and potentially allow for optical control, we treated HeLa cells with *trans* or *cis* isomers of **scaCers** and conducted a cell viability assay comparing cells in the presence or absence of pulsed irradiation (10 ms every 10 s using a Cell DISCO system).[27] While this irradiation protocol did not change the viability in the presence of DMSO or C8-Ceramide, it had significant effects on cells treated with **scaCer1** and **scaCer2** (Figure 3B). In each case, the *trans* isomer (dark-adapted condition) was found be more potent than the *cis* isomer (pulsed irrdatiation). To show that the biological effects observed are due to the induction of apoptosis, we immunoblotted the cell lysates of HeLa cells treated with isomers of **caCers** and **scaCers** for cleaved Poly-ADP-Ribose Polymerase (PARP), which is a well-known downstream marker of apoptotic activity (Figure 3C,D). We found that **scaCers** can induce PARP cleavage in a light-dependent manner (Figure 3D), whereas **caCers** were inactive (Figure 3C). Both assays are consistent and demonstrate that **scaCer1** and **scaCer2** show enhanced apoptogenic activity in their *trans* form. These results show that **scaCers** have increased bioactivity as compared to long-chain analogs and suggest that they could be useful tools for the optical control of ceramide-dependent biological processes in cell culture.

scaCers Are Light-Sensitive Substrates of SM Synthase. We have previously shown that **caCers** are light-dependent substrates for sphingolipid-metabolizing enzymes and were therefore interested to investigate if **scaCers** also act as photoswitchable substrates ("photosubstrates").[19] To this end, we determined their conversion into sphingomyelin by the enzyme sphingomyelin synthase 2 (SMS2) heterologously expressed in *Saccharomyces cerevisiae* (Figure 4). Crude membrane preparations containing SMS2-V5, but not empty vector control membranes, were capable of SM synthesis (Figure 4C). Moreover, SM production was isomer-dependent: the *trans* isomer (kept in the dark) of **scaCer1** and **scaCer2** was converted faster than the corresponding *cis* isomer (pretreated with UV-A light, Figure 4D). Interestingly, for the long-chain analogues **caCer3** and **caCer4**, the opposite trend was observed: under the identical experimental conditions, both were preferably converted as *cis* isomers (Figure 4D). Thus, **scaCer1** and **scaCer2**, like their long-chain analogues, may be amenable for light-controlled manipulation of ceramide biology.

Concluding Remarks. Herein, we report new short-chain bifunctional ceramides (**scaCers**) that incorporate an azobenzene photoswitch to attain optical control of ceramide biology and an alkyne for click chemistry to visualize and quantify these designer lipids or the metabolites thereof. Both chemical modifications are made to the lipid tails, which allows the retention of the headgroup, leading to a functionalized analog with similar bioactivity. We demonstrated that **scaCers** exhibit markedly improved cellular uptake compared to previously reported long-chain analogs. We further showed induction and optical control of ceramide-dependent apoptosis, which notably could not be induced or controlled with the long-chain analogs **caCer3** and **caCer4**. We further demonstrated that **scaCer1** and **scaCer2** are photoswitchable substrates for ceramide metabolism with light-dependent conversion with SMS2. Taken together, these results suggest that **scaCers** could be useful tools for the spatiotemporal control of ceramide biology. Recently, the Wells laboratory published an alternative strategy to the optical control of apoptosis using a photoreceptor engineered caspase.[28] In contrast to this genetically engineered system, **scaCers** do not require genetic manipulations and could therefore be more widely applicable. Optical control of apoptosis could be particularly

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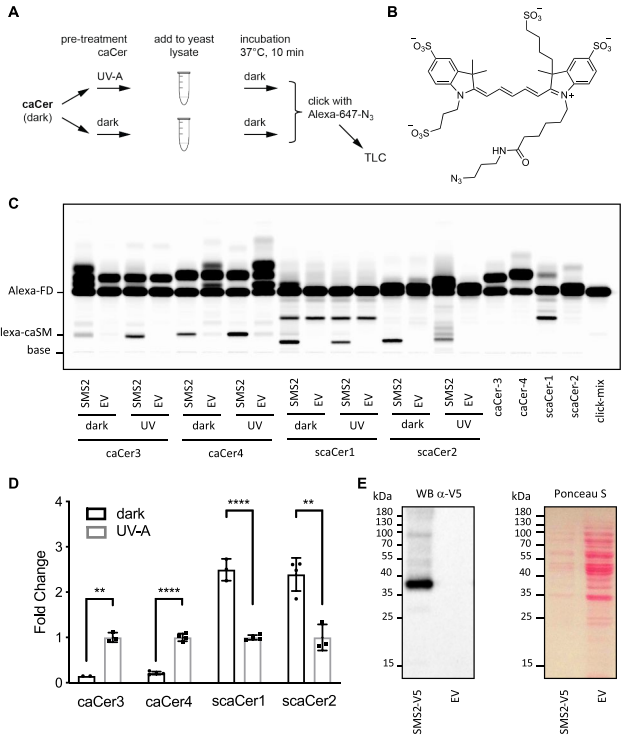

Figure 4. Light-dependent metabolic conversion of **scaCers** by SMS2 in cell lysates. (A) **caCers** and **scaCers** were incubated with lysates, normalized to total protein, of SMS2-expressing yeast or empty vector control cells at 37 °C after preirradiation with UV-A light (365 nm for 1 min) or in the dark. After 10 min, reaction samples were taken, subjected to lipid extraction, click-reacted with Alexa-647-azide (B), and then analyzed by TLC (C). Note that **scaCer1** and **scaCer2** have R_F values that are close to the R_F of the free dye. (D) Quantification of TLC analyses as shown in SI Figure 2, normalized to *cis*, of SM production from **caCers** and **scaCers** in *trans* and *cis*. Error bars represent SEM. ****$p < 0.0001$, **$p < 0.01$, Student's *t*-test. (E) Lysates of yeast cells expressing V5-tagged SMS2 or empty vector control were analyzed by SDS-PAGE and Western blot for V5-SMS2. Total protein content was visualized by Ponceau-S staining of the same blot prior to antibody decoration. Note that for the quality of appearance of both WB and Ponceau panels, the SMS2 containing membranes were diluted stronger than the EV membranes.

interesting in the context of complex cell networks, e.g., for the ablation of cells in development or nervous systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00823.

Experimental details, NMR spectra, photophysical characterization (PDF)

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Notes

The authors declare no competing financial interest.

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