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Combining anticancer agents photodynamic therapy and LCL85 leads to distinct changes in the sphingolipid profile, autophagy, caspase-3 activation in the absence of cell death, and long-term sensitization

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ABSTRACT

Two anticancer agents, LCL85 and photodynamic therapy (PDT) were combined to test whether the combination PDT/LCL85 evokes changes in the sphingolipid (SL) profile and promotes cell death. Treatment of SCCVII mouse squamous carcinoma cells using the silicone phthalocyanine Pc 4 for PDT induced increases in the prodeath global ceramides/dihydroceramides (DHceramides), and no changes in the prosurvival sphingosine-1-phosphate (S1P). In contrast, after LCL85, the levels of most ceramides and DHceramides were reduced, whereas the levels of S1P were increased. After PDT/LCL85 the levels of global ceramides and DHceramides, and of S1P, were restored to resting levels. PDT/LCL85 also enhanced the levels of C18-, C20-, and C20:1-ceramide, and C18-DHceramide. Treatment with PDT, with or without LCL85, led to substantial reductions in sphingosine levels. PDT/LCL85 induced enhanced autophagy and caspase-3 activation. None of the treatments affected short-term viability of cells. In contrast, long-term clonogenic survival was reduced not only after PDT or LCL85, but even more after PDT/LCL85. Overall, our data show that short-term exposure to PDT/LCL85 led to distinct signature effects on the SL profile, enhanced autophagy, and caspase-3 activation without cell death. Long-term exposure to PDT/LCL85 enhanced overall cell killing, supporting translational potential of PDT/LCL85.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality for effective abolishment of malignancies including head and neck cancers. In PDT, a light-absorbing agent (photosensitizer) is activated by highly-focused laser light to trigger oxidative stress and destruction of a cellular target [1]. PDT alone, however, can be ineffective with some tumors. To overcome inefficiency of PDT itself to eradicate tumors, combined treatments become a necessary option.

SL analogs are important for development of combined anticancer treatments [2]. SLs are not only structural components of cell membranes, but can also be involved in modulating biological processes, including apoptosis and macroautophagy (hereafter autophagy) [3,4]. Ceramide is a proapoptotic factor and has been associated with tumor-suppressor functions. S1P is a prosurvival molecule that can have tumor-promoting effects [5,6]. S1P-evoked prosurvival autophagy and ceramide-induced prodeath autophagy in cancer cells are consistent with their roles as tumor-promoting and tumor-suppressor agents, respectively [7]. De novo SL pathway modulates response to anticancer drugs [8,9], including PDT [10–13]. For example, we have shown that silencing sphingomyelin synthase 1, an enzyme that converts de novo ceramide into sphingomyelin, leads to enhanced ceramide and DHceramide accumulation with concomitant promotion of apoptosis [11]. DHceramides have also been implicated in autophagy [14]. Sphingosine, a catabolic product of ceramide, is a proapoptotic factor [15].

We have demonstrated that combining Pc 4–PDT with the cationic C16-ceramide analog LCL30 led to augmented overall killing of mouse SCCVII squamous carcinoma cells (SCCVII cells) [16].

Abbreviations: AO, acridine orange; DEVD, *N*-acetyl-Asp-Glu-Val-Asp; DHceramide, dihydroceramide; LC3, light-chain 3 protein; LCL85, a cationic B13 sphingolipid analog; MS, mass spectrometry; Pc 4, a phthalocyanine photosensitizer; PDT, photodynamic therapy; S1P, sphingosine-1-phophate; SCCVII, mouse squamous cell carcinoma cells; SL, sphingolipids.

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LCL85, a cationic B13 SL analog, is a promising anticancer agent [17]. LCL85-evoked increase in endogenous C16-ceramide is associated with increased cytotoxicity [18]. The purpose of this study was to test the hypothesis that PDT/LCL85 evokes changes in the SL profile and promotes death in SCCVII cells. The model was chosen to use the results of this study for our future experiments in syngeneic mouse SCCVII squamous carcinomas, a recognized mouse model for human head and neck cancers, since intact immune system is an important factor in PDT therapeutic success [1,19].

2. Materials and methods

2.1. Materials

(1*R*,2*R*)-2-*N*-[16-(1′-Pyridinium)-hexadecanoylamino]-1-(4′-nitrophenyl)-1,3-propandiol bromide (LCL85) was synthesized in the Lipidomics Shared Resource (Medical University of South Carolina, MUSC) [17,18]. The phthalocyanine photosensitizer Pc 4, $HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$, was supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). RPMI medium and serum were from Invitrogen and Hyclone, respectively. Anti-LC3 and pan-anti-actin were from MBL International and Neo Markers, respectively. Antibodies against caspases 3, 8 and 9 were from Cell Signaling Technologies.

2.2. Cell culture and treatments

SCCVII cells were grown in RPMI medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 atmosphere, and were treated in the growth medium. For PDT experiments, after overnight incubation with Pc 4 (250 nM) at 37 °C, cells were irradiated with red light (2 mW/cm^2; λ_{max} ~670 nm) using a light-emitting diode array light source (EFOS) at the fluence of 200 mJ/cm^2 at room temperature. The PDT dose (250 nM Pc 4 + 200 mJ/cm^2) used in our studies was chosen based on our previous findings [16]. Pc 4 alone had no effect on any biological effect tested in the presented studies. Following PDT, cells were incubated at 37 °C for 2 h. For PDT/LCL85, LCL85 (20 or 50 μM) was added to the cells 22 h prior to irradiation. For LCL85 or PDT/LCL85, the total incubation time with LCL85 was 24 h. Following treatments, cells were collected on ice and processed for various analyses. For MS analysis, cells were washed twice with cold phosphate-buffered saline (PBS), resuspended in the mixture of ethyl acetate/methanol (1:1, v/v), dried under nitrogen, and shipped overnight on dry ice to the Lipidomics Shared Resource (MUSC) for further processing.

2.3. Electrospray ionization/double mass spectrometry (MS) analysis

Following extraction, SLs were separated by high performance liquid chromatography, introduced to electrospray ionization source and then analyzed by double MS using TSQ 7000 triple quadrupole mass spectrometer (Thermo-Fisher Scientific) as described previously [11].

2.4. Acridine orange (AO) staining

To detect acidic autophagy vesicles, AO was used. AO forms aggregates under acidic conditions that emit red fluorescence (635 nm) [20]. Following treatments cells were stained with AO (5 μg/ml) for 15 min at 37 °C, washed, resuspended in PBS, and analyzed on a BD LSR II flow cytometer (BD Biosciences). A 660/20 band pass filter was used to discriminate AO red fluorescence. Data analysis was done using CellQuest software (BD Biosciences).

2.5. Immunoblotting

Cells were lysed in Laemmli buffer, boiled and then subjected to SDS–PAGE and immunoblotting for LC3, caspase-3, -8 and -9 detection, as reported previously [13,21,22]. Equal protein loading was confirmed following reprobing blots for pan-actin.

2.6. DEVDase (caspase-3-like) activity assay

As described previously [13], DEVDase activity was determined in the cytosol by an assay based on the enzyme's cleavage of a fluorogenic derivative of the tetrapeptide substrate *N*-acetyl-Asp-Glu-Val-Asp (DEVD; Biomol). The fluorescence of the cleaved DEVD substrate was measured using a spectrofluorometer (F-2500 Hitachi; 380 nm excitation, 460 nm emission).

2.7. Trypan blue assay

After treatments cells were harvested, resuspended in cell growth medium, and diluted 1:1 with 0.4% trypan blue stain (Sigma-Aldrich). Stained and unstained cells were counted using a hemocytometer.

2.8. Clonogenic assay

Long-term cell viability was assessed using clonogenic assay according to the modified protocol that we described previously [16]. Plating efficiency was 76% (n = 81) [16].

2.9. Statistical analysis

The effect of each treatment on the levels of SLs was estimated using a linear model in which the dependent variable was the log 2 of the SL level (pmol/mg), while the independent variable was the treatment. Since the MS data came from two experiments, a batch term was included in the linear model to adjust for the batch effects. The nominal p-values were corrected for multiple comparisons using the false discovery rate (FDR) algorithm. Significance was inferred using a 10% threshold on the FDR corrected p-values and the percentage change $\geqslant$20%. Global changes were tested using a one sample t-test based on log 2 ratios (treated/control) of individual SLs within the family of ceramides or DHceramides. All data analysis was performed using the *R* statistical language and environment (www.r-project.org).

3. Results and discussion

3.1. Responses of ceramides to treatments

We have demonstrated that PDT has signature effects on the SL profile in cancer cell lines and in vivo [11,16,22–24]. LCL85 can also have distinct signature effects on the SL profile [18]. In the present study we used MS to determine the effects of the combination, as well as of the individual treatments, on the SL profile in SCCVII cells.

The following patterns in individual ceramide responses were observed: when ceramide levels were not affected by PDT, and were significantly decreased by the drug, they remained below or near resting levels after the combination. Majority of ceramides followed this pattern (Table S1). Among these were the most abundant ceramides, i.e., C16-, C24- and C24:1-ceramide (Fig. 1A; see also Table S2, Fig. S1A). In contrast, a few ceramides, which were among the least abundant (Table S1A, Fig. S1B), followed a different pattern. When PDT significantly increased