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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.

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¹Abbreviations: 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-phosphate; SCCVII, mouse squamous cell carcinoma cells; SL, sphingolipids

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Keywords

autophagy; caspase-3; clonogenicity; LCL85; PDT; sphingolipids

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 be also 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]. 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

(1R,2R)-2-N-[16-(1'-Pyridinium)-hexadecanoylamino]-1-(4'-nitrophenyl)-1,3-propanediol bromide (LCL85) was synthesized in the Lipidomics Shared Resource (Medical University of South Carolina, MUSC) [17, 18]. The phthalocyanine photosensitizer Pc 4, HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)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 caspase 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 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C in a 5% CO₂ 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 milliwatts/cm²; $\lambda_{\text{max}} \sim 670$ nm) using a light-emitting diode array light source (EFOS) at the fluence of 200 mJ/cm² at room temperature. The PDT dose (250 nM Pc 4 + 200 mJ/cm²) used in our studies was chosen based on our previous findings [16]. Pc 4 alone had no effect on any biological outcome tested in the presented studies. Following PDT, cells were incubated at 37°C for two hours. For PDT/LCL85, LCL85 (20 or 50 µM) was added to the cells 22 hours prior to irradiation. For LCL85 or PDT/LCL85, the total incubation time with LCL85 was 24 hours. 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). 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 log2 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 $\geq 20\%$. Global changes were tested using a one sample t-test based on log2 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, including C16-, C24- and C24:1-ceramide followed this pattern (Fig. 1A). In contrast, C18-, C20- and C20:1-ceramide followed a different pattern (Fig. 1B). When PDT significantly increased ceramide levels, and when the effect of LCL85 was minimal, treatment with the combination led to increased or enhanced ceramide accumulation. The exception was C20:1-ceramide, which was significantly increased (76%) in response to LCL85 (50 μ M). Treatment with either combination led to increases in ceramides that were in the case of PDT/LCL85 (50 μ M) significantly greater than after PDT alone. The latter was also true for C22:1-ceramide (Table S2). Globally, ceramides followed the prevailing pattern of individual ceramides, except that their levels were slightly (23%) but significantly elevated after PDT (Fig. 1C, Table S2).

3.2. Responses of DHceramides to treatments

Global DHceramides showed best the prevailing trends: their levels were raised by 20% post-PDT; decreased by 17% and 28% after LCL85 at 20 and 50 μ M, respectively; restored back to resting levels after the combination (Fig. 1C, Table S2). The percent changes were significant. C18- and C22:1-DHceramide, which were among less abundant DHceramides (Table S1, Fig. S2B), were exceptions to the global trends. C22:1-DHceramide is the only DHceramide that was significantly raised (67%) after PDT (Table S2). C18-DHceramide showed enhanced response after PDT/LCL85 (50 μ M) compared to PDT alone (Fig. 1B).

3.3. Responses of other SLs to treatments

The levels of dihydrosphingosine (DHsphingosine), a metabolic precursor of DHceramide were unaffected by individual treatments and were significantly decreased after either combination (Table S2). The levels of sphingosine, a catabolic product of ceramide, were decreased by 58% post-PDT (Fig. 1D, Table S2). LCL85 did not significantly affect sphingosine accumulation, and the combination reduced sphingosine levels by more than 70%. PDT had no significant effect on S1P levels (Fig. 1D, Table S2). LCL85 significantly raised S1P levels, and the combination reversed the effect of the drug by restoring S1P resting levels.

3.4. Short-term viability is not affected by any treatment

To assess the effect of treatments on short-term cell viability, trypan blue exclusion assay was employed. Compared to untreated controls, no treatment was associated with an increase in trypan blue-positive cell fraction (Fig. 2A).

3.5. Caspase-3 is activated after PDT or PDT/LCL85

PDT is a potent apoptotic inducer [25]. To test whether apoptosis is induced after treatments, we used western immunoblotting to test for cleavage of caspase-3, an executioner of apoptosis. We found that the 17 kD-cleavage product of caspase-3 is detectable not only after PDT but also after PDT/LCL85 (50 μ M; Fig. 2B). We also used DEVDase assay to assess the activity of caspase-3. DEVDase activity was increased by 83 and 31% after PDT and PDT/LCL85 (50 μ M), respectively (Fig. 2C).

3.6. AO-positive cells and LC3 processing are observed after PDT or PDT/LCL85

PDT is an inducer of autophagy [26]. Using AO staining to detect acidic vacuoles associated with autophagy, we found a modest increase in AO-positive cells post-PDT, and the effect was enhanced after corresponding combinations (Fig. 2D). AO-positive cells were more abundant after PDT/LCL85 (50 μ M) than after PDT/LCL85 (20 μ M).

Western immunoblotting was used to determine the ratio of LC3-II/LC3-I, an indicator of autophagosome formation associated with autophagy. Following PDT or PDT/LCL85 (50 μ M), corresponding LC3-II/LC3-I ratios were greater than in untreated control (Fig. 2B). LCL85 decreased the LC3-II/LC3-I ratio. The results suggest that autophagy is evoked post-PDT and PDT/LCL85.

3.7. Additional supplemental data

3.7.1 LCL85 is taken up by SCCVII cells—We showed rapid uptake of LCL85 by MCF-7 cells [17]. Here we determined uptake of LCL85 by SCCVII cells using MS. Following a 24 h-incubation of cells with LCL85 at 20 μ M or 50 μ M, there was a significant increase in cell-associated drug levels expressed as pmol/mg (Fig. S1). There was no difference in the uptake between LCL85 and the corresponding combinations.

3.7.2. Effect of LCL85 on short-term cell viability, DEVDase activity, and autophagy—A substantial fraction (0.88) of trypan blue-positive cells was observed only after the highest LCL85 dose (100 μ M; Fig. S3A). No DEVDase activation was detected after various doses of LCL85 (5 –100 μ M; Fig. S3B). A 28% reduction in the enzyme activity was observed after the highest LCL85 dose (100 μ M). Since most of the cells were dead at that concentration (Fig. S3A), DEVDase inactivation is likely a consequence of cell death. We also observed an increase in AO-positive cells with escalating LCL85 doses (Fig. S3C).

3.7.3. No treatment affects caspases 8 and 9—Neither caspase-8 nor caspase-9 was activated after any of treatments (Fig. S4). We did not observe increases in other apoptotic markers (not shown).

3.8. PDT/LCL85 augments total cell killing

To determine the effect of treatments on long-term cell viability, clonogenic assay was used. As shown in Figure 3A, following treatment of cells with 1, 5, and 10 μ M LCL85 led to 7, 19 and 74% total cell killing, respectively. The differences between each dose were significant, supporting a dose-dependent cell killing after LCL85. To test for potential enhancement of cell killing by PDT/LCL85, LDs<30 were chosen for each treatment.

Treatment of cells with LCL85 (5 μ M), PDT, and PDT/LCL85 resulted in 19, 28, and 53% overall cell killing, respectively (Fig. 3B). Thus, PDT/LCL85 led to sensitization of cells to cytotoxic effects of individual treatments.

Since the PDT dose used in these studies was low (LD_{50} <30) and autophagy induced by PDT at low doses is protective [27], we propose that autophagy induced by PDT was a prosurvival response. This idea is further supported by the absence of short-term viability loss. Concomitantly with induction of autophagy, caspase-3 was activated. However, other apoptotic markers, including caspases 8 and 9, tested negative. The data suggest that following PDT, autophagy partially antagonized apoptosis [28]. The complexity of these biological outcomes was paralleled by complex changes in the SL profile, i.e., diverse responses in the prodeath SLs (increases in global ceramides/DHceramides and decrease in sphingosine levels), and no changes in the prosurvival S1P.

Following treatment with LCL85, the levels of most ceramides and DHceramides were reduced, whereas the levels of S1P were increased. Since S1P is involved in the prosurvival autophagy and ceramide in the prodeath autophagy in cancer cells [7], the data suggest that autophagy was a survival response after the drug. This notion is further supported by a dose-dependent increase in autophagic vacuoles, the absence of short-term viability loss, and the absence of caspase-3 activation after LCL85. Since LC3-II tends to be more sensitive than LC3-I in immunoblotting, and this is also dependent on the antibody that is used [29], a decrease in LC3-II/LC3-I ratio observed after LCL85 does not necessarily mean the reduction of autophagy. Autophagy can also be independent of LC3 processing [30].

Treatment with PDT/LCL85 (50 μ M) was associated with increases in autophagy and caspase-3 activation, and no changes in other caspases or short-term viability. The data suggest that autophagy was a prosurvival response that partly opposed apoptosis. Enhanced and selective increases in the proapoptotic ceramides (C18-, C20-, and C20:1-ceramide) and C18-DHceramide correlated, at least in part, with caspase-3 activation. Since C18-DHceramide is a precursor of C18-ceramide, the involvement of de novo ceramide pathway is implicated. This idea is supported by our previous findings [10–13, 31]. A substantial reduction in the levels of the proapoptotic sphingosine post-PDT/LCL85 supports the view of concurrent prosurvival autophagy and defective apoptosis.

Notably, the present study shows enhanced cell killing after long-term exposure to PDT/LCL85 compared to individual treatments. Hence, combining PDT with LCL85 is expected to promote tumor response to PDT, and therefore improve clinical PDT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Two anticancer agents PDT and/or LCL85 affect distinctly the sphingolipid profile
- PDT/LCL85 induces enhanced autophagy, caspase-3 activation, without cell death
- Long-term exposure to PDT/LCL85 leads to enhanced total cell killing

Figure 1.

SL profile after treatments. For PDT/LCL85 treatment, cells were treated with LCL85 (20 or 50 μM) for 22 hours, irradiated with red light (200 mJ/cm^2), and incubated for additional two hours. Exposure to Pc 4 (250 nM) was overnight prior to irradiation. For cells treated with LCL85 alone, the incubation time was 24 hours. Following incubations, cells were collected and processed for MS. The effects of treatments on corresponding SLs are shown as follows: (A) C16-, C24-, C24:1-ceramide; (B) C18-, C20-, C20:1-ceramide, and C18-DHceramide; (C) global ceramides/DHceramides; (D) sphingosine/S1P. The data are shown as the ratios of SL levels in treated vs. control cells. Combined untreated and Pc 4-treated cells were used as controls. For Y-axis, a log scale was used. The boxes contain 50% of the data and the median value is shown as the horizontal thick line within the box. The whiskers extend to the most extreme data point (minimum and maximum value), which is no more than 1.5 times the interquartile range from the box. The data from two independent experiments are shown ($n = 5-7$ per control or each treatment group). The significance $p \leq 0.05$, or for individual SLs, $\text{FDR} \leq 0.1$, and absolute percent change $\geq 20\%$, is indicated as follows: *, treatment is different from control; +, combinations are different from PDT; §, combinations are different from their corresponding LCL85 concentrations; #, P/L50 is different from P/L20; ~, LCL85 doses are different. Con, control; P, PDT; L20, LCL85 (20 μM); P/L20, PDT+LCL85 (20 μM); L50 (50 μM); P/L50, PDT+LCL85 (50 μM). C18-Cer, C18-ceramide, etc. C18-DHCer, C18-DHceramide.

Figure 2.

Effect of treatments on short-term viability, apoptosis and autophagy. (A) None of the treatments has any effect on short-term cell viability (based on trypan blue assay). The data are expressed as fraction of live cells. (B) Caspase-3 is cleaved and LC3 is processed after PDT and PDT/LCL85 (50 μ M). Caspase-3 cleavage products are shown. Representative blots from 3–7 independent Western blot analyses are shown. Equal protein loading was verified using pan-actin immunoblots. (C) DEVDase activity is increased after PDT and PDT/LCL85 (50 μ M). The data are expressed as ratios of treated vs. untreated controls. (D) Enhanced increase in AO-positive cells after the combinations. The data are expressed as (treated minus control) AO-positive cells. (A, C, D) The data are shown as average $\pm$ SEM from 3–8 independent determinations. See Figure 1 Legend for other details.

Figure 3.

Overall cell killing is promoted after PDT/LCL85. Following addition of Pc 4 or LCL85, cells were seeded in the growth medium, preincubated overnight, and irradiated with red light. After eight days of growth at 37°C, colonies (≥ 50 cells) were stained with crystal violet (0.1%) and counted. The data are expressed as percentage of killing and are shown as average $\pm$ SEM from 4–26 independent determinations. (A) Dose-dependent increase in cell killing after LCL85. The significance ($p \leq 0.05$) is shown as follows: *, lower doses (1 or 5 μ M) are different from the highest dose (10 μ M); ~, lower doses (1 and 5 μ M) are different. (B) Total cell killing is augmented after PDT/LCL85. The significance ($p \leq 0.05$) is shown as follows: *, individual treatments are different from the combination; ~, LCL85 is different from PDT.