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C6-pyridinium ceramide sensitizes SCC17B human head and neck squamous cell carcinoma cells to photodynamic therapy

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Abstract

Combining photodynamic therapy (PDT)¹ with another anticancer treatment modality is an important strategy for improved efficacy. PDT with Pc4, a silicon phthalocyanine photosensitizer, was combined with C6-pyridinium ceramide (LCL29) to determine their potential to promote death of SCC17B human head and neck squamous cell carcinoma cells. PDT+LCL29-induced enhanced cell death was inhibited by zVAD-fmk, a pan-caspase inhibitor, and fumonisins B1 (FB), a ceramide synthase inhibitor. Quantitative confocal microscopy showed that combining PDT with LCL29 enhanced FB-sensitive ceramide accumulation in the mitochondria. Furthermore, PDT+LCL29 induced enhanced FB-sensitive redistribution of cytochrome c and caspase-3 activation. Overall, the data indicate that PDT+LCL29 enhanced cell death via FB-sensitive, mitochondrial ceramide accumulation and apoptosis.

Keywords

apoptosis; C6-pyridinium ceramide; death; fumonisins B1; PDT; sphingolipids

¹**Abbreviations:** cyt c, cytochrome c; FB, fumonisins B1; HNSCC, head and neck squamous cancer cell carcinoma; LCL29, C6-pyridinium ceramide; PBS, phosphate-buffered saline; PDT, photodynamic therapy; zVAD, zVAD-fmk.

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1. Introduction

To overcome inefficiency of PDT alone in eradicating some tumors [1–3], combined treatments become a necessary option. The effectiveness of PDT regimens correlates with tumor cell apoptosis [4], and therefore, the proapoptotic sphingolipid ceramide is potentially a good candidate for the combination with PDT. Pyridinium ceramide analogs are water-soluble cationic compounds that have been designed to accumulate preferentially in negatively-charged mitochondria of cancer cells [5–7]. Therapeutically this is important because cancer cells tend to have mitochondria with more negative mitochondrial membrane potential [8]. C6-pyridinium ceramide (LCL29) (Fig. 1, panel A) [7], has been shown to act as an effective anticancer agent, alone or in combination with chemotherapy [5, 6, 9, 10]. We have shown in a syngeneic murine model of head and neck squamous cancer cell carcinoma (HNSCC) that combining PDT with LCL29 augments tumor-associated ceramide accumulation and apoptosis, as well as that the combination improves tumor response to PDT [11–13]. A better understanding of the mechanisms of improved tumor response after the combination is warranted, in order to exploit them to enhance cancer cell killing.

In the present study we used a human HNSCC cell line to address the following questions: does combining PDT with LCL29 advance cell killing? Is cell death mediated by ceramide synthase-dependent ceramide accumulation and apoptosis after the combination? zVAD-fmk (zVAD), a pancaspase inhibitor, was used to assess the role of apoptosis in cell death [14]. Fumonisin B1 (FB), an inhibitor of ceramide synthase, was employed to determine the role of ceramide synthase in ceramide accumulation and apoptotic cell death (Fig. 1, panel B) [15]. Ceramide synthase-dependent reaction in the de novo sphingolipid biosynthesis pathway involves addition of a fatty acyl group to dihydrosphingosine resulting in production of dihydroceramide. Ceramide is generated from dihydroceramide subsequently. FB rescues cells from mitochondrial apoptosis after radiation [16], as well as from cell death after PDT [17]. SCC17B cells were chosen for the study as a clinically-relevant HNSCC model because the cells were derived from a non-metastatic cancer of the larynx, a PDT-treatable HNSCC type [18]. We used Pc4 as the photosensitizer, because our in vitro data showed that combining Pc4PDT with dasatinib, a clinically-approved anticancer agent, enhances cell killing [19]. Also Pc4PDT has been shown to be a promising anticancer treatment in human patients [20, 21].

2. Materials and methods

2.1. Materials

The phthalocyanine photosensitizer Pc4, $\text{HOSiPcOSi}(\text{CH}_3)_2(\text{CH}_2)_3\text{N}(\text{CH}_3)_2$, was kindly provided by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University, Cleveland, OH, USA). C6-pyridinium ceramide [D-erythro-2-N-[6'-1''-pyridinium-hexanoyl] sphingosine bromide; LCL29] was obtained from Avanti Polar Lipids (Alabaster, AL, USA). DMEM/F-12 medium was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum and goat serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors were from the sources indicated in brackets: zVAD-fmk (MBL International Corporation, Woburn, MA, USA), fumonisin B1 (Cayman Chemicals, Ann Arbor, MI, USA). Phosphate-buffered saline (PBS) without calcium and

magnesium used for confocal microscopy was obtained from Life Technologies (Carlsbad, CA, USA).

2.2. Cell culture and treatments

SCC17B cells were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI, USA). Cells were grown in DMEM/F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) in a humidified incubator at 37°C and 5% CO₂. For all experiments, unless indicated otherwise, incubation of cells was carried out in the growth medium under the same conditions as for cell maintenance. All treatments, as well as staining with Mitotracker Red CMXRos (see below) were added to cells in growth medium. After overnight incubation with Pc4, LCL29 was added immediately prior to irradiation. Cells were irradiated at room temperature with red light (200 mJ/cm², 2 mW/cm²; $\lambda_{\text{max}} \sim 670$ nm) using a light-emitting diode array light source (EFOS, Mississauga, ON, Canada), and incubated for 10 h. The inhibitors FB and zVAD-fmk were added 1 h prior to PDT±LCL29.

2.3. Clonogenic assay

Cell survival was assessed using clonogenic assay according to the modified pre-plating protocol, as we described in [22]. Cells were resuspended in growth medium containing Pc4 (20 nM) and seeded (250 cells/well) in a 6-well plate (Thermo Fisher Scientific). After overnight incubation, the cells were irradiated. LCL29 was added immediately prior to irradiation. The inhibitors FB and zVAD were added 1 h prior to PDT±LCL29. After 14 days of incubation, the medium was aspirated, the plates were stained with crystal violet (0.1% in 20% ethanol; Sigma-Aldrich) for 30 seconds, rinsed with water and air-dried. Colonies (> 50 cells) were counted using eCount Colony Counter (VWR International, Radnor, PA, USA). Plating efficiency was 36% (n = 16).

2.4. Quantitative confocal microscopy

Cells were grown on coverslips (Thermo Fisher Scientific) in 6-well plates (Thermo Fisher Scientific). To visualize mitochondria, treated cells were incubated with Mitotracker Red CMXRos (250 nM; Life Technologies) in growth medium for 30 min. After treatments, the coverslips were washed with cold PBS, and fixed by incubation for 15 min in 4% formaldehyde (Thermo Fisher Scientific) in PBS. Cells were washed with PBS, and then permeabilized with ice-cold acetone/methanol (1:1) for 10 min.

Blocking was performed in PBS containing 3% goat serum and 3% fetal bovine serum for 1 h at 4°C. Cells were then incubated at room temperature for 45 min with mouse monoclonal anti-ceramide antibodies (1:30; ALX-804-196; Enzo Life Sciences, Ann Arbor, MI, USA). Cells were washed with cold PBS, incubated at room temperature for 45 min with Alexa 488-conjugated goat anti-mouse monoclonal IgM antibodies (1:200; 115-546-075; Jackson ImmunoResearch, West Grove, PA, USA), and washed again with cold PBS. To visualize nuclei, cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies; 1 µg/ml in cold PBS) for 10 min at room temperature and washed with cold PBS. The coverslips were mounted on slides using the ProLong Antifade Kit (P7481; Life

Technologies). Zeiss LSM780 confocal microscope equipped with a 100×1.4 NA OIL DIC D objective (Carl Zeiss, Thornwood, NY, USA) was used for acquiring the images.

For visualization of cytochrome c (cyt c) distribution, mouse monoclonal anti-cyt c antibodies (1:50; 556432; BD Biosciences, San Jose, CA, USA) were combined with Alexa 594-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch). As a criterion to score the cells positive for the redistribution of cyt c the margin of cyt c staining around the nuclei greater than 10 µm was used. At least 100 cells were assessed for every condition in each experiment. Confocal microscopy imaging was performed for all experiments at the Microscopy, Imaging and Cytometry Resources Core at Wayne State University, School of Medicine.

Quantifications were carried out as follows: ceramide fluorescence associated with the mitochondria were all quantified with MetaXpress software (version 5.00.20; Molecular Devices, LLC; Sunnyvale, CA, USA) as in [23]. Mitotracker antibodies were employed to demarcate the mitochondria. Ceramide intensities associated with the mitochondria were measured with the multiwavelength cell scoring module. The correct mitochondrial identification was verified by visual inspection. All images were corrected for background contribution before quantification. For ceramide associated with the mitochondria a minimum of 195 regions were measured for each data point. Significant differences ($p < 0.05$) were determined using comparison of multiple samples by One-way ANOVA.

2.5. DEVDase (caspase-3) activity assay

As described previously [24], 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; Enzo Life Sciences). The fluorescence of the cleaved DEVD substrate was measured using a spectrofluorometer (F-2500 Hitachi, New York, NY, USA; 380 nm excitation, 460 nm emission).

2.6. Protein determination

Protein content was determined by a modified Bradford assay (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

Significant differences ($p < 0.05$) were determined using Student's t-test or One-way ANOVA.

3. Results and discussion

3.1. Enhanced cell killing after PDT+LCL29 is FB- and zVAD-sensitive

The clonogenic assay was employed to test whether combining PDT with LCL29 improves PDT efficacy. Each treatment was used at LD20, i.e. the dose reducing survival by 20% (or 80% survival), as determined in dose-response studies (not shown). As depicted in Figure 2, combining PDT with LCL29 led to a significant reduction in survival. We have previously shown that FB and the pancaspase inhibitor zVAD protected cells from death after PDT

[17]. Apoptosis is induced after LCL29 [25]. To determine whether apoptosis and ceramide synthase are necessary for enhanced cell killing after PDT+LCL29, we used zVAD and FB. As shown in Figure 2, the inhibitors alone were non-toxic (LD_{50}). FB and zVAD rescued cells from death not only after PDT and LCL29 alone, but also after PDT+LCL29. The data suggest that enhanced cell killing after PDT+LCL29 is mediated by ceramide synthase and caspases.

3.2. PDT+LCL29 enhances FB-sensitive ceramide accumulation in the mitochondria

The biological effects of sphingolipids depend on subcellular site of their generation [26]. We have shown that PDT-induced mitochondrial ceramide accumulation is FB-sensitive [17]. Although it has been shown that LCL29 accumulates in mitochondria of HNSCC cells [6], it is not known whether mitochondrial ceramide accumulation is induced after LCL29 alone or in combination with PDT. Using the anti-ceramide antibody and the mitochondrial marker Mitotracker for quantitative confocal microscopy, we found that PDT and LCL29 alone did induce mitochondrial ceramide accumulation (Fig. 3, panels A and B). Notably, the effect was enhanced after PDT+LCL29. FB inhibited mitochondrial ceramide accumulation after PDT alone or in combination, not after LCL29. Overall, the results suggest ceramide synthase-dependent enhanced mitochondrial ceramide accumulation after PDT+LCL29.

3.3. PDT+LCL29-induced enhanced redistribution of cyt c is inhibited by FB

The mitochondrial apoptosis pathway, including cyt c release, is induced by PDT and LCL29 [17, 25, 27]. Both of these processes are inhibited by FB after PDT [17]. The question is whether cyt c release is regulated by combining PDT with LCL29 and whether the process is ceramide synthase-dependent. Using quantitative confocal microscopy we found that the cells treated with PDT or PDT+LCL29 showed an enhanced redistribution of cyt c staining (Fig. 4, panels A and B). Based on the staining pattern, this could be due to the relocation of mitochondria, mitochondrial swelling, cyt c release into the cytosol, or a combination of these events. In the presence of FB, cyt c redistribution was prevented after PDT alone or in combination, not after LCL29 itself. The data indicate ceramide synthase-dependent cyt c redistribution post-PDT with or without LCL29.

3.4. PDT+LCL29-induced enhanced caspase-3 activation is inhibited by FB

The effect of treatments on caspase-3 activation was tested next. Under conditions used for other short-term studies described thus far, caspase-3 activation was not observed (not shown). At higher doses of PDT and LCL29, however, caspase-3 was activated (Fig. 5). FB inhibited the activation of caspase-3 after PDT alone or in combination, not after LCL29 itself.

Combining PDT with LCL29 is a novel approach, as there have been no reports of this combination, apart from our in vivo studies [11, 12]. The present study shows that enhanced, FB-sensitive mitochondrial ceramide accumulation correlates with enhanced mitochondrial apoptosis and cell killing after combining PDT and LCL29. FB sensitivity of the mitochondrial ceramide accumulation, cyt c redistribution and caspase-3 activation was shown for PDT alone or in combination, but not for LCL29. The data suggest that the FB

sensitivity of these end points after the combination is determined by PDT. The data further support the role for ceramide synthase-dependent mitochondrial ceramide accumulation and apoptosis in advancing cytotoxicity of PDT by combination with LCL29. It remains to be established whether PDT±LCL29 induces formation of ceramide-rich macrodomains in the outer mitochondrial membrane to allow cyt c to be released, as has been shown for radiation-induced, FB-sensitive cyt c release [16].

Mass spectrometry data revealed that cellular levels of ceramides (total and majority of individual ceramides) were increased after PDT (Fig. S1, panel A and panel B), as we showed in [17]. LCL29 did not induce any significant cellular ceramide accumulation. Combining PDT with LCL29 did not raise cellular ceramide levels beyond PDT-induced increases in ceramide. The apparent discrepancy between mass spectrometry and confocal microscopy findings for LCL29 alone and the combination argues for measuring ceramide accumulation not only in whole cells but also at a subcellular level. This is important because the subcellular sites of ceramide accumulation affect the specificity of biological effects of this hydrophobic sphingolipid [26].

We believe that the present study has the following clinical implications: combining PDT with LCL29 is advantageous because lower doses of individual treatments should be required for improved therapeutic benefit. The data presented in this paper, together with our in vivo preclinical data [11, 12] support the translational potential of combining LCL29 with PDT to treat HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- PDT+ C6-pyridinium ceramide (LCL29) enhance caspase-dependent cell death
- Ceramide synthase inhibitor fumonisin (FB) rescues cells from death post-PDT ±LCL29
- PDT+LCL29 enhance FB-sensitive mitochondrial ceramide accumulation
- PDT+LCL29 enhance FB-sensitive cytochrome c redistribution
- PDT+LCL29 enhance FB-sensitive caspase-3 activation

Fig. 1.
Chemical structure of LCL29. Courtesy of Avanti Polar Lipids (panel A). FB inhibits ceramide synthase in the de novo sphingolipid biosynthesis pathway (panel B).

Fig. 2.

Combining PDT with LCL29 enhances loss of clonogenicity in SCC17B cells. The clonogenic potential of PDT±LCL29-treated cells is rescued in the presence of FB and zVAD. FB, zVAD (10 μ M each) were added 1 h prior to PDT (20 nM Pc4+200 mJ/cm²), LCL29 (1 μ M) or the combination. Colonies were stained with crystal violet (0.1%) and counted 14 days after treatments. The data are shown as the average $\pm$ SEM (n = 3–18 samples). Significant differences are shown between: †, treatment and untreated control; #, (treatment + inhibitor) and treatment; *, combination and individual treatments.

Fig. 3.

PDT+LCL29 enhanced FB-sensitive ceramide accumulation in the mitochondria (panels A and B). Cells were treated with FB (10 μ M) 1 h prior to PDT (20 nM Pc4 + 200 mJ/cm²), LCL29 (1 μ M), or the combination, and incubated for 10 h. Incubation with Mitotracker Red CMXRos was carried out prior to immunostaining with anti-ceramide antibodies. Nuclei were visualized with DAPI. All images were acquired by confocal microscopy with identical settings. MetaXpress software was used to quantify ceramide fluorescence located in the mitochondria. Data are shown as the average $\pm$ SEM. The graph depicts ceramide fluorescence/mitochondrial area (panel B). Results were normalized to the untreated control. A minimum of 195 regions were measured for each data point. Significant differences are shown between: †, treatment and untreated control; #, (treatment + FB) and treatment; *, combination and individual treatments. Con, untreated control.

Fig. 4.

PDT+LCL29 enhanced FB-sensitive cyt c redistribution (panels A and B). FB (10 μ M) was added 1 h prior to PDT (20 nM Pc4 + 200 mJ/cm²), LCL29 (1 μ M) or the combination. Incubation time was 10 h post-treatments. After treatments, cells were immunostained with anti-cyt c antibodies. Images were acquired by confocal microscopy using identical settings. The bars show percentages of cells with redistributed cyt c. The data are shown as the average $\pm$ SEM (n=3–4). At least 100 cells were assessed for every condition in each experiment. Significant differences are shown between: †, treatment and untreated control; #, (treatment + FB) and treatment; *, combination and individual treatments (panel B).

Arrow ($\rightarrow$) indicates cell releasing cyt c and arrow head ($\triangleright$) indicates cell with redistributed cyt c. Con, untreated control.

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Fig. 5. PDT+LCL29 enhanced FB-sensitive caspase-3 activation. FB (10 μ M) was added 1 h prior to PDT (250 nM Pc4 + 200mJ/cm²), LCL29 (5 μ M) or the combination. Twenty four hours after treatments, cells were collected and processed for DEVDase assay. The data are shown as the average $\pm$ SEM (n=3–12 samples). Significant differences are shown between: †, treatment and untreated control; #, (treatment + FB) and treatment; *, combination and individual treatments. Con, untreated control. PDT alone data are from [17].