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Enhanced Apoptotic Cancer Cell Killing after Foscan Photodynamic Therapy Combined with Fenretinide via De Novo Sphingolipid Biosynthesis Pathway

Nithin B. Boppana^a, Jeremy Steven DeLor^a, Eric Van Buren^b, Alicja Bielawska^c, Jacek Bielawski^c, Jason S. Pierce^c, Mladen Korbelik^d, and Duska Separovic^{a,b,*}

^aDepartment of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Ave., Detroit, MI 48201, USA

^bKarmanos Cancer Institute, Wayne State University, 4100 John R, Detroit, MI 48201, USA

^cDepartment of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425, USA

^dBritish Columbia Cancer Agency, 675 West 10th Ave., Vancouver, BC V5Z 1L3, Canada

Abstract

We and others have shown that stresses, including photodynamic therapy (PDT)¹, can disrupt the de novo sphingolipid biosynthesis pathway, leading to changes in the levels of sphingolipids, and subsequently, modulation of cell death. The de novo sphingolipid biosynthesis pathway includes a ceramide synthase-dependent reaction, giving rise to dihydroceramide, which is then converted in a desaturase-dependent reaction to ceramide. In this study we tested the hypothesis that combining Foscan-mediated PDT with desaturase inhibitor fenretinide (HPR) enhances cancer cell killing. We discovered that by subjecting SCC19 cells, a human head and neck squamous cell carcinoma cell line, to PDT+HPR resulted in enhanced accumulation of C16-dihydroceramide, not ceramide. Concomitantly, mitochondrial depolarization was enhanced by the combined treatment. Enhanced activation of caspase-3 after PDT +HPR was inhibited by FB. Enhanced clonogenic cell death after the combination was sensitive to FB, as well as Bcl2- and caspase inhibitors. Treatment of mouse SCCVII squamous cell carcinoma tumors with PDT+HPR resulted in improved long-term tumor cures. Overall, our data showed that combining PDT with HPR enhanced apoptotic cancer cell killing and antitumor efficacy of PDT. The data suggest the involvement of the de novo sphingolipid biosynthesis pathway in enhanced apoptotic cell killing after PDT+HPR, identify PDT+HPR as a more effective combination than either treatment alone, and that the combination has potential for cancer treatment.

¹**Abbreviations:** CERS, ceramide synthase; DES, desaturase; DNS, de novo sphingolipid biosynthesis pathway; FB, fumonisins B1; HNSCC, head and neck squamous cancer cell carcinoma; HPR, fenretinide; HPV, human papilloma virus; PDT, photodynamic therapy; zLEHD-fmk, zLEHD; zVAD, zVAD-fmk.

* **Correspondence to:** Dr. Duska Separovic, Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Ave., Detroit, MI 48201, USA. Tel: +1 313 577 8065, dseparovic@wayne.edu.

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Keywords

apoptosis; dihydroceramide; desaturase; fenretinide; PDT; sphingolipids

1. Introduction

Photodynamic therapy (PDT), a cancer treatment modality, can effectively eradicate local malignancies [1]. Because tumors can recur, however, PDT is combined with other anticancer drugs to improve its therapeutic benefit. Sphingolipids include membrane-bound bioactive lipids that can act as anticancer agents [2]. We and others have shown that stresses, including Pc4-mediated PDT, can perturb the de novo sphingolipid biosynthesis (DNS) pathway, leading to changes in the levels of its intermediates, which are potent regulators of cell death [3] [4]. The DNS pathway includes a ceramide synthase (CERS)-dependent addition of a fatty acyl group to dihydrosphingosine resulting in production of dihydroceramide, which is then converted in a desaturase (DES)-dependent reaction to ceramide.

Using Pc4-mediated PDT combined with DNS pathway modulators we demonstrated that enhanced mitochondrial apoptotic cell killing was sensitive to inhibition of CERS, caspase-3, and antiapoptotic Bcl2 [5, 6]. In this study we set out to test the hypothesis that combining Foscan-mediated PDT with fenretinide (HPR), an inhibitor of DES1 [7], the ubiquitous isoform of desaturase, enhances cancer cell killing. The combination includes two clinically relevant agents: HPR, an FDA-approved anticancer drug; Foscan, as the photosensitizer for PDT, a promising treatment of head and neck squamous cell carcinoma (HNSCC) [8–10]. We used SCC19 cells derived from a primary tumor of the base of tongue, a representative of typical HNSCC; they were nonmetastatic, and non-recurrent, and therefore might be representative of types of HNSCC potentially treatable by PDT. Notably, cells were derived from human papilloma virus (HPV)-negative patients [11]. Compared to HPV-positive patients, the prognosis for successful treatment of HPV-negative patients by standard therapies is poor; therefore, using PDT as an alternative, potentially more effective treatment, is all the more relevant [12]. We used the following pharmacological agents to determine their effects on apoptosis and cell death: ABT199 (ABT), an inhibitor of Bcl2 [13]; zLEHD-fmk (zLEHD) and zVAD-fmk (zVAD), a caspase-9 and a pancaspase inhibitor, respectively [14], and fumonisins B1 (FB), an inhibitor of CERS [15].

2. Materials and methods

2.1. Materials

The photosensitizer Foscan (m-tetrahydroxyphenylchlorin, Biolitec AG, Edinburgh, UK) was dissolved (2mg/ml) in a mixture of ethanol:polyethyleneglycol200:water (2/3/5, v/v). HPR [N-(4-hydroxyphenyl) retinamide] and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM/F-12 medium was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Inhibitors were obtained from the sources indicated in brackets: ABT-199 (Selleck Chemicals, Houston, TX, USA), fumonisins B1 (Cayman

Chemicals, Ann Arbor, MI, USA), zLEHD-fmk and zVAD-fmk (MBL International Corporation, Woburn, MA, USA).

2.2. Cell culture and treatments

SCC19 cells were kindly provided by 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 a humidified incubator at 37°C and 5% CO₂. All treatments were added to cells in growth medium. After overnight incubation with Foscan (0.06 µM), HPR (2.5 µM) was added immediately prior to irradiation. Cells were irradiated at room temperature with red light (power density: 2 mW/cm²; fluence: 400 mJ/cm²; $\lambda_{\text{max}} \sim 670$ nm), using a light-emitting diode array light source (EFOS, Mississauga, ON, Canada), and incubated for the desired time. Co-treatments with the inhibitors were performed 1 h prior to PDT±HPR.

2.3. Clonogenic assay

Cell survival was assessed using clonogenic assay according to the protocol that we described in [6]. Plating efficiency was 28% (n = 42).

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

After extraction, sphingolipids 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 we described in [5].

2.5. DEVDase (caspase-3) activity assay

As described previously [16], 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. Tumor model and PDT±HPR treatment

SCCVII squamous cell carcinoma (1×10^6) were implanted subcutaneously in female syngeneic C3H/HeN mice in the sacral lower region on their back, as we showed in [17] [18]. The tumors were allowed to grow until they reached a size of 6–8 mm in largest diameter (7–10 days). The animals were divided into groups for various treatments. Animals were sacrificed when tumor size was 15 mm in diameter. For PDT, mice received intraperitoneal injection of Foscan (0.1 mg/kg). Twenty four hours later tumors were irradiated (power density: 2 mW/cm²; fluence: 50 J/cm²; λ : 650 ± 10 nm), with light

produced by a FB-QTH high throughput illuminator (Sciencetech, London, ON, Canada). HPR was dissolved in dimethylsulfoxide (80 mg/ml), diluted 10 times in an aqueous solution containing 10% polyethylene glycol 400 and 5% Tween-20, and then injected i.p. at 40 mg/kg immediately after PDT. During irradiation animals were kept restrained unanesthetized in metal holders exposing their backs. Thereafter the mice were monitored for tumor regrowth up to 90 days. At that time the mice without visible or palpable tumor were considered cured. The procedures with mice were approved and overseen by the Animal Care Committee of the University of British Columbia.

2.8. Statistical analysis

In vitro studies: significant differences ($p < 0.05$) were determined using Student's t-test. In vivo studies: differences in the response of SCCVII tumors between various treatment groups were compared using the logrank test.

3. Results and discussion

3.1. Accumulation of C16-dihydroceramide is enhanced after FoscanPDT+HPR (The data discussed here are shown in Figure 1 and Supplem Data: Tables I and II, Fig. S1.)

Biological roles of dihydroceramides, intermediates in the DNS pathway, have been recognized [19]. We showed in SCC22A cells, an HNSCC cell line, that knockdown of DES1 suppresses dihydroceramide and ceramide accumulation after Pc4PDT [20]. Using HPR for combination with Pc4PDT we found that HPR induced accumulation of C16-dihydroceramide without affecting the levels of total ceramides [5]. Combining HPR with Pc4PDT led to enhanced C16-dihydroceramide accumulation. In contrast, Pc4PDT+HPR did not enhance the levels of total ceramides [5]. It is not known whether FoscanPDT, alone or in combination with HPR, affects accumulation of these sphingolipids. Here we set out to characterize the signature of FoscanPDT±HPR on the sphingolipid profile in SCC19 cells using mass spectrometry.

Treatment with HPR led to an increase in C16-dihydroceramide levels and a decrease in total ceramides. The levels of the three most abundant ceramides, C16-, C24- and C24:1-ceramide, were all decreased after HPR. Apart from a significant decrease in the accumulation of C22-ceramide, the levels of other ceramides did not significantly change compared to their corresponding resting levels. Similar results were obtained at both time points. The data are consistent with the idea that HPR, as a DES1 inhibitor, by increasing the accumulation of its substrate dihydroceramide, decreases the levels of its product, ceramide.

After FoscanPDT the levels of C16-dihydroceramide, as well as individual and total ceramides, were elevated at 2 and 24 h. However, combining FoscanPDT with HPR led to enhanced accumulation of C16-dihydroceramide, but not ceramides, either individual or total. Specifically, the predominant trend among individual ceramides was that their levels were lower after the combination compared to PDT alone but higher when compared to HPR alone. These trends were also reflected in the levels of total ceramides. Overall, the data suggest the induction of DES1/DNS pathway after FoscanPDT+HPR.

3.2. PDT+HPR enhance mitochondrial depolarization

We showed that Pc4PDT±HPR-induced mitochondrial apoptosis in SCC17B cells [5]. Here we tested whether combining FoscanPDT with HPR induces mitochondrial apoptosis. Mitochondrial depolarization was used as a mitochondrial apoptotic marker. As depicted in Figure 2, FoscanPDT+HPR induced enhanced mitochondrial depolarization in SCC19 cells.

The effect of treatments on caspase-3 activation was tested next. FB inhibited the enhanced activation of caspase-3 after FoscanPDT±HPR at 2 and 24 h (Fig. 3). Overall, the results show that FoscanPDT+HPR induce enhanced mitochondrial depolarization and caspase-3 activation. The data also suggest CERS-dependent caspase-3 activation after FoscanPDT ±HPR.

3.3. Enhanced cell killing after PDT+HPR is CERS-, caspase- and Bcl2-sensitive

We have previously shown that Pc4PDT+HPR-induced enhanced cell death is CERS-, caspase- and Bcl2 inhibition-sensitive [5]. The clonogenic assay was used to determine whether combining FoscanPDT with HPR promotes PDT efficacy. FoscanPDT and HPR were used at their corresponding LD20s determined in dose-response studies (not shown). As shown in Figure 4, combining PDT with HPR led to a significant reduction in survival.

To determine whether mitochondrial apoptosis and ceramide synthase are necessary for enhanced clonogenic cell killing after PDT+HPR, we used FB, ABT, and caspase inhibitors, zLEHD, a caspase-9 inhibitor, and zVAD, a pancaspase inhibitor. The results demonstrated that PDT+HPR induced enhanced clonogenic cell killing, was inhibited by FB, zLEHD, and zVAD and was further increased by ABT (Fig. 4). The data show that enhanced cell killing after PDT+HPR is mediated by CERS and caspases.

3.4. HPR augments cure rates of SCCVII tumors treated by PDT

The enhancement of PDT effects by HPR in vitro can be also demonstrated with mouse tumor models in vivo (Fig. 5). The chosen dose of HPR (40 mg/kg), shown previously to be effective when used three times per week to slow down growth of A549 xenograft mouse model [21], was found not curative with mouse SCCVII tumors upon use in a single dose. However, this HPR treatment produced a significant improvement in tumor cure rates when combined with a PDT dose that was marginally curative when used alone (Fig. 1). Thus HPR shares with several other sphingolipid metabolism-modulating drugs the potential to enhance PDT-mediated tumor cures [22, 23].

In conclusion, our data show that enhanced clonogenic cell killing after PDT+HPR is Bcl2 inhibition-, CERS- and caspase-dependent, and that enhanced accumulation of C16-dihydroceramide correlates with enhanced, FB-sensitive, apoptosis. Notably, FoscanPDT +HPR improved the therapeutic outcome in a mouse model of HNSCC. The data suggest the involvement of the DNS pathway in enhanced apoptotic cell killing after PDT+ HPR, identify PDT+HPR as a more effective combination than either treatment alone, and that the combination has potential for promoting the therapeutic benefit of PDT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Dihydroceramide is produced de novo via fumonisin-sensitive ceramide synthase
- Dihydroceramide then gives rise to ceramide via fenretinide-sensitive desaturase
- Fenretinide + Foscan-mediated PDT (PDT) enhances dihydroceramide accumulation
- Fenretinide + PDT enhances fumonisin-sensitive apoptotic cell death
- Fenretinide + PDT improves long-term tumor cures

Fig. 1. PDT+HPR enhance dihydroceramide accumulation in SCC19 cells

Cells were treated with PDT (0.15 μ M Foscan + 400 mJ/cm²), HPR (2.5 μ M), or PDT+HPR, incubated for 2 or 24 h, collected, and processed for mass spectrometry (MS). The levels of C16-dihydroceramide were calculated as pmoles/mg protein and are shown as the mean $\pm$ SD (n=2–3). Significant differences are between †treated and untreated control (Con); *PDT +HPR and individual treatments; and §2 h and 24 h.

Fig. 2. PDT+HPR enhance mitochondrial depolarization in SCC19 cells

Cells were treated with PDT (0.15 μ M Foscan+400 mJ/cm²), HPR (2.5 μ M), or PDT+HPR, incubated for 2 h, collected, and processed for flow cytometry. Post-harvested cells were stained with JC1 and DAPI, a mitochondrial and nuclear stain, respectively, and were analyzed by flow cytometry. JC1 (-), cells with depolarized mitochondria; DAPI (-), nonnecrotic, apoptotic cells. The data are shown as the mean $\pm$ SD (n=7–12). Significant differences are between † treated and untreated control (Con); * (PDT+HPR) and HPR.

Fig. 3. PDT+HPR enhance FB-sensitive caspase-3 activation

Cells were treated with PDT (0.15 μ M Foscan + 400 mJ/cm²), HPR (2.5 μ M), or PDT+HPR, incubated for 2 or 24 h, collected, and processed for DEVDase assay. FB (10 μ M) was added 1 h prior to PDT $\pm$ HPR. The enzyme activity is expressed in arbitrary units (a.u.), and the data are shown as the mean $\pm$ SD (n=2–6). Significant differences are between † treated and untreated control (Con); * (PDT+HPR) and individual treatments; and # (treatment + FB) and treatment.

Fig. 4. Enhanced cell killing after PDT+HPR is CERS-, caspase- and Bcl2-sensitive
 CERS (FB)-, caspase-9 (ZLEHD)-, pancaspase (zVAD)- and Bcl2 (ABT) inhibitors were used at their corresponding non-toxic doses, and were added 1 h prior to PDT (0.15 μ M Foscan + 400 mJ/cm²) $\pm$ HPR (2.5 μ M). Ten days after treatments, colonies were stained with crystal violet (0.1%) and counted. The data are shown as the mean $\pm$ SD (n=3–42). Significant differences are between: † treated and untreated control; * (PDT+HPR) and individual treatments; and # (treatment + inhibitor) and treatment.

Fig. 5. HPR augments cure rates of SCCVII tumors treated by PDT

Mice bearing SCCVII tumors were treated by PDT (Foscan 0.1 mg/kg i.p. followed 24 h later by exposure of tumors to 80 J/cm² of light at 650 ± 10 nm). HPR (40 mg/kg i.p.) was administered either alone or immediately after PDT. Mice remaining tumor-free 90 days after therapy were considered cured. Each treatment group consisted of 8 mice. Based on the log rank test, the difference between the response to PDT alone and PDT+HPR is statistically significant (p<0.05).