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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 S. DeLor [a], Eric Van Buren [b], Alicja Bielawska [c], Jacek Bielawski [c], Jason S. Pierce [c], Mladen Korbelik [d], Duska Separovic [a,b,*]

[a] Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Ave., Detroit, MI 48201, USA
[b] Karmanos Cancer Institute, Wayne State University, 4100 John R, Detroit, MI 48201, USA
[c] Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425, USA
[d] British Columbia Cancer Agency, 675 West 10th Ave., Vancouver, BC V5Z 1L3, Canada

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ABSTRACT

We and others have shown that stresses, including photodynamic therapy (PDT), 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, and identify the combination as a novel more effective anticancer treatment than either treatment alone.

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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, and Foscan-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 non-metastatic, 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

Abbreviations: ABT, ABT-199; CERS, ceramide synthase; DES, desaturase; DNS, de novo sphingolipid biosynthesis pathway; FB, fumonisin B1; HNSCC, head and neck squamous cancer cell carcinoma; HPR, fenretinide; HPV, human papilloma virus; MS, mass spectrometry; PDT, photodynamic therapy; zLEHD-fmk, zLEHD; zVAD, zVAD-fmk.

* Corresponding author at: Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Ave., Detroit, MI 48201, USA.
E-mail address: dseparovic@wayne.edu (D. Separovic).

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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: ABT-199 (ABT), an inhibitor of Bcl2 [13]; zLEHD-fmk (zLEHD) and zVAD-fmk (zVAD), a caspase-9 and a pancaspase inhibitor, respectively [14], and fumonisin 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 (2 mg/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). The inhibitors with the corresponding commercial sources indicated in parentheses were: ABT-199 (Selleck Chemicals, Houston, TX, USA), fumonisin 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% CO_2. For all experiments, incubation of cells was carried out in a humidified incubator at 37 °C and 5% CO_2. 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/cm^2; fluence: 400 mJ/cm^2; λ_{max} ~ 670 nm), using a light-emitting diode array light source (EFOS, Mississauga, ON, Canada), and incubated for the desired time. The inhibitors were added 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. Mitochondrial Depolarization Measurement

The lipophilic cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyanine iodide; BD Biosciences, San Diego, CA, USA) was used to determine mitochondrial membrane potential by flow cytometry. In normal mitochondria with a high negative membrane potential, JC-1 produces aggregates that emit a red fluorescence. In mitochondria with low membrane potential (depolarized mitochondria), the dye generates monomers in the cytosol that emit a green fluorescence. After PDT ± HPR, cells were harvested and processed for flow cytometry according to the manufacturer's instructions (BD Biosciences). JC-1 aggregates were excited at 561 nm. BD LSR II flow cytometer was used for analysis (BD Biosciences).

2.8. 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/cm^2; fluence: 50 J/cm^2; 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.

2.9. 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 log-rank test.

3. Results and Discussion

3.1. Accumulation of C16-dihydroceramide Is Enhanced after FoscanPDT + HPR

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 MS.

Treatment with HPR led to an increase in C16-dihydroceramide levels (Fig. 1) and a decrease in total ceramides in SCC19 cells (Supplementary Data). 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

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