Published in final edited form as:

Photochem Photobiol Sci. 2014 November ; 13(11): 1621–1627. doi:10.1039/c4pp00292j.

Ceramide synthase inhibitor fumonisin B1 inhibits apoptotic cell death in SCC17B human head and neck squamous carcinoma cells after Pc4 photosensitization

Nithin B. Boppana^a, Mohamed Kodiha^b, Ursula Stochaj^b, Ho-sheng Lin^c, Adrianna Haimovitz-Friedman^d, Alicja Bielawska^e, Jacek Bielawski^e, George W. Divine^f, John A. Boyd^a, Mladen Korbelik^g, and Duska Separovic^a

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

^bDepartment of Physiology, McGill University, Montreal, QC H3G 1YC, Canada

^cDepartment of Otolaryngology-Head and Neck Surgery, School of Medicine, Wayne State University, Detroit, MI 48201, USA

^dDepartment of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

^eDepartment of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA

^fDepartment of Public Health Sciences, Henry Ford Hospital, Detroit, MI 48202, USA

^gBritish Columbia Cancer Agency, Vancouver, BC V5Z 1L3, Canada

Abstract

The sphingolipid ceramide modulates stress-induced cell death and apoptosis. We have shown that ceramide generated via de novo sphingolipid biosynthesis is required to initiate apoptosis after photodynamic therapy (PDT). The objective of this study was to define the role of ceramide synthase (CERS) in PDT-induced cell death and apoptosis using fumonisin B1 (FB), a CERS inhibitor. We used the silicon phthalocyanine Pc4 for PDT, and SCC17B cells, as a clinically-relevant model of human head and neck squamous carcinoma. zVAD-fmk, a pan-caspase inhibitor, as well as FB, protected cells from death after PDT. In contrast, ABT199, an inhibitor of the anti-apoptotic protein Bcl2, enhanced cell killing after PDT. PDT-induced accumulation of ceramide in the endoplasmic reticulum and mitochondria was inhibited by FB. PDT-induced Bax translocation to the mitochondria and cytochrome c release were also inhibited by FB. These novel data suggest that PDT-induced cell death via apoptosis is CERS/ceramide-dependent.

1. Introduction

Photodynamic therapy (PDT), a minimally invasive, nonsurgical cancer treatment modality, utilizes a light-absorbing photosensitizer, molecular oxygen and visible light to generate reactive oxygen species and destroy malignant cellular targets.¹ The effectiveness of PDT regimens correlates with tumor cell apoptosis.² The mitochondrial pathway of apoptosis, characterized by the translocation of the pro-apoptotic protein Bax from the cytosol to the

mitochondria and the release of cytochrome c (cyt c) from the mitochondria to the cytosol has been observed after PDT.^{3–7} On the other hand, the anti-apoptotic protein Bcl2 protects against PDT-induced cell death in apoptosis-competent cells.⁸

The bioactive sphingolipid (SL) ceramide regulates apoptosis and cell death.^{9, 10} The subcellular localization of ceramide correlates with the specificity of its biological effects. Ceramide can be generated via de novo sphingolipid biosynthesis in the endoplasmic reticulum (ER). This pathway includes ceramide synthase (CERS)-dependent acylation of dihydrosphingosine, giving rise to dihydroceramide, which is then converted to ceramide by desaturation [Fig. 1]. CERS/ceramide has been associated with ER stress and apoptosis.¹¹ FB-sensitive mitochondrial ceramide accumulation has been linked to radiation-induced apoptosis.¹² The CERS inhibitor FB induces resistance to cell death and apoptosis after stress.^{10, 13–15}

We have shown previously that PDT-induced ceramide accumulation involves the de novo SL synthesis pathway and CERS.^{16–18} This implies (a) that PDT induces ceramide generation in the ER, and (b) that PDT-induced apoptosis requires de novo SL synthesis and CERS.^{16–18} The following questions, however, remain to be addressed: (i) Is CERS required for PDT-induced cell death? (ii) Are the ER and mitochondria the subcellular sites of PDT-induced ceramide accumulation? (iii) Are PDT-induced Bax mitochondrial translocation and cyt c release CERS-dependent? (iv) Is apoptosis critical for PDT-induced cell death in human head and neck squamous carcinoma (HNSCC) cells? (v) Can inhibition of Bcl2 sensitize HNSCC cells to PDT?

The objectives of this study were to address the above with established pharmacological compounds: the CERS inhibitor FB, the pan-caspase inhibitor zVAD-fmk (zVAD), and the Bcl2 inhibitor ABT199 (ABT).^{19–22} For PDT, we used the silicon phthalocyanine Pc4. We used SCC17B cells, an HNSCC cell line, as a main model system. This cell line was derived from larynx, a typical HNSCC, and of clinical relevance for PDT. Colony formation assays were performed to determine cell death. Quantitative confocal microscopy was used to measure the subcellular localization of ceramide, Bax mitochondrial translocation and cyt c release. In addition, mass spectrometry (MS) was used to identify various ceramide species produced by PDT.

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 supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University, Cleveland, OH, USA). DMEM/F-12 medium and fetal bovine and goat serum were purchased from Thermo-Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich (Atlanta, GA, USA), respectively. Inhibitors were from the sources indicated; zVAD-fmk (MBL International, Woburn, MA, USA), fumonisins B1 (Cayman Chemicals, Chicago, IL, USA) and ABT199 (Selleck Chemicals, Houston, TX, USA).

2.2. Cell culture and PDT

The HNSCC cell lines SCC17B and SCC22A, kindly supplied by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI, USA) were cultured in DMEM/F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in a humidified incubator at 37°C and 5% CO₂. For PDT experiments, after overnight incubation with Pc4 at 37°C, cells were irradiated at room temperature with red light (2 mW/cm²; $\lambda_{\text{max}} \sim 670$ nm) using a light-emitting diode array light source (EFOS, Mississauga, ON, Canada) at the fluence of 200 mJ/cm², and then incubated at 37°C for indicated periods of time and processed for various analyses.

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

After treatments, cells were collected on ice, washed with cold phosphate-buffered saline (PBS; Corning Life Sciences, New York, NY, USA), resuspended in a mixture of ethyl acetate/methanol (1:1, v/v; EMD Chemicals, Billerica, MA, USA), dried under nitrogen, and shipped overnight on dry ice to the Lipidomics Shared Resource Facility (Medical University of South Carolina, Charleston, SC, USA) for further processing. After extraction, SLs were separated by high performance liquid chromatography, introduced to the electrospray ionization source and then analyzed by double MS using TSQ 7000 triple quadrupole mass spectrometer (Thermo-Fisher Scientific) as described previously.²³

2.4. Quantitative confocal microscopy

Cells grown on coverslips in 6-well plates were treated with PDT ± FB, washed with cold PBS and fixed with 4% formaldehyde in PBS for 15 min. After washing with PBS, cells were permeabilized with ice-cold acetone/methanol (1:1) for 10 min. After blocking with 3% goat serum and 3% fetal bovine serum in PBS for 1 h at 4°C, cells were incubated with monoclonal anti-ceramide antibodies (1:30; Enzo Life Sciences, Farmingdale, NY, USA) at room temperature for 45 min. After washing with cold PBS, cells were incubated with Alexa 488-conjugated goat anti-mouse IgM antibodies (1:200; Jackson ImmunoResearch, Westgrove, PA, USA) at room temperature. After 45 min, cells were washed with PBS, and mounted on slides using the ProLong Antifade Kit (Invitrogen). Where indicated, mitochondria were stained with Mitotracker Red CMXRos (250 nM; Invitrogen) which was added to the culture medium at 37°C for 30 min before fixation. Images were acquired with a Zeiss LSM 780 confocal microscope equipped with a 100×1.4 NA OIL DIC D objective (Carl Zeiss, Thornwood, NY, USA). For ER and ceramide detection, anti-KDEL (1:50; Abcam, Cambridge, MA, USA) and anti-ceramide antibodies were combined with Alexa 594-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-mouse IgM antibodies (both 1:200; both from Jackson ImmunoResearch), respectively. Anti-Bax antibodies (1:50; Abcam) were combined with Alexa 488-conjugated goat anti-mouse IgG antibodies (1:200; Jackson ImmunoResearch). Anti-cyt c antibodies (1:50) (BD Pharmingen, Chicago, IL, USA) were combined with Alexa 594-conjugated goat anti-mouse IgG (1:200) (Jackson ImmunoResearch). In all experiments nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml, 10 min at room temperature). Confocal microscopy imaging was performed at the Microscopy, Imaging and Cytometry Resources Core at Wayne State

University, School of Medicine. To quantify the percentage of cells that release cyt c, 100 cells were assessed in each experiment. Cells scored positive, if the margin of cyt c staining around nuclei was greater than 10 μm .

Ceramide fluorescence located in the ER or mitochondria, and Bax fluorescence located in the mitochondria was quantified with MetaXpress software essentially as described.²⁴ Mitochondria or the ER were demarcated with Mitotracker or anti-KDEL antibodies. Ceramide or Bax-pixel intensities located in these compartments were measured with the multiwavelength cell scoring module. The correct compartment identification was verified by visual inspection. To determine the relative amount of PDT-induced ceramide in the ER and mitochondria, colocalization of ceramide and ER or mitochondrial fluorescence were determined with the colocalization module of MetaXpress software. Images were corrected for background contribution and then analyzed with the software module.

2.5. Clonogenic assay

Long-term cell viability was assessed using clonogenic assay according to the modified pre-plating protocol that we described previously.²⁵ Plating density was 250 cells/plate. Plating efficiency was 36% ($n = 16$).

2.6. Protein determination

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

2.7. Statistical analysis

Data are shown as the average $\pm$ SEM. Student's t-tests assuming unequal variances were used to compare two samples. For quantitative confocal microscopy using MetaXpress multiple samples were compared by One-way ANOVA. Differences with P -values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. PDT-induced cell death is inhibited by FB and zVAD and enhanced by ABT

We have previously shown that PDT-induced apoptosis requires de novo SL synthesis and CERS in cell lines other than SCC17B cells.¹⁶⁻¹⁸ However, it is not known whether the PDT-induced cell death requires CERS. Bcl2 protects against PDT-induced cell death in apoptosis-competent cells (e.g. human breast cancer MCF-7 cells overexpressing human pro-caspase 3).⁸ However, it has yet to be determined whether in SCC17B cells apoptosis is essential for PDT-induced death. It is also not known whether inhibition of Bcl2 sensitizes these cells to PDT. To define the requirement for CERS, caspases, and Bcl2 inhibition in PDT-induced cell death, different pharmacological inhibitors were employed: the CERS inhibitor FB, the pan-caspase inhibitor zVAD, and the Bcl2 inhibitor ABT. Colony formation assays were used to determine cell death after treatments in SCC17B cells. All compounds were used at non-toxic doses. PDT was used at LD20 (20 nM Pc4 + 200 mJ/cm^2), as determined in dose-response studies (not shown). FB and zVAD inhibited cell killing after PDT by 68 and 56%, respectively [Fig. 2]. ABT enhanced PDT-induced cell

killing by 61% [Fig. 2]. Moreover, in SCC22A cells PDT-induced cell death is inhibited by FB and zVAD [Fig. S4]. Together the data indicate that PDT-induced cell death depends on CERS, caspase activation, and inhibition of Bcl2.

3.2. PDT-induced ceramide accumulation in the ER and mitochondria is inhibited by FB

The subcellular localization of ceramide correlates with the specificity of its biological effects. CERS-dependent ceramide accumulation has been associated with ER stress and apoptosis.¹¹ On the other hand, FB-sensitive mitochondrial ceramide accumulation has been linked to radiation-induced apoptosis.¹² We have previously shown that PDT-induced ceramide accumulation involves the de novo SL biosynthesis and CERS,^{16–18} which implies generation of ceramide in the ER. However, experimental evidence for PDT-induced ceramide accumulation in the ER is missing. Also, evidence that PDT induces mitochondrial ceramide accumulation is lacking.

Here, we used for the first time quantitative confocal microscopy to measure ceramide in mitochondria and the ER. To this end, ceramide was detected by immunostaining; Mitotracker and KDEL served as markers of mitochondria and the ER, respectively. As depicted in Figure 3, we determined the accumulation of ceramide in the ER and mitochondria at 2, 10 and 24 h post-PDT. Ceramide accumulation at both subcellular sites peaked at 10 h post-PDT, as quantification data revealed (not shown). PDT treatment for 10 h raised the colocalization with the ER and mitochondria by 8 and 29% of the total cellular ceramide, respectively (not shown). In addition, we have used the mitochondrial protein TOM20/immunocytochemistry and confirmed our Mitotracker data [Fig. S1].

Next we used FB to test whether PDT-induced ceramide accumulation is CERS-dependent. FB reduced ceramide accumulation in the ER and mitochondria post-PDT at all time points [Fig. 3A and B]. This was also confirmed by the quantification analysis (not shown). Together these results reveal that PDT induces ceramide accumulation in the ER and mitochondria. The data suggest that PDT-induced ceramide accumulation at these subcellular sites is CERS-dependent.

3.3. MS reveals increases in ceramides post-PDT

To validate confocal microscopy findings, we used MS to identify ceramide species accumulated in response to PDT. The levels of total ceramides and most of the individual ceramides were significantly increased after PDT [Table 1]. PDT also increased the levels of C16-dihydroceramide, a precursor of ceramide in de novo SL biosynthesis. Overall, our data demonstrate increases in the levels of ceramides and C16-dihydroceramide post-PDT. They also suggest the involvement of the de novo SL biosynthesis.

3.4. PDT-induced Bax translocation to mitochondria and cyt c release are inhibited by FB

The translocation of the pro-apoptotic protein Bax from the cytosol to mitochondria and the release of cyt c from the mitochondria to the cytosol are hallmarks of the mitochondrial pathway of apoptosis and have been demonstrated in response to PDT.^{3–7} However, it is not known whether PDT induces Bax mitochondrial translocation and cyt c release in SCC17B cells. It is also not clear whether these processes are CERS-dependent. Using quantitative

confocal microscopy we determined whether Bax mitochondrial translocation and cyt c release are induced by PDT and sensitive to FB. As shown in Figure 4 (A and B), Bax translocation to the mitochondria was induced at 2 and 10 h post-PDT. In the presence of FB, this process was inhibited. Cyt c release was observed at 10 h post-PDT [Fig. 4C, D] but not earlier (not shown). Co-treatment of cells with FB and PDT led to inhibition of cyt c release. Together these results indicate that PDT induces Bax mitochondrial translocation and cyt c release in SCC17B cells. Moreover, both processes are CERS-dependent.

The present studies demonstrate that inhibition of CERS and caspases protect cells from PDT-induced cell death, indicating that PDT induces apoptotic cell death in SCC17B cells via CERS. In addition, inhibition of Bcl2 enhances PDT-induced cell death in these cells further supporting the view that apoptosis is involved. These findings highlight the functional significance of apoptosis and CERS in cell death in response to PDT. Accordingly, Bcl2 has been shown to be a critical factor determining the effectiveness of PDT in cells capable of undergoing apoptosis.⁸

Here we demonstrate that PDT-induced accumulation of ceramide in the ER and mitochondria is inhibited by FB. The results support the notion that FB-sensitive CERS is localized at these subcellular sites. Similar data were reported in response to other stress inducers.^{12, 26} The link between CERS-dependent ceramide production in the ER and mitochondria, and apoptotic cell death, is further supported by the evidence that either or both subcellular sites are involved in PDT-induced apoptotic cell death.^{7, 27, 28}

Neither caspase-3 (casp3; not shown) nor nuclear apoptotic changes (by DAPI staining; Fig. 3) were observed post-PDT under given conditions. At higher PDT doses, however, casp3 was activated and FB inhibited the activation [Figs. S2, S3].

The current studies show that PDT-induced Bax translocation to the mitochondria, and cyt c release, are sensitive to FB. As shown previously, radiation-induced Bax activation and cyt c release are sensitive to FB.¹² Radiation induces the formation of ceramide-rich macrodomains within the mitochondrial outer membrane, into which Bax inserts and oligomerizes to form a pore allowing cyt c release.¹² Future studies will test whether such a scenario is operative in response to PDT.

4. Conclusion

Our present study indicates that CERS-dependent ceramide accumulation is required for apoptotic cell death after PDT (Fig. 5). These novel findings suggest that CERS is a potential druggable target that could control the effectiveness of PDT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by U.S. Public Health Service Grants: to DS, R01 CA77475 from the National Cancer Institute (NCI), National Institutes of Health (NIH) and Wayne State University Bridge funding; to the MS-related

work at the Lipidomics Shared Resource Facility (Medical University of South Carolina), NCI Grants IPO1CA097132 and P30 CA 138313, NIH/NCRR SC COBRE Grant P20 RR017677, C06 RR018823 from the Extramural Research Facilities Program of the National Center for Research Resources. The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by NIH Center grant P30 CA022453 to the Karmanos Cancer Institute at Wayne State University, and the Perinatology Research Branch of the National Institutes of Child Health and Development at Wayne State University.

References

1. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. *CA Cancer J Clin.* 2011; 61:250–281. [PubMed: 21617154]
2. Henderson BW, Gollnick SO, Snyder JW, Busch TM, Kousis PC, Cheney RT, Morgan J. Choice of oxygen-conserving treatment regimen determines the inflammatory response and outcome of photodynamic therapy of tumors. *Cancer Res.* 2004; 64:2120–2126. [PubMed: 15026352]
3. Usuda J, Chiu SM, Azizuddin K, Xue LY, Lam M, Nieminen AL, Oleinick NL. Promotion of photodynamic therapy-induced apoptosis by the mitochondrial protein Smac/DIABLO: dependence on Bax. *Photochem Photobiol.* 2002; 76:217–223. [PubMed: 12194220]
4. Srivastava M, Ahmad N, Gupta S, Mukhtar H. Involvement of Bcl-2 and Bax in photodynamic therapy-mediated apoptosis. Antisense Bcl-2 oligonucleotide sensitizes RIF 1 cells to photodynamic therapy apoptosis. *J Biol Chem.* 2001; 276:15481–15488. [PubMed: 11278320]
5. Chiu SM, Xue LY, Usuda J, Azizuddin K, Oleinick NL. Bax is essential for mitochondrion-mediated apoptosis but not for cell death caused by photodynamic therapy. *Br J Cancer.* 2003; 89:1590–1597. [PubMed: 14562036]
6. Chiu SM, Oleinick NL. Dissociation of mitochondrial depolarization from cytochrome c release during apoptosis induced by photodynamic therapy. *Br J Cancer.* 2001; 84:1099–1106. [PubMed: 11308261]
7. Lam M, Oleinick NL, Nieminen AL. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. Reactive oxygen species and mitochondrial inner membrane permeabilization. *J Biol Chem.* 2001; 276:47379–47386. [PubMed: 11579101]
8. Xue LY, Chiu SM, Azizuddin K, Joseph S, Oleinick NL. Protection by Bcl-2 against apoptotic but not autophagic cell death after photodynamic therapy. *Autophagy.* 2008; 4:125–127. [PubMed: 18025862]
9. Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell.* 1995; 82:405–414. [PubMed: 7634330]
10. Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, Obeid LM. Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. *J Biol Chem.* 2011; 286:15929–15942. [PubMed: 21388949]
11. Senkal CE, Ponnusamy S, Manevich Y, Meyers-Needham M, Saddoughi SA, Mukhopadhyay A, Dent P, Bielawski J, Ogretmen B. Alteration of Ceramide Synthase 6/C16-Ceramide Induces Activating Transcription Factor 6-mediated Endoplasmic Reticulum (ER) Stress and Apoptosis via Perturbation of Cellular Ca²⁺ and ER/Golgi Membrane Network. *J Biol Chem.* 2011; 286:42446–42458. [PubMed: 22013072]
12. Lee H, Rotolo JA, Mesicek J, Penate-Medina T, Rimner A, Liao WC, Yin X, Ragupathi G, Ehleiter D, Gulbins E, Zhai D, Reed JC, Haimovitz-Friedman A, Fuks Z, Kolesnick R. Mitochondrial ceramide-rich macromolecules functionalize Bax upon irradiation. *PLoS One.* 2011; 6:e19783. [PubMed: 21695182]
13. Ueda N, Camargo SM, Hong X, Basnakian AG, Walker PD, Shah SV. Role of ceramide synthase in oxidant injury to renal tubular epithelial cells. *J Am Soc Nephrol.* 2001; 12:2384–2391. [PubMed: 11675414]
14. Biswal SS, Datta K, Acquah-Mensah GK, Kehrer JP. Changes in ceramide and sphingomyelin following fludarabine treatment of human chronic B-cell leukemia cells. *Toxicology.* 2000; 154:45–53. [PubMed: 11118669]

15. Sauane M, Su ZZ, Dash R, Liu X, Norris JS, Sarkar D, Lee SG, Allegood JC, Dent P, Spiegel S, Fisher PB. Ceramide plays a prominent role in MDA-7/IL-24-induced cancer-specific apoptosis. *J Cell Physiol.* 2010; 222:546–555. [PubMed: 19937735]
16. Wispriyono B, Schmelz E, Pelayo H, Hanada K, Separovic D. A role for the de novo sphingolipids in apoptosis of photosensitized cells. *Exp Cell Res.* 2002; 279:153–165. [PubMed: 12213223]
17. Separovic D, Breen P, Joseph N, Bielawski J, Pierce JS, Van Buren E, Gudz TI. Ceramide synthase 6 knockdown suppresses apoptosis after photodynamic therapy in human head and neck squamous carcinoma cells. *Anticancer Res.* 2012; 32:753–760. [PubMed: 22399588]
18. Separovic D, Breen P, Joseph N, Bielawski J, Pierce JS, Van Buren E, Gudz TI. siRNA-mediated down-regulation of ceramide synthase 1 leads to apoptotic resistance in human head and neck squamous carcinoma cells after photodynamic therapy. *Anticancer Res.* 2012; 32:2479–2485. [PubMed: 22753704]
19. Wang E, Norred WP, Bacon CW, Riley RT, Merrill AH Jr. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *J Biol Chem.* 1991; 266:14486–14490. [PubMed: 1860857]
20. Wu WL, McDonough VM, Nickels JT Jr, Ko J, Fischl AS, Vales TR, Merrill AH Jr, Carman GM. Regulation of lipid biosynthesis in *Saccharomyces cerevisiae* by fumonisin B1. *J Biol Chem.* 1995; 270:13171–13178. [PubMed: 7768913]
21. Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA. Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem.* 1998; 273:32608–32613. [PubMed: 9829999]
22. Souers AJ, Levenson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, Dayton BD, Ding H, Enschede SH, Fairbrother WJ, Huang DC, Hymowitz SG, Jin S, Khaw SL, Kovar PJ, Lam LT, Lee J, Maecker HL, Marsh KC, Mason KD, Mitten MJ, Nimmer PM, Oleksijew A, Park CH, Park CM, Phillips DC, Roberts AW, Sampath D, Seymour JF, Smith ML, Sullivan GM, Tahir SK, Tse C, Wendt MD, Xiao Y, Xue JC, Zhang H, Humerickhouse RA, Rosenberg SH, Elmore SW. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med.* 2013; 19:202–208. [PubMed: 23291630]
23. Separovic D, Semaan L, Tarca AL, Awad Maitah MY, Hanada K, Bielawski J, Villani M, Luberto C. Suppression of sphingomyelin synthase 1 by small interference RNA is associated with enhanced ceramide production and apoptosis after photodamage. *Exp Cell Res.* 2008; 314:1860–1868. [PubMed: 18374917]
24. Kodiha M, Brown CM, Stochaj U. Analysis of signaling events by combining high-throughput screening technology with computer-based image analysis. *Science Signaling.* 2008; 1:pl2. [PubMed: 18799422]
25. Separovic D, Saad ZH, Edwin EA, Bielawski J, Pierce JS, Van Buren E, Bielawska A. C16-ceramide analog combined with Pc 4 photodynamic therapy evokes enhanced total ceramide accumulation, promotion of DEVDase activation in the absence of apoptosis, and augmented overall cell killing. *J Lipids.* 2011; 2011:1–9.
26. Yao J, Bi HE, Sheng Y, Cheng LB, Wendu RL, Wang CH, Cao GF, Jiang Q. Ultraviolet (UV) and Hydrogen Peroxide Activate Ceramide-ER Stress-AMPK Signaling Axis to Promote Retinal Pigment Epithelium (RPE) Cell Apoptosis. *Int J Mol Sci.* 2013; 14:10355–10368. [PubMed: 23685869]
27. Mak NK, Li KM, Leung WN, Wong RN, Huang DP, Lung ML, Lau YK, Chang CK. Involvement of both endoplasmic reticulum and mitochondria in photokilling of nasopharyngeal carcinoma cells by the photosensitizer Zn-BC-AM. *Biochem Pharmacol.* 2004; 68:2387–2396. [PubMed: 15548385]
28. Buytaert E, Callewaert G, Hendrickx N, Scorrano L, Hartmann D, Missiaen L, Vandenheede JR, Heirman I, Grooten J, Agostinis P. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. *Faseb J.* 2006; 20:756–758. [PubMed: 16455754]

Fig. 1.
CERS-dependent ceramide production is inhibited by FB.

Fig. 2.

PDT-induced cell death is sensitive to FB, zVAD and ABT. FB, zVAD (10 μ M each) and ABT (0.1 μ M) were added 1 h prior to PDT (20 nM Pc4+200 mJ/cm²; LD20). Colonies were stained with crystal violet (0.1%) and counted 14 days after treatments. The data are shown as the average $\pm$ SEM (n = 6–18 samples). Differences were significant between PDT- and (PDT + inhibitor)-treated cells (*). Untreated controls had 0% loss of clonogenicity.

Fig. 3A.

Fig. 3B.

Fig. 3.

PDT-induced ceramide accumulation in the ER (A) and mitochondria (B) is time-dependent and inhibited by FB. Where indicated, cells were treated with FB (10 μ M) 1 h prior to PDT (20 nM Pc4+200 mJ/cm²; LD20), incubated for indicated periods of time and immunostained with anti-ceramide and anti-KDEL antibodies. Nuclei were visualized with DAPI. (B) Incubation with Mitotracker Red CMXRos was carried out prior to immunostaining with anti-ceramide antibodies. All images were acquired by confocal microscopy with identical settings. Con, untreated control.

Fig. 4A.

Fig. 4B.

Fig. 4C.

Fig. 4D.

Fig. 4.

PDT-induced Bax translocation to mitochondria (A, B) and cyt c release (C, D) are inhibited by FB. FB (10 μ M) was added 1 h prior to PDT (20 nM Pc 4 + 200 mJ/cm²; LD20). Cells were incubated for indicated periods of time (A, B) or for 10 h (C, D), and then immunostained with anti-Bax or anti-cyt c antibodies. Where indicated, incubation with Mitotracker Red CMXRos was carried out prior to immunostaining with anti-Bax antibodies. Images were acquired by confocal microscopy using identical settings. (B) Bax fluorescence located in the mitochondria was quantified with MetaXpress software. The data were calculated as Bax-pixel intensities/mitochondrial area. A minimum of 277 regions were measured for each data point. Results are shown as the average $\pm$ SEM. (D) Percentages of cells releasing cyt c were calculated by scoring 100 cells for every sample and were plotted as bar graph. Each data point represents 3–4 samples and the data are shown as the average $\pm$ SEM. Significant differences were observed between treated- and untreated-cells (#), or between PDT- and (PDT + FB)-treated cells (*). Con, untreated control.

Fig. 5.
PDT-induced apoptotic cell death is CERS/ceramide dependent: a model.

Table I

Effect of PDT on SLs in SCC17B cells.

Sphingolipid	Untreated	PDT
Total ceramides	638.8 ± 42.3	1057.8 ± 87.8 (1.7) *
C14-Ceramide	16.1 ± 0.9	26.9 ± 2.8 (1.7) *
C16-Ceramide	91.0 ± 11.9	129.6 ± 8.5 (1.4) *
C18-Ceramide	17.8 ± 2.1	61.6 ± 4.1 (3.5) *
C18:1-Ceramide	7.6 ± 0.3	25.5 ± 2.6 (3.3) *
C20-Ceramide	5.2 ± 0.7	21.4 ± 1.8 (4.2) *
C20:1-Ceramide	1.5 ± 0.2	5.3 ± 0.6 (3.5) *
C22-Ceramide	51.6 ± 2.7	132.7 ± 10.6 (2.6) *
C22:1-Ceramide	18.7 ± 1.7	49.6 ± 4.5 (2.7) *
C24-Ceramide	180.8 ± 20.0	252.6 ± 22.4 (1.4) *
C24:1-Ceramide	199.3 ± 9.4	305.4 ± 28.6 (1.5) *
C26-Ceramide	15.8 ± 3.6	13.9 ± 1.3 (0.9)
C26:1-Ceramide	33.4 ± 3.2	33.4 ± 2.3 (1.0)
C16-Dihydroceramide	42.1 ± 4.8	100.2 ± 6.3 (2.4) *

SCC17B cells were treated with PDT (20 nM Pc4+200 mJ/cm²; LD20), and 10 h later were collected and processed for MS. The levels of SLs were calculated as pmoles/mg protein and are shown as the average ± SEM (n=4). The ratios of PDT-treated vs. untreated controls are shown in the brackets.

* PDT-treated cells are significantly different compared to untreated controls.