Increased tumour dihydroceramide production after Photofrin-PDT alone and improved tumour response after the combination with the ceramide analogue LCL29. Evidence from mouse squamous cell carcinomas

Photodynamic therapy (PDT) has been proven effective for treatment of several types of cancer. Photodynamic therapy alone, however, attains limited cures with some tumours and there is need for its improved efficacy in such cases. Sphingolipid (SL) analogues can promote tumour response in combination with anticancer drugs. In this study, we used mouse SCCVII squamous cell carcinoma tumours to determine the impact of Photofrin-PDT on the in vivo SL profile and the effect of LCL29, a C6-pyridinium ceramide, on PDT tumour response. Following PDT, the levels of dihydroceramides (DHceramides), in particular C20-DHceramide, were elevated in tumours. Similarly, increases in DHceramides, in addition to C20:1-ceramide, were found in PDT-treated SCCVII cells. These findings indicate the importance of the de novo ceramide pathway in Photofrin-PDT response not only in cells but also in vivo. Notably, co-exposure of SCCVII tumours to Photofrin-PDT and LCL29 led to enhanced tumour response compared with PDT alone. Thus, we show for the first time that Photofrin-PDT has a distinct signature effect on the SL profile in vitro and in vivo, and that the combined treatment advances PDT therapeutic gain, implying translational significance of the combination.

Keywords: C6-pyridinium ceramide; dihydroceramide; plasma; PDT; Photofrin; tumour

Ceramide mimetics and drugs targeting sphingolipid (SL) metabolism have made major advances towards cancer treatment (Fox et al, 2006; Zeidan and Hannun, 2007). To overcome the low solubility of ceramide, cationic pyridinium ceramide analogues have been developed, which are water soluble (Szluc et al, 2006). These analogues have effective anticancer activity at relatively low concentrations (Novgorodov et al, 2005; Rossi et al, 2005; Dindo et al, 2006; Senkal et al, 2006; Zeidan and Hannun, 2007; Dahm et al, 2008). LCL124, alone or in combination with the chemotherapeutic agent gemcitabine, inhibits substantially the growth of human head and neck squamous cell carcinomas in vitro and in vivo (Senkal et al, 2006). LCL30 has potent antitumour activity against colorectal cancer in mice (Dahm et al, 2008).

The oxidative stress inducer photodynamic therapy (PDT) uses a photosensitiser, visible light and oxygen to generate reactive oxygen species that can destroy malignant cells by apoptosis (Dougherty et al, 1998; Miller et al, 2007). We have shown in Jurkat cells with downregulated sphingomyelin synthase that an enhanced accumulation of ceramide and dihydroceramide (DHceramide) correlates with the promotion of apoptosis post-Pc 4-PDT (Separovic et al, 2008). We have also demonstrated that the combination of Pc 4-PDT with exogenous C16-ceramide increases mitochondrial depolarisation and apoptosis in Jurkat cells (Dolgachev et al, 2003). There is need to verify the relevance of our cell culture findings in tumour models. A novel effective ceramide mimetic LCL29 (Szluc et al, 2006; Bielawska et al, 2008), a structural analogue of LCL124, was used to test its ability to enhance the response of mouse SCCVII squamous cell carcinomas to Photofrin-PDT. The photosensitiser Photofrin was used because of its clinical relevance as it is the only photosensitiser approved by the Food and Drug Administration for cancer treatment in the United States. The main objectives of this study were (i) to determine signature effects of Photofrin-PDT on endogenous ceramide and DHceramide in SCCVII cells and tumours and (ii) to determine the therapeutic effect of the combination PDT + LCL29 in SCCVII tumours.

MATERIALS AND METHODS

Tumour-related cell model and PDT treatment

As shown earlier (Cecic and Korbelik, 2006), mouse SCCVII squamous carcinoma cells were grown in Alpha Minimal Essential
Medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% heat-inactivated foetal bovine serum (Hyclone, Logan, UT, USA). The photosensitiser Photofrin (Axxon Pharma, Mont-Saint-Hilaire, QC, Canada) was dissolved in 5% dextrose. For the experiment, following overnight incubation with Photofrin (20 μg ml⁻¹), SCCVII cells were exposed to light (0.5 or 1.0 mJ cm⁻²) and incubated at 37°C for 4 h before collection for MS analysis. Fold increase refers to an increase in the level of each SL after PDT per 1 mJ cm⁻² of light. *P-value, nominal P-value. **P-value, FDR-adjusted P-value. Bold type values indicate statistical significance.

Tumour model and PDT treatment

As described earlier (Sun et al, 2002), female C3H/HeN mice were implanted with syngeneic SCCVII squamous cell carcinoma tumours (Khurana et al, 2001) by a subcutaneous injection of 1 × 10⁶ SCCVII cells at a lower dorsal site. After 7–8 days, the tumours reached a size of 6–8 mm in largest diameter. At that point treatment began. The cohort of SCCVII tumour-bearing mice was divided into groups for various treatments. Tumour growth was monitored and recorded by measuring three orthogonal dimensions, a, b and c using a caliper. Tumour volume (V) was calculated using the formula: \( V = \pi \times ab \times c/6 \). The measurement was taken every second day. Mice were killed before starting to suffer from tumour burden, that is, at the point when tumour size was 15 mm in diameter. For PDT, tumours were treated 24 h after photosensitiser administration with light produced by an FB-QTH high-throughput illuminator (Scientech, London, ON, Canada). Light was delivered through a 630 ± 10 nm interference filter for superficial tumour illumination with a fluence rate of 80–90 mW cm⁻². During treatment of tumours with light, the mice were immobilized with metal holders. Thereafter, the mice were monitored up to 14 days post-therapy and the presence of tumours or their absence recorded every 2 days. The ceramide analogue d-erythro-2-N-[6-((1⁴R)-pyridinium)-hexanoyl]-sphingosine bromide (LCL29, also known as C₆-pyridinium ceramide) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). For experiments, LCL29 was dissolved in distilled water and injected intraperitoneally (20 mg kg⁻¹) daily over a period of 7 days. The first injection was administered 2 h before PDT light treatment. Blood obtained from mice by cardiac puncture was collected into EDTA-containing tubes, and plasma samples together with samples of tumour tissue homogenates were analysed for the SL profile using MS. The procedures with mice were approved and overseen by the Animal Care Committee of the University of British Columbia.

Measurement of SLs by electrospray ionisation/double MS

As we have shown earlier (Separovic et al, 2007; Separovic et al, 2008) following extraction, SLs were separated by high-performance liquid chromatography, introduced to electrospray ionisation source and then analysed by double MS using TSQ 7000 triple quadrupole mass spectrometre (Thermo-Fisher Scientific, San Jose, CA, USA) which allows the simultaneous determination of various SLs, including various ceramide and DHceramide species, dihydroxyphosphosine (DHphosphosine), sphingosine and sphingosine-1-phosphate (S1P) (Bielawski et al, 2006). Specifically, samples obtained from cells or tissues were fortified with the internal standards (C17-base-d-e-sphingosine, C17-sphingosine-1-phosphate, DHsphingo-sine-1-phosphate, N-palmitoyl-d-e-C13-sphingosine and C17-d-e-sphingo-sine) and extracted with ethyl acetate/isopropanol/water (60/30/10, v/v). After evaporation and reconstitution in methanol, samples were injected into the HP1100/TSQ 7000 LC/MS system and gradient eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3-μm particle size column, with 1 ml methanolic ammonium formate/2 ml aqueous ammonium formate mobile phase. Peaks corresponding to the target analytes and internal standards were collected and processed using the Xcalibur software system. Quantitative analysis is based on the calibration curves generated by spiking an artificial matrix with known amounts of the target analyte synthetic standards and an equal amount of the internal standards. For the calibration curves, the target analyte/internal standard peak area ratios are plotted against analyte concentration. The target analyte/internal standard peak area ratios from the samples are similarly normalised to their respective internal standards and compared with the calibration curves, using a linear regression model.

Statistical analysis

For statistical analysis of MS data, unless indicated otherwise, the mass of SLs in pmol mg⁻¹ was log₂ transformed to improve the normality of the distributions and allow computation of more robust means and standard deviations. As 26–29 SLs were measured and tested for differences at the same time, besides nominal P-values obtained by t-test, adjusted P-values (P < 0.1) using the false discovery rate (FDR) method were used to infer significance (Benjamini and Hochberg, 1995). The R statistical environment (www.r-project.org) was used for all computations. For in vitro studies, the analysis of the interaction between Photofrin and light dose, in addition to assessing the effect of time, was performed by linear models (Wilkinson and Rogers, 1973). For MS in vivo studies, a two-tailed t-test was employed to compare concentration levels of SLs between treatment groups, as well as to test whether fold changes for the entire family of SLs (e.g., ceramides) were different than 1. To assess the effect of the combined treatment on tumour reduction, a linear mixed-effects model was used (Pinheiro and Bates, 2000).
RESULTS AND DISCUSSION

Signature effects of Photofrin-PDT on the SL profile of in vitro cultured SCCVII cells

SCCVII cells were exposed to low and high PDT doses corresponding to \( \text{LD}_{30} \) and \( \text{LD}_{80} \), respectively (Cecic and Korbelik, 2006), incubated for 2 or 4 h at 37 °C, and collected for MS. Neither Photofrin nor light alone had significant effects on basal levels of SLs (not shown). Dose responses of SLs to PDT were statistically evaluated against their corresponding controls. The levels of C20:1-ceramide, C14-, C16-, C18:1- and C22-DHceramide significantly rose to 7.3-, 10.5-, 11.8-, 9.6- and 5.3-fold, respectively, by 4 h per 1 mJ cm\(^{-2}\) of light fluence (Table 1). A dose-dependent increase in C22-DHceramide is shown in Figure 1. A three-dimensional plot of C22-DHceramide levels as a function of light dose (Figure 1). A dose-dependent increase in C22-DHceramide levels is shown. Unlike Photofrin or light alone, PDT triggered a significant increase in C22-DHceramide levels.

Individual ceramides did not show significant time-dependent changes post-PDT. However, there was a significant, 12.5% average increase per hour post-PDT in the levels of all 11 ceramides. The chance that this was a random event is only 0.00048 (not shown).

The levels of other SLs were also evaluated with respect to PDT dose. DHsphingosine-1-phosphate was increased 9.8-fold per unit of PDT light dose.

Overall, these are important findings because they demonstrate that the accumulation of ceramides and DHceramide is not only limited to in vitro cultured SCCVII cells.
50% of total ceramides. Comparative levels of tumour and plasma C16-DHceramide followed the same pattern as C16-ceramide (Figure 2B). The very-long fatty acyl chain DHceramides, C22-, C22:1- and C24-DHceramide were among the most abundant in both tumours and plasma. Overall, ceramides and DHceramides with long and very-long fatty acyl chains were the most abundant species in these tissues.

Global increase in tumour DHceramides after Photofrin-PDT

SCCVII tumours were treated with a therapeutic dose of Photofrin-PDT (Korbelik and Cecic, 2008). Tumour and plasma SL profiles were identified by MS (Figure 3). Among plasma ceramides, only the levels of C24-ceramide were 29% lower after PDT compared with Photofrin alone (Figure 3B). Notably, following PDT the levels of tumour C20-DHceramide increased to 5.4-fold of Photofrin alone (Figure 3C). There were no significant differences in the levels of specific tumour ceramides or plasma DHceramides after PDT compared to Photofrin alone (Figures 3A and D).

In addition, overall fold changes were calculated for averages of all ceramides or DHceramides after PDT relative to averages of all corresponding SLs in untreated or Photofrin-treated mice. Compared with untreated controls, the levels of total tumour ceramides were reduced by 67 and 38% after Photofrin and PDT, respectively (Figure 4A). Compared with Photofrin, a 21% attenuation of tumour ceramide response to PDT was significant (Figure 4B). A significant 1.3-fold total increase in plasma ceramides was observed following either Photofrin alone or PDT (Figures 4A and B).

Relative to untreated controls, a 34% total decrease in tumour DHceramides was observed post-Photofrin (Figure 4A). In contrast, exposure of mice to PDT led to a 15% total increase in...
tumour DHceramides relative to untreated controls (Figure 4A). Thus, compared with Photofrin alone, there was an overall significant 1.6-fold increase in tumour DHceramides post-PDT (Figure 4B). Total increases in plasma DHceramides were similar in mice exposed to either treatment (Figures 4A and B).

No significant effect on tumour S1P levels was observed after PDT. In plasma, Photofrin alone or PDT decreased S1P levels by 31% so that there was no difference between the two (not shown). Neither tumour nor plasma sphingosine levels were significantly affected by PDT (not shown).

In summary, these are significant data not only because they confirm our cell culture findings but also because they show for the first time that a therapeutic dose of Photofrin-PDT triggers a significant, potentially selective, build-up in tumour DHceramides, supporting the involvement of the de novo ceramide pathway in PDT in vivo response. Photofrin alone also affects the SL profile, but the impact of PDT is distinctly different, particularly in triggering more dramatic increases in DHceramide levels. The effects of photosensitiser alone on SLs are expected to be less pronounced, with more potent sensitising agents administered at much lower doses than Photofrin.

Enhanced tumour response after Photofrin-PDT + ceramide analogue LCL29

To test whether the ceramide analogue LCL29 can improve PDT tumour response, SCCVII tumours were treated with a moderately therapeutic dose of Photofrin-PDT + non-toxic dose of LCL29 (Senkal et al., 2006). LCL29 was administered daily over 7 days, starting as the combination with PDT. As shown in Figure 5 (insert), LCL29 alone had no effect on tumour growth. Remarkably, growth retardation of mouse tumours attained by PDT was enhanced by adjuvant LCL29 (P < 0.012; Figure 5). An average of 24% decrease in tumour volumes was detected across all time points. It is conceivable that this effect can be improved by optimising LCL29 treatment protocol, for example, by escalating LCL29 doses, and/or by other SL agents. These are important data showing for the first time that PDT tumour response is promoted by an SL-modulating agent.

This study demonstrates for the first time that Photofrin-PDT has a definitive effect on the SL profile not only in SCCVII cells but also in vivo. Specifically, DHceramides are elevated in vitro and in vivo Photofrin-PDT and sphingolipids

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Figure 5 Enhanced response of SCCVII tumours to Photofrin-PDT + LCL29. SCCVII tumours were treated with PDT (Photofrin 5 mg kg-1 plus 150 J cm-2 24 h later), LCL29 (seven daily injections of 20 mg kg-1 i.p.) or their combination (initial LCL29 injection 2h before PDT light was followed by additional six daily injections). A control group and PDT-only group received vehicle injections as in the LCL29 treatment protocol. The therapy response was monitored by tumour size measurement. Depicted in the insert are the results (presented as the means ± s.d.) for LCL29 treatment alone and the control group with vehicle (p) injections. The results in the main graph are presented as separate growth curves for individual tumours. Tumours in PDT + LCL29 group grew slower than the majority of those in PDT-only group. On the basis of the linear mixed-effects model, the difference between these two groups is significant with P < 0.012 (n = 7). Volume normalisation was performed by dividing the tumour volume data by the tumour volume measured on day 0.
tumours after PDT. These novel findings support the notion that the de novo SL pathway is a PDT target. Others (Bose et al, 1995; Perry et al, 2000; Charles et al, 2001; Min et al, 2007; Senkal et al, 2007; Wang et al, 2008) and we (Wispiyono et al, 2002; Dolgachev et al, 2004; Dolgachev et al, 2005; Separovic et al, 2007; Separovic et al, 2008) have shown the involvement of de novo SLs in response to anticancer therapeutics. DHceramide synthase 1 (LASS1)/C18-ceramide have been associated with chemotherapy-triggered killing of human head and neck squamous cell carcinomas (Senkal et al, 2007). We observed a significant increase in C18:1-DHceramide, the product of DHceramide synthase 1. Our findings in SCCVII cells support the notion that, besides DHceramide synthase 1, other DHceramide synthases, such as DHceramide synthase 2 and DHceramide synthase 4, might be involved in increasing DHceramide levels after PDT. A marked increase in tumour C20-DHceramide further supports the role of DHceramide synthase 2 and DHceramide synthase 4, which have specificity for C20–C26 and C20 ± 2 fatty acyl CoA, respectively (Pewzner-Jung et al, 2006; Laviad et al, 2008). These findings imply that the accumulation of tumour DHceramide might serve as a biomarker of tumour response to PDT and that targeting the de novo ceramide pathway might be a strategy for drug development to advance PDT tumour response.

Another key finding of this study is that co-exposure of SCCVII tumours to Photosensin-PDT and LCL29 leads to enhanced retardation of tumour growth compared with Photosensin-PDT alone. Similarly, LCL124, a structural analogue of LCL29, together with the anticancer agent gemcitabine, effectively inhibits tumour growth of human head and neck squamous cell carcinomas in vivo (Senkal et al, 2006). Endogenous ceramide levels were not changed after 24 days, that is, at the end of in vivo studies after LCL124 or after the combination with gemcitabine (Senkal et al, 2006), suggesting no long-term effect of the treatments on ceramide metabolism. Notably, LCL29 evokes a time-dependent increase in total ceramide levels over 24 h in MCF-7 cells (Szulc et al, 2006). Selective increases in C16-, C14- and C18-ceramides and decreases in C24- and C24:1-ceramides were observed (Szulc et al, 2006). The signature effects of LCL29 alone or in combination with Photosensin-PDT on the SL profile in SCCVII cells and tumours remain to be determined.

In summary, this is the first report demonstrating that following Photosensin-PDT, definite changes in the SL profile in SCCVII cells and tumours are triggered, and that tumour response is improved after the combined treatment with Photosensin-PDT + ceramide analogue LCL29. Thus, our study validates the therapeutic efficacy of LCL29 in combination with Photosensin-PDT. These findings indicate that ceramide analogues hold the potential of a new class of adjuvants for advancing PDT therapeutic successes.

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