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CATIONIC CERAMIDES AND ANALOGUES, LCL30 AND LCL85, AS ADJUVANTS TO PHOTODYNAMIC THERAPY OF TUMORS

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Abstract

Photodynamic therapy (PDT) is known to alter the expression of various genes in treated cells. This prompted us to examine the activity of genes encoding two important enzymes in sphingolipid (SL) metabolism, dihydroceramide desaturase (DES) and sphingosine kinase (SPHK), in mouse SCCVII tumor cells treated by PDT using either the porphyrin-based photosensitizer Photofrin or silicon phthalocyanine Pc4. The results revealed that PDT induced an upregulation in the expression of two major isoforms of both genes (DES1 and DES2 as well as SPHK1 and SPHK2). While the changes were generally moderate (2-3 fold gains), the increase in DES2 expression was more pronounced and it was much greater with Photofrin-PDT than with Pc4-PDT (over 23-fold vs. less than 5-fold). Combining either Photofrin-PDT or Pc4-PDT with the cationic C16-ceramide LCL30 (20 mg/kg i.p.) for treatment of subcutaneously growing SCCVII tumors rendered important differences in the therapy outcome. Photofrin-PDT, used at a dose that attained good initial response but no tumor cures, produced 50% cures when combined with a single LCL30 treatment. In contrast, the same LCL30 treatment combined with Pc4-PDT had no significant effect on tumor response. The optimal timing of LCL30 injection was immediately after Photofrin-PDT. The therapeutic benefit was lost when LCL30 was given in two 20 mg/kg injections encompassing intervals before and after PDT. LCL85, the cationic B13 ceramide analogue and SL-modulating agent, also increased cure rates of Photofrin-PDT treated tumors, but the therapeutic benefit was less pronounced than with LCL30. These results with LCL30 and LCL85, and our previous findings for LCL29 (another SL analogue), assert the potential of SLs for use as adjuvants to augment the efficacy of PDT-mediated tumor destruction.

Keywords

Sphingolipid analogs; Squamous cell carcinoma SCCVII; Photodynamic therapy; Dihydroceramide desaturase; Sphingosine kinase

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1. Introduction

Although photodynamic therapy (PDT) is a clinically established treatment for various cancers [1,2], further advances in the understanding of its anti-tumor effects and their modulators are expected to lead to additional improvements in therapeutic benefits with this modality. Among biomolecules with potential to strongly influence PDT response are sphingolipids (SLs). They comprise about 30% of total plasma membrane lipids and are organized in liquid ordered micro domains (rafts)[3,4]. Through these specialized structures SLs mediate the interaction of cells with their microenvironment by modulating the behavior of cellular proteins and receptors and participating in signal transduction [5]. Key SL metabolites (i.e. ceramide, sphingosine and sphingosine-1-phosphate) are critical mediators of cellular stress responses and have prominent roles in cell apoptosis, autophagy, as well as immune cell functions [6-8]. Our earlier research has shown that SLs are important participants in response to PDT at both cellular and tumor levels [9-11]. Similar to chemotherapy and some other types of cancer therapy, PDT has a distinct signature effect on the SL profile owing in particular to induction of the *de novo* ceramide biosynthesis [11,12]. *De novo* ceramide appears to be involved in the initiation of apoptosis of cancer cells after PDT [13,14]. We reported that, compared to cancer cells, considerably higher ceramide and S1P levels can be found in tumor-associated macrophages both before and after tumor PDT. This prompted us to suggest that SLs could have an important role in PDT-associated host immune responses [15].

Cancer cells exhibit a higher sensitivity than normal cells to lethal effects of increased endogenous cellular ceramide levels induced by exogenous ceramide or SL analogues [16]. This motivated the development of various SL analogues as prospective anti-cancer agents [17,18]. We have investigated the efficacy of one such agent, C6-ceramide analogue LCL29, as adjuvant to PDT. Our results show that cure rates of PDT-treated mouse squamous cell carcinomas SCCVII can be significantly increased by adjoined treatment with a single dose of LCL29 [11]. Further investigation revealed that the combined LCL29 treatment strongly amplified apoptosis in cancer cells of PDT-treated tumors [14].

Two additional SL modulators, LCL30 (C16-ceramide analogue)[19] and LCL85 (acid ceramidase inhibitor B13 analogue)[20] were examined *in vitro* combined with PDT and were both found to augment PDT-mediated killing of SCCVII cells [21,22]. These cationic, mitochondrially-targeted compounds were originally developed as potential anticancer agents [23,24]. The present study with SCCVII tumor-bearing mice evaluated the efficacy of LCL30 and LCL85 in enhancing the curative outcome of PDT.

2. Materials and methods

2.1. Cells and tumor model

Mouse squamous cell carcinoma SCCVII is a recognized model for poorly immunogenic head and neck cancer of spontaneous origin [25]. Alpha minimal essential medium (Sigma Chemical Co., St. Louis MO, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan UT, USA) was used for culturing SCCVII cells *in vitro*. The cells were regularly screened for mycoplasma contamination. *In vivo*, SCCVII tumors were maintained in syngeneic immunocompetent C3H/HeN mice. Cells obtained by enzymatic digestion of maintenance tumors were used for implanting cohorts of experimental tumors by a subcutaneous injection of 1 million cells into the lower dorsal region of 7-9 week old mice. The mouse protocols were approved by the Animal Care Committee of the University of British Columbia.

2.2. Photodynamic treatment and SL analogues

Mice received Photofrin (Axcan Pharma, Mont-Saint-Hilaire QC, Canada) by intraperitoneal injection of 10 mg/kg and 24 hours later their tumors were exposed to superficial illumination while they were restrained unanaesthetized in holders exposing their backs. The light (wavelength 630 ± 10 nm) was produced by a FB-QTH high throughput illuminator (Sciencetech, London ON, Canada) based on a 150 W QTH lamp and equipped with integrated ellipsoidal reflector. It was delivered through an 8-mm core diameter liquid light guide (Oriel Instruments, Stratford CT, USA) at an irradiance-derived fluence rate of 80-90 mW/cm². The light dose used was 150 J/cm².

Alternatively, the treatment of tumors by PDT was done by administering Pc4, HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)₂, at 1.25 mg/kg i.p and using a 665 ± 10 nm filter with the same light source. This phthalocyanine photosensitizer was supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). For injection, Pc4 was delivered in cremophor:ethanol:saline 0.5:0.5:9.0. The light dose used was 200 J/cm² at 80 mW/cm².

For *in vitro* PDT, SCCVII cells cultured in 3-cm diameter Petri dishes were incubated either with Photofrin (20 µg/ml) and 18 hours later treated with 630 ± 10 nm light (1 J/cm²), or with Pc4 (0.179 µg/ml, 250 nM) and 18 hours later exposed to 665 ± 10 nm light (0.5 J/cm²). Immediately before light treatment, photosensitizer-containing medium was removed, the dishes (containing around 1 million cells) were washed and PBS left during illumination. The cells were then kept in culture at 37°C with full growth medium for 4 hours before they were taken for RNA extraction. Cell survival levels were deducted from flow cytometry-based assessment of the incidence of apoptotic/necrotic death [14].

Both SL analogs, D-erythro-2-*N*-[16-(1-pyridinium)-hexadecanoyl]-sphingosine bromide (LCL30) and D-threo-2-*N*-[16-(1-pyridinium)-hexadecanoyl]amino-1-(4-nitrophenyl)-1,3-propanediol bromide (LCL85), were synthesized by previously reported methods [19,20]. For injection into mice, LCL30 was initially dissolved in ethanol-cremophor and then diluted in physiological saline, while LCL85 was dissolved in 5% dextrose. The effect of these drugs on tumor growth rate was monitored by measuring three orthogonal tumor diameters with a caliper.

2.3. Gene expression analysis

Expression of genes encoding two major isoforms of mouse dihydroceramide deasaturase (DES) [26,27] and mouse sphingosine kinase (SPHK) [28] was determined by extracting from SCCVII cells total RNA that served for creating complementary strand DNA transcript subsequently amplified by quantitative real-time RT-PCR (qRT-PCR) in the presence of gene-specific primers. Protocol details were described in detail in a previous report [29]. Briefly, 0.5 µg of total RNA was processed using the Superscript VILO cDNA synthesis kit (#11754-050, Invitrogen Canada Inc., Burlington ON, Canada) in total reaction volume of 15 µl. To perform qRT-PCR, 1 µl of cDNA from each sample was used with EXPRESS SYBR GreenER qPCR Supermix (Invitrogen) in triplicates in a total reaction volume of 10 µl. The primers were designed and tested in our laboratory, and their concentrations optimized using the PerfeCta SYBR Green Master Mix (Quanta Biosciences, Gaithersburg MD, USA). Their sequences (forward and reverse, respectively) for DES1 were TTC GAG GGC TGG TTC TTC TG and GGG ATT GAT GAA CAG GGG, for DES2 were TTC GAG GGC TGG TTC TTC TG and AGG GCG TTG AGG ATC TCC AT, for SPHK1 were ACT CAC CGA ACG GAA GAA CC and AGT CTG GCC GTT CCA TTA GC, and for SPHK2 were AGA ACG ACA GAA CCA TGC CC and TCA GCA CCT CGT AAA GCA GC. The

expression level of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured and used for normalizing the expression of studied genes.

2.4. Flow cytometry

Determining the levels of apoptotic cells in SCCVII tumors was performed as described earlier [14]. Briefly, the host mice were injected FITC-VAD-FMK (MBL International Corp., Woburn MA, USA) at 10 µg/mouse intravenously at 2.5 hours after PDT. The mice were sacrificed 30 minutes later, tumors excised and disaggregated into single cell suspensions, which were stained with anti-mouse GR1 antibody conjugated with fluorescent fluorophore PE-Cy5 (eBioscience Inc., San Diego CA, USA). Flow cytometry analysis was performed on a Coulter Epics Elite ESP (Coulter Electronics, Hialeah FL, USA) with at least 20,000 cells per sample.

2.5. Statistical analysis

In vivo treatment groups had at least 8 mice and *in vitro* samples were in quadruplicates. Statistical evaluation of tumor response results was done using log-rank test, while the analysis of other data was performed based on Mann-Whitney test. The threshold for statistical significance was set at 5%.

3. Results

3.1. Upregulation of DES and SPHK genes in PDT-treated cells

The effect of PDT on the expression in treated SCCVII cells of mouse genes encoding two important enzymes in the sphingolipid metabolism, DES and SPHK [27,28], was determined using qRT-PCR. The two major isoforms of each enzyme, DES1 and DES2 plus SPHK1 and SPHK2, were examined. *In vitro* growing cells were exposed to either Photofrin-PDT or Pc4-PDT at a dose resulting in 70-80% cell kill. Control samples included untreated, light only and photosensitizer only treated cells. The cells were collected for analysis at 4 hours after PDT, a time-point shown to be informative for various genes in our related studies [29,30]. The data was GAPDH-normalized and presented relative to untreated cells. The results for DES show that treatment with PDT induced an upregulation in the expression of both isoforms, but the effect was much more pronounced with DES2 (Fig. 1a). The maximal response of about 24-fold increase in DES2 expression was seen after Photofrin-PDT. The corresponding response to Pc4-PDT was also prominent but not as strong (close to 5-fold). These effects were PDT-specific because treatments by light only or either photosensitizer alone produced no significant change in DES2 expression; however, Pc4-PDT effect compared to Pc4 was not statistically different. On the other hand, both Photofrin-PDT and Pc4-PDT induced around two-fold upregulation of DES1 gene (Fig. 1a). Interestingly, a small but statistically significant upregulation of this gene was seen with Pc4 only treatment with consequent loss of the statistical significance for Pc4-PDT effect when compared to photosensitizer only.

A moderate upregulation of SPHK genes was also induced by PDT treatment. The effects on SPHK1 and SPHK2 expression were more pronounced after Pc4-PDT than after Photofrin-PDT (2.6- and 2.2-fold vs. 1.9- and 1.5-fold respectively, Fig. 1b). A significant upregulation of these genes was also observed with Pc4 alone treatment but its extent was lower than with Pc4-PDT. The light treatment alone produced no significant gene expression changes.

3.2. LCL30 and LCL85 elevate PDT-mediated tumor cures

Subcutaneous SCCVII tumors growing in C3H/HeN mice were treated with either Pc4 or Photofrin PDT with or without additional treatment with LCL30. The response is presented

using a modified Kaplan-Meier plot with ordinate denoting the percentage of mice with no palpable tumor. Typically, the tumors showed signs of edema and inflammation during the first several hours after PDT treatment and became necrotic and impalpable by the next day. The injected 20 mg/kg of LCL30 (i.p.) was chosen as a safe maximum dose in mice. None of the tested single LCL30 treatments (2 hours before PDT, immediately after PDT, and 24 hours after PDT) produced any significant benefit on the response of tumors to Pc4-PDT (Fig. 2a). The chosen Pc4-PDT dose given either alone or in combination with LCL30 produced initially a 100% tumor ablation but no permanent cures because of tumor recurrence within 18 days after PDT. Single LCL30 administration without PDT retarded tumor growth but produced no cures (Fig. 2 inset). Similar to Pc4-PDT, the selected Photofrin-PDT dose was close to curative range and produced an initial ablation of tumors that re-grew later (Fig. 2b). However, adjuvant LCL30 treatment given immediately after light delivery for Photofrin-PDT prevented recurrence in 50% of treated tumors (since one half of treated tumors remained impalpable at the end of monitoring period of 90 days). Somewhat less effective but still beneficial was the regimen with LCL30 injection at 2 hours before PDT. No significant benefit was seen when LCL30 was administered at 24 hours after Photofrin-PDT. For comparative purpose, both Pc4 and Photofrin were administered intraperitoneally with the more hydrophobic photosensitizer (Pc4) distributing more slowly to the tumor tissue. However, with mice the chosen 24-hour interval for light treatment should allow a reasonable time for adequate tumor tissue distribution for both photosensitizers.

In further testing we opted for the SL analog injection immediately after photodynamic light treatment and to use Photofrin for PDT. In addition to LCL30, LCL85 was included in the study at the maximal non-toxic dose (10 mg/kg i.p.). As in the previous experiment, single LCL30 treatment rendered non-curative PDT treatment into 50% curative (Fig. 3). Combining PDT with LCL85 produced also a therapeutic benefit, albeit less pronounced than with PDT plus LCL30 combination. Treatment with LCL85 alone had no detectable effect on tumor growth (not shown), and there were no tumor-free mice at any time after either LCL30 or LCL85 treatment. Since both these drugs produced no cures it can be concluded that their interaction with PDT is greater than additive.

Further experiments examined whether two doses rather than single LCL30 treatment can achieve additional therapeutic benefit. Hence, the tested groups in addition to PDT alone (A group) and single LCL30 injection immediately post PDT (B group) featured LCL30 given either 24 hours before PDT plus immediately post PDT (C group), or immediately post PDT plus 48 hours post PDT (D group). Each administered LCL30 dose was 20 mg/kg. Interestingly, treating with two LCL30 doses effectively reduced (C group) or even completely eliminated the therapeutic benefit gained with a single LCL30 injection (D group)(Fig. 4).

Unlike ceramide analogue LCL29 [14], LCL30 given as a single injection had apparently not significantly affected the induction of apoptosis in tumors treated by Photofrin-PDT. At 3 hours after treatment, the percentage of apoptotic cells in SCCVII tumors were 17.7 ± 5.0 and 14.9 ± 5.6 (means $\pm$ SD) in PDT alone and PDT plus LCL30 groups, respectively. The LCL30 treatment alone had not produced significant changes in the percentage of apoptotic cells in these tumors.

4. Discussion

It is well established that PDT induces changes in the expression of a variety of genes in treated cells [30,31]. The present study demonstrates that this includes also the expression of DES and SPHK genes. The former, which facilitates the last step of *de-novo* biosynthesis of

ceramide, has two isoforms DES1 and DES2 that are highly expressed in various organs in mammals [27,32,33]. Phosphorylation of the primary hydroxyl group on sphingosine, which is necessary for enabling its catabolism, is catalyzed by SPHK [34]. The product, S1P, is a key member of the “sphingolipid rheostat” contributing together with ceramide and sphingosine to cell fate [28]. Hence, SPHK is a critical regulator of the sphingolipid rheostat as it produces the anti-apoptotic mediator S1P while decreasing the levels of the pro-apoptotic ceramide and sphingosine. Two isoforms of this enzyme, SPHK1 and SPHK2, are found in various tissues in humans, mice and other mammals [35].

The presented results reveal that PDT induces a very strong upregulation of DES2 gene in treated cells, especially if PDT is mediated by Photofrin. The activity of the other isoform, DES1, also increased after PDT but not more than twofold. A moderate upregulation of SPHK1 and SPHK2 genes was also detected in PDT-treated cells. These gene expression changes could, at least in part, be responsible for elevated ceramide and S1P levels found in Photofrin-PDT treated SCCVII cells [10].

Our earlier studies and work by other investigators has shown that PDT triggers an increase in signal transduction activity, which can to a large extent be attributed to response triggered by oxidative stress inflicted by this modality [36-38]. The engaged signal transduction pathways involve notably PI3K/Akt and various gene transcription factors as downstream targets including NF- κ B, AP-1, STAT3, and HIF-1 [38-40]. Genes involved in *de novo* ceramide biosynthesis were shown to be activated through TLR4-NF- κ B signaling [41]. Distinct patterns of intracellular microlocalization of Photofrin (wide spread in various membrane structures) and Pc4 (mitochondrial membrane but also a broader spectrum) are responsible for the differences in the site of singlet oxygen and oxygen radical action within cells for PDT with these photosensitizers [2]. The consequent disparity in signal transduction initiating sites could be largely responsible for the difference in the extent of DES2 upregulation between Photofrin-PDT and Pc4-PDT. Such deviations could be expected to result in differences in the SL profile changes after PDT mediated by different photosensitizers. This may become relevant particularly in the response of tumors to PDT combined with specific SL-modulating agents. This was shown for the agent LCL30, which exhibited beneficial effect on SCCVII tumor cures when combined with Photofrin-PDT but a subadditive effect with Pc4-PDT (Fig. 2).

The C16-ceramide analogue LCL30 belongs to pyridinium salts-containing cationic ceramide conjugates synthesized to target mitochondria as this organelle plays an important role in ceramide-induced cell death [19,23,42,43]. Mitochondrial targeting is advantageous for cancer treatment because cancer cells tend to have mitochondria with more negative mitochondrial membrane potential [44]. Exogenous ceramide and its derivatives (including LCL30) were shown to induce cell death in various human cancer cell types [45,46] and these cells seem to be more susceptible to this toxic effect than normal cells [16]. Studies with mouse tumor models have demonstrated that daily injections of LCL30 significantly reduced growth of established colon carcinomas [24]. Our results show that a Photofrin-PDT treatment of SCCVII tumors producing no cures was boosted to attain 50% cures when combined with a single LCL30 treatment. Injecting LCL30 immediately after photodynamic light treatment was identified as optimal timing for this combination. While the therapeutic benefit was still evident (although smaller) if LCL30 was given 2 hours before PDT, it was completely lost if delayed to 24 hours after PDT. This finding suggests that LCL30 modulates the early response to PDT, most likely because of the critical timing of the impact of elevated global ceramide levels and autophagy expression induced by this agent [47].

The adjuvant effect of ceramide analogue LCL29 correlates with enhanced PDT-mediated cancer cell apoptosis [14]. This appears not to be the case with LCL30 since similar

percentages of apoptotic cancer cells were found in SCCVII tumors treated by PDT alone or PDT combined with LCL30. Our *in vitro* studies with SCCVII cells revealed that combining PDT with LCL30 enhanced global ceramide level increase, caspase-3 activation, and overall cell killing in the absence of apoptosis [21]. However, these *in vitro* results were obtained with Pc4-PDT and it remains to be seen to what extent they are mimicked by Photofrin-PDT.

For the interaction of LCL30 with PDT it is also characteristic that the therapeutic benefit with SCCVII tumors obtained by a single LCL30 injection after PDT was lost when LCL30 was administered twice (encompassing intervals either before or after PDT) (Fig. 4). This supports the suggestion that LCL30 can have timing-dependent contrasting effects on PDT response. Further experimentation, including testing the effects of reduced LCL30 dose in multiple treatments, seem merited for shedding light on the underlying mechanistic issues.

In addition to LCL30, this study examined LCL85, another SL-modulating agent, as adjuvant to PDT. Developed as a cationic analogue of B13, an established acid ceramidase inhibitor [24], LCL85 is considered a promising anticancer agent [20]. Our work with *in vitro* cultured SCCVII cells revealed that LCL85 promoted the rise in global ceramide levels in Pc4-PDT-treated cells, while S1P levels that were significantly raised by LCL85 alone were restored to resting status by PDT plus LCL85 [22]. It was also shown that combining these two agents resulted in an enhanced autophagy and caspase-3 activation with reduced clonogenic survival of these cells. However, for interpreting our tumor response results with Photofrin-PDT the insight from this *in vitro* data with Pc4-PDT is uncertain. Notwithstanding the unresolved issues on the underlying *in vivo* mechanisms, the results of present study demonstrate that LCL85 produces a therapeutic benefit when combined with Photofrin-PDT. However, it appears that the full potential of this drug cannot be reached because mice did not tolerate the doses greater than 10 mg/kg. It can be speculated that if it was possible to use the 20 mg/kg dose of LCL85 the efficacy in promoting PDT-mediated tumor cures would be similar to the same dose of LCL30.

In conclusion, the results with SL analogs LCL30 and LCL85 confirm our previous finding with LCL29 that the adjuvant modulation of the SL profile can augment the efficacy of PDT-mediated tumor destruction. Further investigations are warranted for identifying the agents of this class that can serve as the most potent PDT adjuvant. This study also reveals that PDT mediated by two different photosensitizers (Photofrin and Pc4) causes different changes in the expression of genes encoding enzymes involved in the SL metabolism, and that such differences may dictate the choice of photosensitizer used for PDT combined with SL-modulating agents.

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Highlights

Photodynamic therapy induces photosensitizer-dependent upregulation of dihydroceramide desaturase and sphingosine kinase

Non-curative Photofrin-PDT attained 50% tumor cures when combined with LCL30 but no such benefit was obtained with Pc4-PDT

Similar gain as with LCL30 was seen with LCL85 and LCL29; hence sphingolipid modulation augments the efficacy of tumor PDT

a

b

Figure 1. PDT-induced changes in the expression of DES and SPHK genes in SCCVII cells

The cells were incubated with either Photofrin (20 $\mu\text{g/ml}$) or Pc4 (0.170 $\mu\text{g/ml}$) for 18 hours and then exposed to 1 J/cm^2 of 630 ± 10 nm light for Photofrin-PDT or to 0.5 J/cm^2 of 665

± 10 nm light for Pc4-PDT. Control samples included cells treated only with Photofrin, Pc4 or light. After treatment, the cells were kept in culture for additional 4 hours and then collected for analysis of the expression of **(a)** DES and **(b)** SPHK genes (two isoforms each) using qRT-PCR. Target gene expression was GAPDH-normalized and presented relative to the corresponding gene expression level in untreated samples. Bars depict SD, N = 4; *values are statistically different than with untreated cells. Please note the scale differences in (a) and (b).

Figure 2. Response of SCCVII tumors to Pc4-PDT or Photofrin-PDT combined with LCL30

The mice were given either (a) Pc4 (1.25 mg/kg i.p.) or (b) Photofrin (10 mg/kg i.p.) and 24 hours later their tumors were treated with either 200 J/cm² (Pc4-PDT) or 150 J/cm² (Photofrin-PDT). A single LCL30 dose (20 mg/kg i.p.) was administered either 2 hours before, immediately after or 24 hours after photodynamic light treatment. Mice were thereafter monitored up to 90 days for signs of tumor regrowth. Treatment with LCL30 alone achieved tumor growth-rate retardation but no ablation (inset). *statistically significant difference compared to PDT only ($p < 0.05$).

Figure 3. Response of SCCVII tumors to Photofrin-PDT combined with either LCL30 or LCL85
 The mice were administered Photofrin (10 mg/kg i.p.) followed by 150 J/cm². Adjuvant treatment with LCL30 (20 mg/kg) or LCL85 (10 mg/kg) was given intraperitoneally immediately after photodynamic light exposure. Tumor response was determined as described for Fig. 2. *statistically significant difference compared to PDT only ($p < 0.05$).

Figure 4. Response of SCCVII tumors to Photofrin-PDT combined with either single or dual LCL30 treatment

The PDT treatment was performed as described for Fig. 3. Single injection of LCL30 (20 mg/kg i.p.) was done immediately after PDT, while the additional injection for dual treatment was administered either 24 hours before PDT or 48 hours after PDT. Tumor response was determined as described for Fig. 2. *statistically significant difference compared to PDT only ($p < 0.05$).