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Letter

Evaluation of laser irradiance on photodynamic therapy using talaporfin sodium-induced glioblastoma T98G cell death

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ABSTRACT — In photodynamic therapy (PDT) for glioma patients, apoptosis not necrosis is the desirable mode of cell death, as necrotic cell death induces late appearance of obstacles following PDT. We previously demonstrated that increase in both treatment dose of photosensitizer talaporfin sodium (NPe6) and laser fluence (laser energy density) changes the dominant cell death process from apoptosis to necrosis in glioblastoma T98G cells. Here, we investigated the effect of laser irradiance (laser power density), which is another important parameter of PDT, on PDT-induced cell death modalities in cultured T98G cells. When fluence was fixed at 10 J/cm^2, NPe6 dose-dependently reduced the cell viability, regardless of irradiance (11, 22, 33, and 44 mW/cm^2). Morphological observations and biochemical analysis (measurement of caspase-3 activity, staining of cell surface-exposed phosphatidylserine, and staining of propidium iodide) further confirmed that increase in dose of NPe6 changed the dominant cell death process from apoptosis to necrosis, regardless of irradiance. We also noted no influence of irradiance level on the leakage of lactate dehydrogenase from T98G cells following PDT treatment. Taken together, our present and previous findings suggest that dose of NPe6 and laser fluence but not laser irradiance are important parameters to consider in PDT using NPe6 in T98G cells.

Key words: Apoptosis, Glioma, Necrosis, Photodynamic therapy, Talaporfin sodium

INTRODUCTION

Photodynamic therapy (PDT) is a light-mediated treatment method that involves activation of a photosensitizer to generate singlet oxygen and radical species, which are toxic to cells (Dougherty *et al*., 1998; Dolmans *et al*., 2003). Since photosensitizers selectively localize in neoplastic tissue, subsequent light-treatment induces tumor-selective cell death (Wilson, 1992). As such, PDT is widely used both clinically and preclinically to treat range of tumor types, including lung, skin, liver neoplasms (Agostinis *et al*., 2011). We previously demonstrated the efficacy of PDT using the photosensitizer talaporfin sodium (mono-L-aspartyl chlorine e6, NPe6; NPe6-PDT) to treat glioma patients. When NPe6 was administered to mice via the tail vein, NPe6 was accumulated in the transplanted glioma, and subsequent light-irradiation induced apoptosis and necrosis in transplanted glioma (Matsumura *et al*., 2008; Namatame *et al*., 2008). *In vitro* study has also shown that NPe6-PDT induces cytochrome c release from mitochondria, caspase-9 and caspase-3 activation that were the features of mitochondrial pathway-mediated apoptosis (Miki *et al*., 2013). In addition, clinical study has shown that NPe6-PDT with surgical resection markedly prolonged 1 year survival rate than conventional protocols (Akimoto *et al*., 2012).

Induction of apoptotic cell death in tumor tissues is considered important for effective NPe6-PDT in glioma patients, as necrotic cell death causes leakage of cytosolic components into extracellular space that may induce undesirable obstacles such as occlusion or shut-down of blood vessels and inflammatory response. With apoptosis, in contrast, the cellular components are safely sequestered by plasma membranes (Fietta, 2006; Hanayama *et al*., 2004; Maderna and Godson, 2003; Ren and Savill, 1998; Savill and Fadok, 2000). Apoptotic cell death is therefore

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the ideal mode of cell death with NPe6-PDT.

We have recently showed that, when laser irradiance (laser power density) was fixed at 33 mW/cm^2, increasing in both dose of NPe6 (0-50 μg/mL) and laser fluence (laser energy density) (0-30 J/cm^2) changes the dominant cell death process from apoptosis to necrosis in NPe6-PDT-treated glioblastoma T98G cells (Miki *et al*., 2014). Therefore, to optimize PDT-treatment conditions, we investigated the effect of laser irradiance level (0-44 mW/cm^2), which is another important parameter for PDT, on cell death modalities in NPe6-PDT-treated glioblastoma T98G cells.

MATERIALS AND METHODS

Materials

NPe6 was provided by Meiji Seika Pharma Co., Ltd (Tokyo, Japan). The cell counting kit-8 was obtained from Dojindo (Kumamoto, Japan). The MEBCYTO apoptosis kit (Annexin V-FITC with propidium iodide [PI] kit) was purchased from Medical and Biological Laboratories (Nagoya, Japan). NUCView™ 488 caspase-3 assay kit for live cells was obtained from Biotium, Inc (Hayward, CA, USA). The lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

PDT treatment of glioma cells

PDT treatment was performed as previously described (Miki *et al*., 2014). Briefly, human glioblastoma T98G cells (Riken Cell Bank, Tsukuba, Japan) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (10% FBS-RPMI 1640) in 96-well culture plates at 37°C in 5% CO_2 atmosphere. After 24 hr incubation, T98G cells were pretreated with 0-50 μg/mL NPe6 for 4 hr and then washed with culture medium and incubated for an additional 1 hr. The cells were then immersed in fresh 10% FBS-RPMI 1640 and subjected to laser irradiation (wave length: 664 nm, laser fluence: 10 J/cm^2; laser irradiance: 0, 11, 22, 33, or 44 mW/cm^2) using a semiconductor laser irradiator (Panasonic Healthcare Co., Ltd., Ehime, Japan). Laser fluence was calculated as follows:

$$\text{Fluence (J/cm}^2\text{)} = \text{Irradiance (mW/cm}^2\text{)} \times \text{irradiation time (sec)} / 1000.$$

In the present experiments, laser fluence was maintained at 10 J/cm^2, and laser irradiance and irradiation time were adjusted as follows, respectively: (1) 0 mW/cm^2 and 0 sec (nonirradiated control), (2) 11 mW/cm^2 and 909 sec, (3) 22 mW/cm^2 and 454 sec, (4) 33 mW/cm^2 and 303 sec, and (5) 44 mW/cm^2 and 227 sec.

Measurement of cell viability and observation of cell morphology

After 24 hr of PDT treatment, T98G cell viability was measured using a Cell counting kit-8. Briefly, T98G cells were incubated with Cell counting kit-8 solution at a final concentration of 10% at 37°C for 1 hr. After incubation, the reaction was stopped by addition of HCl at a final concentration of 0.1 M, and absorbance was immediately measured at 450 nm (reference wavelength: 600 nm) using a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA, USA). Separately, morphology of T98G cells after PDT treatment was observed under a phase contrast microscope (μ-Radiance; BioRad, Hercules, CA, USA).

Detection of apoptosis and necrosis

After 24 hr of PDT treatment, all cells (floating cells, lightly adherent cells, and adherent cells) were collected, as described previously (Miki *et al*., 2014). Capase-3 activity, cell surface-externalized phosphatidylserine and/or PI staining in T98G cells were measured in the collected cells using commercial assay kits (caspase-3: NucView™ 488 caspase-3 assay kit for live cells, exposed phosphatidylserine and PI: MEBCYTO apoptosis kit), as described previously (Miki *et al*., 2014).

Determination of LDH activity

After 24 hr of PDT treatment, the conditioned medium was harvested and an aliquot was used to determine LDH activity. LDH activity was measured using an LDH cytotoxicity assay kit in accordance with the manufacturer's instructions. Absorbance of each sample was measured at 490 nm (reference wavelength: 680 nm) using a Varioskan Flash microplate reader (Thermo Scientific).

Statistical analysis

Statistical significance of the data was determined using analysis of variance (ANOVA) and Bonferroni's multiple *t*-test. *P* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We first examined the relationship between laser irradiance and NPe6 dose in NPe6-PDT-induced cell death in human glioblastoma T98G cells. As shown in Fig. 1A, when laser fluence was fixed at 10 J/cm^2, NPe6 decreased the viability of T98G cells in a dose-dependent manner at 24 hr after NPe6-PDT treatment, regardless of laser

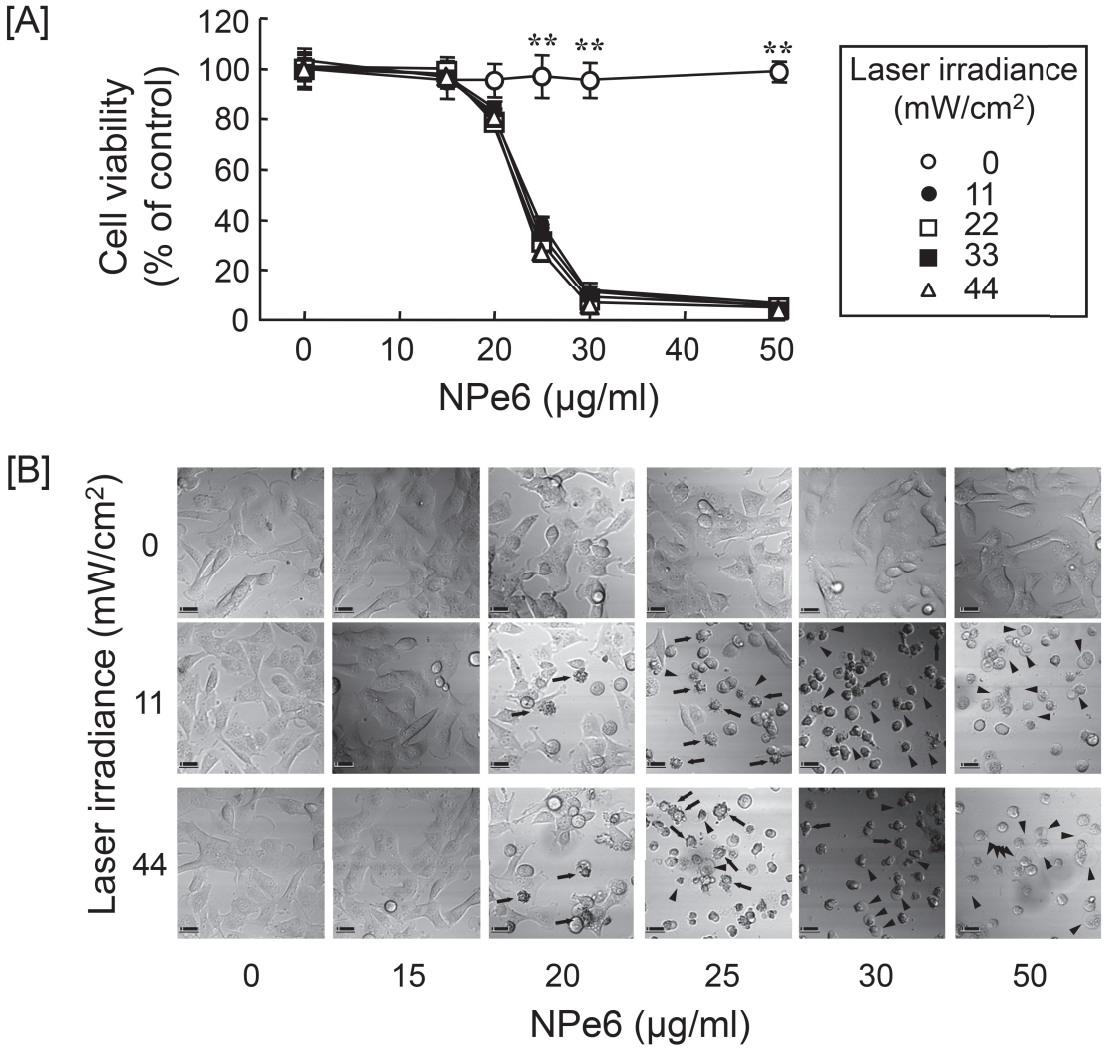

Fig. 1. Effect of laser irradiance on cell viability and cell morphology in NPe6-PDT-treated T98G cells. T98G cells were pretreated with or without NPe6 (15, 20, 25, 30, and 50 μg/mL) for 4 hr and then subjected to laser irradiation (wave length: 664 nm, laser fluence [laser energy density]: 10 J/cm^2, laser irradiance [laser power density]: 11, 22, 33, and 44 mW/cm^2 [irradiation time: 909, 454, 303, and 227 sec, respectively]). [A]: Cell viability of T98G cells was measured with a Cell counting kit-8. Values are means ± S.D. of at least three samples. ** $P < 0.01$ compared with the corresponding "11 mW/cm^2 laser irradiance". [B]: T98G cell morphologies after 24 hr NPe6-PDT-treatment were observed using phase contrast microscopy. Scale bars = 25 μm. *Arrows*: membrane-blebbing cells; *arrow heads*: swelling cells.

irradiance levels (11, 22, 33 and 44 mW/cm^2). Fig. 1B shows the morphology of T98G cells at 24 hr after NPe6-PDT treatment. No changes in morphology were noted in non-irradiated cells with NPe6 alone. However, on exposure to a laser at 10 J/cm^2 fluence and 11 mW/cm^2 irradiance, incidence of plasma membrane blebbing (apoptotic morphology) was increased in cells pretreated with 20 and 25 μg/mL NPe6, and incidence of swelling (necrotic morphology) was increased in cells pretreated with 30 and 50 μg/mL NPe6. Similar morphological changes were observed in cells exposed to a laser at 10 J/cm^2 fluence and 44 mW/cm^2 irradiance, suggesting that laser irradiance did not influence the cytotoxicity of NPe6-PDT in T98G cells.

We next investigated the numbers of apoptotic and necrotic cells by biochemical evaluation following NPe6-PDT treatment. Caspase-3 activity, measured as an index for apoptosis, was relatively unchanged in cells treated with NPe6 without laser irradiation (Fig. 2A). However, when T98G cells were treated with NPe6 at ≥ 20 μg/mL

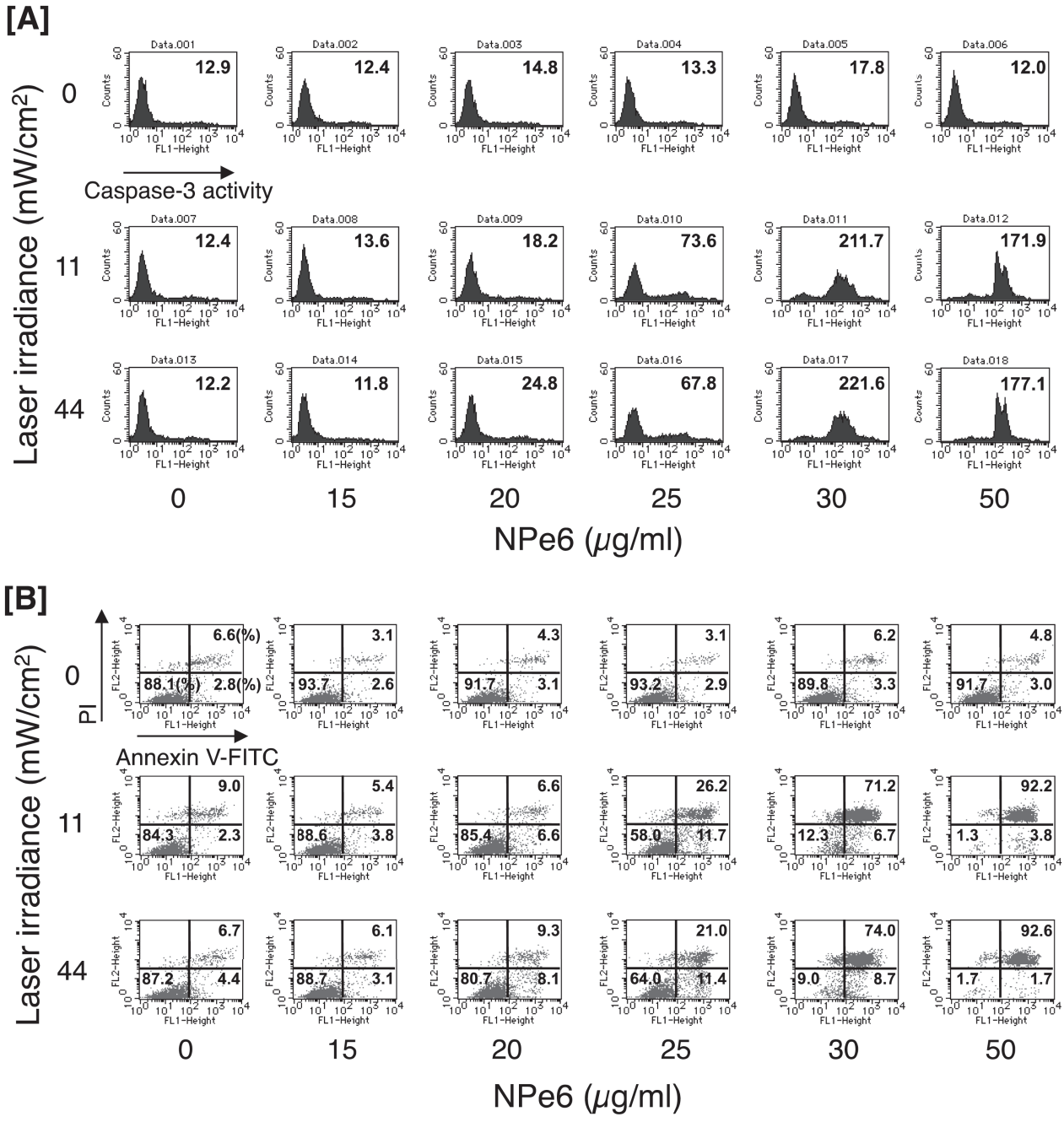

Fig. 2. Effect of laser irradiance on cell death modality in NPe6-PDT-treated T98G cells. T98G cells were pretreated with or without NPe6 (15, 20, 25, 30, and 50 μg/mL) for 4 hr and then subjected to laser irradiation (wave length: 664 nm, laser fluence [laser energy density]: 10 J/cm^2, laser irradiance [laser power density]: 11 and 44 mW/cm^2 [irradiation time: 909 and 227 sec, respectively]). At 24 hr after NPe6-PDT-treatment, indices of apoptosis and necrosis were calculated by measuring fluorescence using a flowcytometer. [A]: Caspase-3 activity was measured as apoptosis index. Mean fluorescence intensity (caspase-3 activity) is displayed in each figure. [B]: T98G cells were stained with Annexin V-FITC (externalized phosphatidylserine) and PI. The cells showing fluorescence above the basal level were regarded as positive cells. The lower right area (Annexin V-FITC-positive, PI-negative cells) is regarded as apoptotic cells. The upper right area (Aannexin V-FITC-positive, PI-positive cells) is regarded as necrotic cells. Proportions of apoptotic and necrotic cells are displayed in each figure.

and exposed to a laser at 10 J/cm^2 fluence and 11 mW/cm^2 irradiance, caspase-3 activity increased in an NPe6 dose-dependent manner, although activity dipped slightly at 50 μg/mL NPe6. Similar results were obtained after laser exposure at 10 J/cm^2 fluence and 44 mW/cm^2 irradiance.

We also determined the numbers of apoptotic and necrotic cells after NPe6-PDT. Apoptosis and necrosis were distinguished using annexin V and PI staining. Cells positive for annexin V but negative for PI were regarded as apoptotic, while cells positive for both annexin V and PI were regarded as necrotic (Vermes *et al*., 2000). Numbers of apoptotic cells and necrotic cells were relatively unchanged when treated with NPe6 without laser irradiation (Fig. 2B). However, when T98G cells were treated with NPe6 at ≥ 20 μg/mL and exposed to a laser at 10 J/cm^2 fluence and 11 mW/cm^2 irradiation, the number of apoptotic cells increased in an NPe6 dose-dependent manner, although numbers were slightly lower with 30 and 50 μg/mL NPe6 than with 25 μg/mL NPe6. Numbers of necrotic cells were increased after NPe6-PDT at ≥ 25 μg/mL. Similar results were obtained after laser exposure at 10 J/cm^2 fluence and 44 mW/cm^2 irradiance, indicating that cell death modality was not also affected by irradiance in NPe6-PDT-treated T98G cells.

Suppression of necrotic cell death is important in ensuring effective treatment of NPe6-PDT in glioma patients, as leakage of cytosolic constituents from necrotic cells induces occlusion or shut-down of blood vessels, resulting in injury of brain tissue (Akimoto *et al*., 2012). LDH leakage following NPe6-PDT treatment did not differ significantly between 11 and 44 mW/cm^2 laser irradiance (Fig. 3), a finding which supports the above-mentioned observations.

Robinson *et al.* (1998) found that the efficacy of clinical treatment depends on several parameters, including photosensitizer type, wavelength of applied light, light fluence, light irradiance, and tumor type. Wang *et al.* (2007) also reported that high laser-irradiance PDT using the photosensitizer photofrin was effective against tumor tissue with no significant toxicity against normal tissue. These findings indicate that laser irradiance is a critical parameter in PDT treatment, whereas our present findings showed that laser irradiance did not influence either of the cytotoxic effect and cell death modalities. Baran and Foster (2012) showed that neither high- nor low-irradiance PDT with the photosensitizer Pc 4 had any significant effect on tumor growth delay in intradermal mouse mammary EMT6 tumor transplanted-mice. NPe6 and Pc 4 may therefore be irradiance-independent photosensitizers.

We previously showed that both laser fluence and NPe6 dose affect both the cytotoxic and cell death modalities of NPe6-PDT in T98G cells (Miki *et al*., 2014). Therefore, the present data show that NPe6 dose and laser fluence are more influential parameters than laser irradiance on the efficacy of NPe6-PDT in glioblastoma T98G cells. We believe that clarifying the mechanism of NPe6-PDT-induced cell death in glioma would be leading to the potential application of this treatment in human glioma patients.

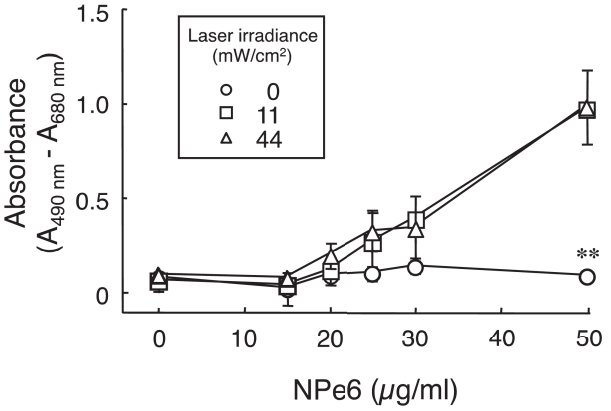

Fig. 3. Effect of laser irradiance on leakage of cellular LDH in T98G cells. T98G cells were pretreated with or without NPe6 (15, 20, 25, 30, and 50 μg/mL) for 4 hr and then subjected to laser irradiation (wave length: 664 nm, laser fluence [laser energy density]: 10 J/cm^2, laser irradiance [laser power density]: 11 and 44 mW/cm^2 [irradiation time: 909 and 227 sec, respectively]). LDH activity in the conditioned medium was measured using an LDH cytotoxicity assay kit and the activity was evaluated by absorbance data. Values are means ± S.D. of at least three samples. ** $P < 0.01$ compared with the corresponding "11 mW/cm^2 laser irradiance".

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Talaporfin sodium (NPe6) was provided by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). The semiconductor laser irradiation device was provided by Panasonic Healthcare Co., Ltd. (Ehime, Japan).

Conflict of interest---- The authors declare that there is no conflict of interest.

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