Synergic Effect of Photodynamic Therapy using Talaporfin Sodium with Conventional Anticancer Chemotherapy for the Treatment of Bile Duct Carcinoma

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Running title: PDT with Anticancer Chemotherapy in Biliary Carcinoma

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ABSTRACT

Background. Photodynamic therapy (PDT) is an effective laser treatment for locally treating advanced bile duct carcinoma. The study objective was to evaluate the synergic effect of PDT using a new photosensitizer, talaporfin sodium (Laserphyrin®), in combination with conventional anticancer drug treatments.

Methods. The range of the necrotic area, the percent of apoptosis-positive cells, VEGF (vascular endothelial growth factor) expression quantification and the PCNA (proliferating cell nuclear antigen)-labeling index (LI), as treatment effects, were examined in the bile duct carcinoma cell line (NOZ) in vitro and in vivo (4-week-old male BALB/c mice).

Results. Tumor viability was determined by an in vitro MTS assay. PDT with a single treatment of 5-fluorouracil, gemcitabine, oxaliplatin and CDDP showed a significantly lower viability compared with the control group or the PDT alone group (p<0.05). Furthermore, administering PDT combined with two anticancer drugs showed a further decline in tumor viability. A treatment of PDT combined with oxaliplatin and gemcitabine showed the least viability (p<0.05). Thus, this regimen was administered in the in vivo study. The tumor necrotic area, Apoptosis positivity and the VEGF expression rate were higher in the PDT with anticancer drug group compared with those of the other group (p<0.05). The PCNA LI results in the PDT with the anticancer drugs group were significantly lower than those of the other groups (p<0.05).

Conclusions. A treatment of PDT combined with gemcitabine and oxaliplatin showed the best synergic effect for necrosis, apoptosis and cytostatic alterations for the treatment of bile duct carcinoma.

Key Words: Bile duct carcinoma; Photodynamic therapy; Talaporphyn sodium; Apoptosis; Synergic effect
INTRODUCTION

Photodynamic therapy (PDT), a cancer-specific treatment based on using light-activated photosensitizers and inducing cytotoxicity in targeted cancer cells, has been widely applied in various cancer treatments.\(^1\) PDT is technically feasible and is a useful modality for treating non-resectable or resectable bile duct carcinomas (BDC).\(^2\)\(^\text{-}^5\) Remarkably, PDT treatment induces a powerful anti-tumor immunological response.\(^6\) In two randomized controlled trials, PDT provided a longer survival.\(^4\)\(^,\)\(^5\) PDT treatment benefits have been reported for treating the targeted area in BDC patients who are receiving chemotherapy or adjuvant chemotherapy after surgery.\(^7\)\(^,\)\(^8\) Thus, PDT should be a promising treatment modality to augment the conventional anticancer chemotherapy and brachytherapy as recommended in the 2009 Japanese BDC treatment guidelines.\(^9\)

The first clinically approved photosensitizer, porfimer sodium (Photofrin\(^8\)), is a hematoporphyrin derivative and has a very powerful cytocidal effect in BDC.\(^1\)\(^\text{-}^8\) However, the anti-tumor effect was limited to the shallow bile duct wall because the 630 nm laser used in treatment had a low permeability.\(^10\) Furthermore, the long period of skin photosensitivity required the patients to be kept away from strong sunlight for several weeks following the drug administration.\(^10\) Therefore, we evaluated a new and effective photosensitizer, mono-L-aspartyl chlorin e6 (talaporfin sodium; NPe6, Laserphyrin\(^8\)), which has been used for treating malignant tumors, such as bronchial cancer.\(^11\)\(^\text{-}^13\) The 664 nm semiconductor laser light activates talaporfin sodium and penetrates into deep tissue to a depth of more than 10 mm.\(^14\) Furthermore, laserphyrin-PDT (L-PDT) has a lower skin phototoxicity compared with photofrin-PDT (P-PDT) because talaporfin sodium degrades rapidly in vivo.\(^15\)\(^,\)\(^16\) Based on the demonstrated clinical effectiveness and the photosensitivity principles, a study that compared L-PDT and P-PDT for the treatment of human biliary cancer cells was examined.\(^17\)\(^,\)\(^18\) The
study demonstrated that L-PDT was a more powerful and effective anticancer treatment, had a higher percent of tumor necrosis and apoptosis, a lower cancer cell proliferation activity and a higher anti-angiogenic activity. Based on these results, a clinical trial evaluating the L-PDT treatment in BDC patients has begun (not published in English). In the BDC patients, various anticancer drugs, such as gemcitabine, have been adopted worldwide, which has resulted in a longer survival period for patients with non-resectable BDC.\textsuperscript{19,20}

In the future, combining a systemic chemotherapy and an effective local treatment, such as PDT, would be feasible and necessary for BDC treatment. Therefore, we hypothesized that L-PDT combined with a systemic anticancer treatment would show a greater synergic effect to control cancer tissue compared with PDT alone or systemic chemotherapy alone treatments. To evaluate our hypothesis, the cytotoxic and angiogenic effects of L-PDT combined with various well-known anticancer drugs were examined in a BDC cell line (NOZ). The percent of tumor necrosis, the TUNEL assay to assess the extent of apoptosis, the PCNA labeling index (LI) to determine the cancer proliferative activity, and vascular endothelial growth factor (VEGF) expression quantification as an index of oxygenation of tumor tissue \textit{in vitro} and \textit{in vivo} were the effects evaluated in the present study.
MATERIALS and METHODS

In vitro studies of photosensitizer properties

Cancer cell culture

NOZ cells, a human biliary cancer cell line (JCRB1033: Japanese Collection of Research Bioresources, Tokyo, Japan), were cultured in Dulbecco’s modified Eagle medium (DMEM: Nissui Centical Co., Tokyo) with 10% fetal bovine serum, glutamine (0.6 mg/ml), penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37°C under a humidified atmosphere of 5% CO₂ in air.

Cell viability assay

The effect of PDT on NOZ cell viability was investigated using a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega Co., Madison, WI, USA.). Cells were cultured in 96-well microplates and were irradiated for 24 hours. Subsequently, 20 μl of a MTS solution (317 μg of MTS/ml phosphate buffered saline [PBS]) was added to each well, followed by a 4-hour incubation. After a complete dye solubilization by vortexing the plate, the absorbance was read on an Immunoreader (model NJ-2000, Nihon Inter Med, Tokyo) at 490 nm. A cell suspension (1×10⁵ cells/ml) was made, and 100 μl/well of this suspension was incubated in 16 wells/plates at 37°C overnight. The cells were decanted and washed twice in PBS. A 100-μl aliquot of each anticancer drug was diluted with DMEM and fetal bovine serum and placed in well plate followed by another overnight incubation at 37°C.
Photodynamic therapy

At day 3, PDT was performed for 24 hours on the incubated NOZ cell mixture that had previously been treated with an anticancer drug. The NOZ cell mixture was exposed for 24 hours to 20 μg/ml of talaporfin sodium (NPe6, Laserphyrin® (Meiji Seika Pharma. Co. Ltd., Tokyo)) diluted with DMEM and irradiated using a semiconductor laser apparatus (ZH-L5011HJP, Panasonic Healthcare Co. Ltd., Tokyo, Japan) that was tuned to 664±2 nm and a 10 Hz frequency (energy density range: total 60 J/cm²). The estimated talaporfin sodium dose was 20 μg/ml as determined by our preliminary study. The administered solution was incubated at 37°C for 3 hours, later decantated and then washed three times in PBS. To induce LD₅₀ PDT conditions, a laser power of 12 J/cm² was chosen to irradiate the NOZ cells. The laser was irradiated for 1 minute. Apoptotic induction of the NOZ cells in each group was investigated following the cell treatment under the LD₅₀ conditions as determined in our preliminary study. Each sample was again incubated overnight at 37°C, and 20 μl of MTS solution (CellTiter96® AQueous One Solution Cell Proliferation Assay Promega Co. Madison, WI, USA) was added. Incubation at 37°C for 4 hours was performed, and the viability of each sample of 16 wells/plate was analyzed three times using a 96-well plate reader at a 490 nm absorbance.

The anticancer chemotherapeutic drugs that were co-administered in vitro with PDT were 5-fluorouracil (5-FU, Kyowa Hakko Kogyo Co Ltd., Tokyo), gemcitabine (Eli Lilly and Co., Indianapolis, IN), oxaliplatin (Tokyo Chemical Industry, Co., Ltd, Tokyo)) and cisplatin (Bristol Co., Ltd, Tokyo). The anticancer effects of these drugs, used as a single treatment or in multiple treatments, in BDC patients were previously reported. The adequate concentration of each of the anticancer drugs was determined using the MTT assay.
Animal experiments *(in vivo)*

*Tumor xenograft*

In these experiments, $1 \times 10^7$ NOZ cells were inoculated subcutaneously into the backs of 4-week-old nude mice ($n=16$, BALB/cANcrj nu/nu, Charles River Inc., Japan). The tumors that grew to an approximate $8 \times 8$ mm size in approximately 21 days after inoculation were used as the experimental models.

*PDT protocol*

A power meter (30 A-P Ophir Optics, Jerusalem, Israel) was used to measure the light intensity. Talaporfin sodium was injected intraperitoneally into BALB/cANcrj nu/nu mice. The time interval between the photosensitizer injection and the light exposure for talaporfin sodium was 2 hours. Each tumor received a total energy fluency of 10 mJ/cm$^2$/pulse (total 60 J/cm$^2$) for 10 minutes. During the laser light exposure, the animals were anesthetized with sodium pentobarbital (40 mg/kg body weight) intraperitoneal injection. The PDT was directed to the transplanted tumor on the back of the animal (Fig. 1). Mice were sacrificed at 72 hours following the PDT treatment. Neither the photosensitizer nor the laser light were used to treat the control animals ($n=4$). The adequate administrative dose of talaporfin sodium was 5 mg/kg based on our preliminary study.  

*Anticancer drugs*
Based on the *in vitro* results, the co-administration of anticancer chemotherapeutic drugs, gemcitabine (Eli Lilly) and oxaliplatin (Tokyo Chemical Industry, Co., Ltd.), with PDT were used *in vivo* in replicates. The adequate dose for each drug in combination with PDT was determined. Nude mice were sacrificed at day 7 following the treatment administration (*n*=4 for each dose).

*Analysis of tumor necrotic area*

Four mice per treatment were sacrificed with ether inhalation at 24 hours following the continuous PDT treatment. The tumors were excised and fixed in 3.7% neutral buffered formalin for 24 hours, followed by a routine paraffin embedding process. Three 4-μm sections were prepared from each specimen, mounted on the silanized slides (DAKO Japan Co., Tokyo), and dried overnight on a hot plate at 37°C to promote adhesion. In the first section from each of the specimens, routine hematoxylin and eosin (H&E) staining was carried out (Fig. 2a). In each of the specimens, the tumor necrotic area was measured using computer-assisted image analysis software (Macintosh Image 1.62 program, Apple Computer Power Book G4). The proportion of the necrotic area relative to the total cross-sectional tumor area was computed.25

*TUNEL assay*

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed using an Apoptosis Detection Kit (Wako Co. Ltd., Osaka, Japan) in accordance with the manufacturer’s instructions. The slides were assessed using light microscopy; the TUNEL-positive nuclei (stained deep brown, Fig. 2b) were counted in
3 randomly selected microscopic fields (400X; field size: 0.08 mm²) per slide containing the necrotic areas. The TUNEL-positive nuclei were expressed as a percentage of the total nuclei counted. At least 1,000 nuclei were counted in each slide.

*Immunohistochemistry for proliferating cell nuclear antigen (PCNA)*

PCNA immunohistochemical staining (ABC Kit, Vector Laboratories, Burlingame, CA) was carried out \( n=4 \) for each group, Fig. 2c) using a mouse anti-PCNA monoclonal antibody (PC10, dilution 1:100, Dako, Glostrup, Denmark). The sections that were incubated with the normal mouse serum instead of the PCNA served as the negative controls. Both the labeled and the unlabeled tumor cells were counted with the aid of a squared eyepiece reticule (Nikon, Tokyo) \( 0.0625 \text{ mm}^2/\text{field} \) at a 400X magnification. The PCNA labeling index (LI) of the tumor cells was defined as the percentage of the PCNA-positive cells among 1,000 tumor cells counted from three randomly selected fields.

*VEGF immunohistochemistry*

VEGF immunohistochemical staining (ABC Kit, Vector Laboratories, Burlingame, CA) was performed as described below \( n=4 \) for each group, Fig. 2d). The tissue sections were deparaffinized and rehydrated in water. The sections were treated with a 0.1% trypsin solution in 0.05 M Tris buffer (pH 7.6) at room temperature for 15 minutes, followed by three PBS washes. Endogenous peroxidase was inhibited by treating the sections with 0.3% \( \text{H}_2\text{O}_2 \) in methanol for 30 minutes. After three PBS washes, the sections were incubated overnight at 4°C with an anti-human VEGF polyclonal antibody (dilution 1:200, #A-20, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS. The sections were then treated with a diluted
biotinylated secondary antibody for 30 minutes, followed by an ABC reagent treatment for 30 minutes. Immunohistochemical reactions were stained using a diaminobenzidine (DAB) solution (20 mg/100 ml in a 0.05 M Tris buffer that contains 17 μl of 30% H₂O₂). The sections were lightly counterstained using Mayer’s hematoxylin. The negative controls, prepared by substituting a normal goat serum for the primary antibody, resulted in no detectable staining.

The VEGF expression was reported as the percentage of the VEGF immunopositive area (PVIA), which was quantified using computer-assisted image analysis software (Macintosh Image 1.62 program, Apple Computer). The sections were photographed using a Nikon digital camera (Coolpix 4500, Nikon Co., Tokyo), at a 50X magnification. After saving the captured image from three randomly selected fields to a personal computer, the image was cropped to 512×512 pixels. After reducing the noise and enhancing the edges, the image was analyzed following the method of Wu et al., and the PVIA for each specimen was determined.

Statistical analysis

The data are expressed as the mean ± the standard deviation (SD). The statistical significance was determined using the independent samples t-tests. A P value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Science (SPSS) software, version 18.0 (SPSS, Chicago, IL).
RESULTS

*In vitro studies*

The cell viability at each dose setting for each of the anticancer drugs using MTT assays was examined (n=4 for each dose). The anticancer drug doses that show an equal anti-tumor effect were CDDP at 50 μg/ml, oxaliplatin at 50 μg /ml, gemcitabine at 100 μg /ml and 5-FU at 100 μg /ml.

Figure 3 shows the cell viability when administrating PDT combined with one or two anticancer drugs. Compared to the control group (no treatment), the NOZ cell viability was significantly decreased using the treatments with PDT alone and PDT with anticancer drugs (p<0.01). In comparison with the PDT alone treatment (48.3±3.0%), the cell viability was significantly decreased by co-administering PDT with one of the following: 5-fluorouracil (40.3±1.4%) or gemcitabine (37.8±1.2%) or oxaliplatin (37.0±1.1%) or CDDP (36.5±1.1%) (p<0.05). With respect to combination with two anticancer drugs, compared to the control group (no treatment), the NOZ cell viability was significantly decreased by the PDT alone and the PDT combined with an anticancer drug treatments (p<0.01). In comparison with the PDT alone treatment, the cell viability was significantly decreased by administering PDT with each of the following combinations of anticancer drugs: CDDP+5-fluorouracil: 41.7±1.4%; oxaliplatin+5-fluorouracil: 44.0±1.3%; CDDP+gemcitabine: 31.9±0.9%; oxaliplatin+gemcitabine: 28.5±0.8% (p<0.05). Among these combinations, the treatment co-administering PDT with gemcitabine and oxaliplatin showed the lowest *in vitro* NOZ cell viability. Upon comparing the treatment co-administering PDT with 5-fluorouracil and oxaliplatin with the co-administration of PDT with gemcitabine and oxaliplatin, the cell viability was decreased, but was not significantly different (p=0.34). The co-administration of
oxaliplatin and gemcitabine and PDT showed a significantly lower cell viability compared with the single anticancer drug and PDT treatments ($p<0.05$). Based on this result, the most effective regimen for the synergic treatment effect, PDT combined with gemcitabine and oxaliplatin, was administered in the subsequent in vivo study.

*In vivo animal study*

*Establishment of administrative dose of talaporfin sodium*

Using 5 mg/kg of talaporfin sodium, we examined the adequate oxaliplatin dose. The intraperitoneal administration of 0.5, 1, 5, 10, 20, 30 mg/kg of oxaliplatin was evaluated ($n=4$ for each dose). All mice died when dosed with 20 and 30 mg/kg of oxaliplatin. Additionally, no tumor necrosis was observed using the 0.5 and 1 mg/kg doses. Using a dose less than 5 mg/kg, only 5% of the tumor necrosis was observed. Using a dose less than 10 mg/kg, 15-20% of the tumor necrosis was observed. Therefore, 10 mg/kg of oxaliplatin was selected as the dose for this study. In the second step, the adequate dose of gemcitabine was also evaluated. The intraperitoneal administration of 1, 5, 10, 20, 50, 100 mg/kg of gemcitabine was evaluated ($n=4$ for each dose). All mice died when dosed with 100 mg/kg of gemcitabine; therefore, the tumor necrosis could not be evaluated. Additionally, no tumor necrosis was observed using the 1, 5 and 10 mg/kg doses. Using a dose less than 20 mg/kg, a small number of necrotic tumors were observed. Using a dose less than 50 mg/kg, 15-20% of the tumor necrosis was observed. Although cell toxicity was insufficient using the 50 mg/kg dose, a gemcitabine dose of 50 mg/kg was selected for this study based on previously reported results.27,28,29
Eventually, the combination dose regimen of 5 mg/kg of talaporfin sodium, 10 mg/kg of oxaliplatin and 50 mg/kg of gemcitabine was selected for use in the final study.

Histological findings

Figure 4 shows the tumor necrotic area, the percentage of apoptosis positive cells, PCNA LI and PVIA (%) when co-administering PDT with the anticancer drugs oxaliplatin and gemcitabine. No tumor necrosis was observed in the control group (no treatment). The PDT alone (28.3±12.8%) and the PDT with oxaliplatin and gemcitabine groups (33.3±7.1%) showed a significantly higher tumor necrotic area ($p<0.05$) than the combined oxaliplatin and gemcitabine treatment group (17.2±4.8%). However, there was no significant difference in the necrotic areas comparing the PDT alone to the PDT with anticancer drugs groups ($p=0.52$).

Compared with the control group (no treatment) (0.2±0.1%), the oxaliplatin and gemcitabine combination (14.7±5.8%), the PDT alone (17.4±3.6%) or the PDT with these anticancer drugs (30.0±5.1%) groups showed a significantly higher cell apoptosis percentage ($p<0.05$). There were no significant differences in the apoptosis-positive percentage between the PDT alone and anticancer drugs alone groups ($p=0.46$).

PCNA LI in all of the other groups was significantly lower than the PCNA LI of the control group (no treatment) (41.4±3.1%) ($p<0.05$). The PDT alone (25.9±4.4%) or PDT with oxaliplatin and gemcitabine (13.7±2.5%) treatments showed a significantly lower PCNA LI than the PCNA LI observed when co-administering oxaliplatin and gemcitabine alone (36.3±3.6%) ($p<0.05$). Furthermore, the PDT combined with oxaliplatin and gemcitabine group showed a significantly lower PCNA LI than that of the PDT alone group ($p=0.015$).
In comparison with the control group (no treatment) (7.1±0.9%), the PVIA in the other
groups showed a significantly lower PVIA ($p<0.05$). The PVIA of the PDT combined with
oxaliplatin and gemcitabine (30.7±2.5%) was significantly higher than that of the
co-administration of oxaliplatin and gemcitabine alone (23.5±1.3%) or the PDT alone
(17.9±1.3%) treatments ($p<0.05$).
DISCUSSION

Our institute will administer PDT for neoadjuvant or adjuvant chemotherapy for the treatment of resected cases of bile duct carcinomas. Bile duct cancer often metastasizes in the lymph nodes, invades perineurally, anticancer drugs treatments are necessary to treat the spread of this cancer. Chemotherapy alone using novel anticancer drugs did not show an acceptable survival period (the median overall survival was 4.7-15.4 months), and the tumor response rate was 9-37% (median progression free survival was 3-7.2 months). In Japan, gemcitabine has been mainly used to treat bile duct carcinoma and is now a key drug in the treatment of bile duct carcinoma.

Previous our studies using cultured cells showed that the main effect of PDT is apoptosis, evidenced by assays that measured either DNA fragmentation or chromatin condensation. PDT induced NOZ cell death by apoptosis, detected in the Hoechst 33342-stained sections, in a light dose-dependent manner. Based on this preliminary study, the photosensitizer dose and light dose were already determined, which was applied in the present study. Talaporfin sodium (TPS)-PDT has been used for lung cancer treatment, and the strong cytotoxicity in cancer cells was experimentally evaluated, which is the most adequate for the PDT treatment of bile duct carcinoma at this stage. Using the current laser apparatus, the necrotic tumor area, percent of apoptotic positivity, proliferative activity and VEGF expression were similar to that of the previous apparatus (data not shown).

In the present study, we compared the synergic cytotoxic effects of PDT using talaporfin sodium and PDT combined with various anticancer drugs on biliary cancer cells, in vitro and in vivo. Our in vitro results showed that the combined treatment of PDT and a single dose of an anticancer drug showed lower tumor viability compared to the PDT alone treatment. Furthermore, the combination of the two drugs showed an increased synergic cytocidal effect. Our in vivo study also showed a more potent apoptotic induction, lower proliferative activity
and a higher VEGF expression administering PDT combined with anticancer drugs compared with chemotherapy alone or PDT alone treatments. PDT has a direct cytotoxic effect and an indirect effect on the tumor microenvironment, and rapidly induces apoptosis, an inflammatory reaction, tumor-specific and/or non-specific immune reactions and damages the tumor bed microvasculature. An enhanced apoptotic response, as evidenced by the high Bax to Bcl-2 protein ratio in LLC-IL-6 cells, and IL-6 expression are important determinants of the antitumor effect of PDT. A synergic cytotoxic effect by a cytokine or genetic alterations induced by the anticancer drugs might promote the PDT effect. Although the tumor necrotic area of the PDT alone and the PDT with anticancer drugs groups was similar, apoptosis positivity area was higher in the PDT with chemotherapy treatment than that in the PDT alone in the present result. This synergic cytotoxicity might be due to apoptosis.

In the present in vivo study, the adequate dose of oxaliplatin and gemcitabine was determined by balancing the resulting cellular toxicity and safety profile. The oxaliplatin dose was similar to that used in the previous study. Although the tumor necrosis effect was the highest at a gemcitabine dose of 100 mg/kg, animal safety could not be determined. Previous animal (mouse) study showed that approximately 50 mg/kg of gemcitabine was used in the lung or pancreas carcinoma model experimentally to determine the cytocidal effect of gemcitabine. Therefore, we chose a 50 mg/kg dose of gemcitabine for the present in vivo study.

In the present study, the PCNA LI was examined to evaluate the tumor proliferative activity. More profound suppression of cancer cell proliferation activity in the PDT with anticancer drugs group than in the PDT alone was observed. Song et al. reported that NPe6-PDT promoted a greater tumor regression in comparison with HpD-PDT, with a long lasting effect on the tumor growth inhibition in a human cholangiocarcinoma model. NPe6-PDT induces complement activation with the subsequent expression of various
leukotrienes and cytokines.\textsuperscript{52} The above-specified changes in the immune system may mediate the suppressive actions of PDT on the re-growth of residual tumors and its early-to-late cytostatic effects, compared with the conventional PDT using porfimer sodium. Anticancer drugs may induce synergic affect DNA damage in the various cell cycle mechanisms.\textsuperscript{53}

PDT induces a severe tumor tissue hypoxia immediately after its application, which is linked to the induction of a photochemical reaction.\textsuperscript{54, 55} VEGF secretion is induced in cells under hypoxic conditions.\textsuperscript{56, 57} As PDT consumes oxygen, it may generate hypoxic conditions \textit{in vivo}. Hypoxia-induced stabilization of HIF (hypoxia inducible factor)-1a, followed by its binding to the HRE (hypoxia responsive element) in the VEGF promoter, is a major regulator of VEGF gene expression.\textsuperscript{58} As our preliminary study indicates, VEGF expression has been used as an index of tumor tissue oxygenation, and the observed overexpression of VEGF after PDT was likely due to the hypoxia induced by photochemical reactions.\textsuperscript{18} Jiang \textit{et al.} demonstrated that VEGF expression increased within the PDT-treated lesions and remained elevated for some weeks.\textsuperscript{59} By noting the hypoxic effect of the PDT alone treatment, VEGF expression might increase by PDT combined with anticancer drug treatments as in our result. Anticancer drugs also have an anti-angiogenic effect,\textsuperscript{60} and an anti-angiogenic effect might be synergically influenced by oxaliplatin or gemcitabine.

In the present study, treatment using PDT with a combination of anticancer drugs, such as oxaliplatin and gemcitabine, showed more cytotoxic effects in comparison with PDT alone or PDT with other regimens. Although Oxaliplatin has not been permitted to use for bile duct carcinomas in Japan, the combination of gemcitabine and oxaliplatin with PDT would be a better regimen for future new chemotherapy to treat bile duct carcinoma. Recently, anti-angiogenic chemotherapy drugs, such as bevacizumab, have been used for the treatment of colorectal cancer.\textsuperscript{61} Although the molecular targeting drugs cannot be currently used for
the treatment of bile duct carcinoma at this stage, PDT with such a molecular targeting drug would be still a more powerful chemotherapeutic modality in the future.

In conclusion, the tumor viability measured by the MTT assay showed a significantly higher cytocidal effect in bile duct carcinoma cell line, NOZ, when treated with PDT using talaporfin sodium combined with oxaliplatin and gemcitabine in vitro. In the in vivo study using a nude mouse model, the tumor necrotic area, the percent of apoptosis positive cell and the percentage of VEGF cells were significantly higher in the group treated with PDT combined with the anticancer drugs compared with the chemotherapy alone or the PDT alone treatment groups. Proliferative activity, measured by PCNA LI, was significantly lower in the group treated with PDT combined with anticancer drugs compared with the chemotherapy alone or the PDT alone treatment groups. PDT using talaporfin sodium appears to have activities against bile duct cancer cells in pre-clinical model, and represents an important area for the future clinical trials.

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FIG. 1 The *in vivo* model. (a) Nude mouse with a transplanted NOZ cell tumor on its back. (b) The anti-cancer drug was administrated subcutaneously in the intraperitoneal; subsequently, talaporfin sodium administration was followed by a 10-minute laser irradiation. (c) The semiconductor laser apparatus.

FIG. 2 Histological findings. (a) The arrows show the necrotic tumor area treated with PDT (40X). (b) The arrow shows the TUNEL positive cells (apoptotic cell) (200X). (c) The arrow shows the PCNA positive cells (200X). (d) The arrows show the VEGF positive cells (100X).

FIG. 3 Synergic effect of cell viability measured using the MTT assay to evaluate the synergic effect of PDT and anticancer drug treatments *in vitro* is shown. The data are the mean ± SD (The mean value was indicated on each bar). PDT: photodynamic therapy; gem: gemcitabine; oxa: oxaliplatin; 5FU: 5-fluorouracil; CDDP: cisplatin (n=16 for each bar).

FIG. 4 The percentage of the tumor necrotic area (NA), apoptosis positive cells (APC), proliferative activity (by PCNA LI) and VEGF positive area (PVIA) indicates the synergic effect of the PDT combined with oxaliplatin and gemcitabine treatment *in vivo*. The data are presented as means ± SD (The mean value was indicated on each bar). PDT: photodynamic therapy; oxa: oxaliplatin; gem: gemcitabine (n=4 for each bar). * p<0.05 vs. oxa+gem, # p<0.05 vs. oxa+gem and PDT only, ** p<0.05 vs. oxa+gem, ¶ p<0.05 vs. PDT only, § p<0.05 vs. oxa+gem, † p<0.05 vs. PDT only.