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Abstract. Photodynamic therapy (PDT) is a photochemical modality approved for cancer treatment. PDT has demonstrated efficacy in early stage lung cancer and esophageal cancer. The accumulation of photosensitizers in cancer cells is necessary to enhance the therapeutic benefits of PDT; however, photosensitizers have low uptake efficiency. To overcome this limitation, a drug delivery system, such as the hemagglutinating virus of Japan envelope (HVJ-E) vector, is required. In this study, the combination of PDT and HVJ-E was investigated for enhancing the efficacy of PDT. The photosensitizers that were evaluated included 5-aminolaevulinic acid (5-ALA), protoporphyrin IX (PPIX), and HVJ-PPIX. The uptake of the photosensitizers as increased twenty-fold with the addition of HVJ-E. The cytotoxicity of conventional 5-ALA was enhanced by the addition of HVJ-E vector. In conclusion, HVJ-E vector improved the uptake of photosensitizers and the PDT effect. © *2012 Society of Photo-Optical Instrumentation Engineers (SPIE).* [DOI: 10.1117/1.JBO.17.7.078002]

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1 Introduction

Photodynamic therapy (PDT) is a photochemical modality approved for the treatment of various cancers, skin diseases, and diseases with neovascularization.[1,2] The PDT process comprises injection of a photosensitizer, which selectively accumulates at the lesion site, followed by local illumination of the site with a laser of appropriate wavelength to activate the photosensitizer. Irradiation of the photosensitized area with light at a specific wavelength leads to singlet oxygen generation.[3]

The PDT treatment method is associated with minimal injury to patients because photosensitizers have low cytotoxicity and the excitation light is harmless in the visible light range. Therefore, PDT is expected to be a less invasive treatment method for early lung cancers and stomach carcinoma. Moreover, this treatment method also helps preserve organ function and limits pain compared to existing surgical treatments or anti-cancer drug therapies. PDT is an ideal treatment for elderly patients, and it may also improve the patient's quality of life.

Selective accumulation of the photosensitizer in the cancer cells is necessary to improve the therapeutic efficacy of PDT. However, a limitation of this therapy is the difficulty in photosensitizers reaching the diseased site, which reduces the efficiency of PDT therapy. To improve the effectiveness of PDT therapy, a new drug delivery system (DDS) needs to be developed for improving the selectivity of photosensitizers. The DDS is a technology designed to optimize drug therapy by controlling the drug disposition and selectively transporting drugs to a target site at a preferred density or time. The DDS should also effectively reduce side effects and improve the safety of a given drug.

For the accumulation of drugs in cancer cells, active targeting DDS using monoclonal antibodies were developed. However, conventional photosensitizers are mostly hydrophobic, and therefore, conjugation of photosensitizers to antibodies without compromising the immunoreactivity of the antibodies and thereby *in vivo* target accumulation is difficult. Various combinations of conventional photosensitizers and monoclonal antibodies have been tested to improve their selectivity, albeit with limited success, especially with regard to *in vivo* therapeutic efficacy.[4–10]

Currently, liposomes are used clinically as drug carriers, and polymer micelles, silica nanoparticles, and carbon nanohorns are potential carriers that have also been extensively studied.[11,12] These carriers have also been investigated as photosensitizer carriers for PDT and have some advantages compared with the administration of the photosensitizer alone.[13–16] The carriers are composed of two defined layers: an outer envelope and an inner shell. The interaction between the living organism and the outer envelope of the carrier influences the characteristics of drug disposition, and the inner shell of the carrier contains the drug. These carriers can be used as active targeting systems by adding monoclonal antibodies to the outer envelope; the effect of adding monoclonal antibody was determined in *in vivo* experiments.[17,18] hemagglutinating virus of Japan envelope (HVJ-E) is a nonviral DDS carrier having the same bilayer structure and capability as the active targeting system with

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antibody.[19–23] In this study, the effectiveness of a new PDT DDS using a vector based on HVJ-E was examined.

HVJ-E not only encloses drugs and carries them to target sites but also has some advantages over other carriers. For example, HVJ-E is effective in fusing with other cells[24] and is therefore expected to improve accumulation of photosensitizers within the target tumor cells compared with other carriers. The construction of tissue-specific HVJ envelope vectors was described in Ref. 25 (However the data was not published). HVJ-E alone has been shown to have an antitumor effect.[26–28] In addition, a clinical grade HVJ envelope vector is currently being produced for use in clinical trials.[29] Consequently, HVJ-E is a suitable drug carrier for PDT.

We constructed HVJ-PPIX by adding protoporphyrin IX (PPIX) to HVJ-E; PPIX is being widely used as a carrier in PDT for brain tumors.[30,31] PDT with PPIX is based on the endogenous accumulation of PPIX after topical or systemic administration of 5-aminolaevulinic acid (5-ALA).[32] The cytotoxicity of PPIX and 5-ALA are low because they are normal products in healthy tissue. PPIX has absorption peaks at 405, 510, 545, 580, and 630 nm.[33,34] Very high absorption was observed at 405 nm, and high therapeutic effectiveness is expected at this wavelength. Further, the light at a wavelength of 630 nm is highly transmissible and appropriate for application to deep lesions.[35] PPIX is s synthesized inside mitochondria. The amount of PPIX in the cell depends on the amount of intracellular 5-ALA and the synthesis ability.[36] For this reason, there is a limit on the accumulation of PPIX induced 5-ALA. HVJ-E can transport PPIX directly and achieve the high concentration.

The membrane structure of HVJ-E is the same as that of biomembranes, and hence, small molecules leak out from HVJ-E. Synthetic compounds with their molecular weight more than 1000 Da can be incorporated into HVJ-E.[37] Therefore, encapsulation of 5-ALA is difficult. 5-ALA must not be added any molecules for increasing the molecular size because it is required for the synthesis of PPIX for PDT. Therefore, we constructed HVJ-PPIX from PPIX-C17 (diheptadecanoyl-substituted protoporphyrin IX) using the PPIX-C17[38] solution technique.

We evaluated the amount of photosensitizer taken up by lung cancer and melanoma cells *in vitro* and the photocytotoxic effect of PDT on the cultured cells by using HVJ-E with added PPIX. The benefits of using HVJ-E as a DDS for PDT are an enhanced uptake efficiency of photosensitizer by tumor cells and a selective photocytotoxic effect because of the cytotoxicity of HVJ-E as well as the PDT.

2 Materials and Methods

2.1 Photosensitizer and Light Source of PDT

5-ALA (A7793, Sigma-Aldrich Inc.) and PPIX (Sigma-Aldrich Inc.) were used as photosensitizers. HVJ-PPIX was generated from PPIX-C17. A continuous wave semiconductor laser generator was used for PDT at a wavelength of 405 nm, at which the photosensitizers showed a maximum peak of absorption. The cells were grown on a 96-well culture plate (Black with Clear Bottom 96-well Microtest™ Optilux™ Plate, BD Bioscience Inc.), and then irradiated by a laser via a fiber attached to the bottom of the culture plate. An optical instrument with an automated stage for positioning was purchased from Sigma Koki Co. Ltd., Japan.

2.2 HVJ-E Vector

The HVJ-E vector is a nonviral vector. The viral activities of the HVJ-E vector were abrogated by exposure to ultraviolet rays, but the vector still had cell fusion abilities. DNA, RNA, and proteins can be incorporated into HVJ-E and delivered efficiently both *in vitro* and *in vivo* based on its virus-to-cell fusion ability, which protects the contents of the HVJ-E vector from degradation by endosomes and lysosomes.[28] Virus replication was lost completely, but hemagglutinating activity was not affected.[24]

2.3 Synthesis of HVJ-PPIX

An HVJ suspension of 10,000 HAU was inactivated by UV irradiation (99 J/cm^2). Phosphate buffered saline solution (300 μL) (PBS, pH 7.4, Sigma-Aldrich Inc.) and PPIX-C17 solution (87 μL at a concentration of 11.5 mg/mL) were added to the 10,000 HAU HVJ-E suspension. After centrifuge separation (15,000 rpm, 4°C, 5 min), the supernatant was removed and 200 μL of PBS was added. The PPIX-C17 was prepared according to the protocol of El-zaria.[38] The incorporation ratio of PPIX in HVJ-E was 2.5×10^{18} mol per one HVJ-E vesicle (mean vesicle volume is about 0.11 mm^3).

2.4 Cell Culture

The human lung cancer cell line A549 and the murine melanoma cell line B16 were used. The A549 cells were cultured in RPMI-1640 medium (Sigma-Aldrich Inc.) containing 10% fetal bovine serum (FBS, Biowest Inc., France), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Nacalai Tesque, Japan). The B16 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich Inc.) containing 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. The cells were incubated at 37°C with 5% CO$_2$.

2.5 PPIX Uptake by Cells

For adherent cultures, 100 μL (2×10^4 cells/well) of the A549 cells were plated in a 96-well plate. Three hours later, when the cells had adhered to the plate, the medium was removed and replaced with FBS-free DMEM containing 100 μL of 5-ALA (800 μM), PPIX (100 μM), and HVJ-PPIX (100 μM) solution. Conjugation of eight ALA molecules yields PPIX in a mitochondria; ALA dehydratase synthesizes porphobilinogen (PBG) from two 5-ALA molecules. PBG deaminase converts four PBG molecules into hydroxymethylbilane. Linear hydroxymethylbilane is fused into a ring to make uroporphyrinogen III. Four carboxyl groups are removed to make coproporphyrinogen III, and a coproporphyrinogen oxidase converts coproporphyrinogen III to protoporphyrinogen IX.[39] After 2 h, the supernatant was removed and the cells were washed once in PBS, lysed with 100 μL of 0.05% sodium dodecyl sulfate solution at 37°C for 30 min, and the intracellular uptake of PPIX was quantified using a multimode microplate reader (SpectraMax M5®, Molecular Devices Co. Ltd.) with excitation and emission wavelengths of 405 and 635 nm, respectively.

2.6 Fluorescence Imaging of Intracellular PPIX

A549 cells were exposed to 5-ALA (800 μM), PPIX (100 μM), and HVJ-PPIX (100 μM) dissolved in FCS-free DMEM as described above. After the supernatant was removed and the cells were washed once in PBS, the distribution of PPIX in

the cells was observed using fluorescent microscopy (BZ-9000, KEYENCE, Japan). The excitation wavelength was 405 nm, and fluorescence emission was detected at 650 nm. 4,6-Diamidino-2-phenylindole hydrochloride (DAPI) (Nacalai Tesque, Japan) was used to locate the nucleus.

2.7 Evaluation of the Difference of PPIX Uptake between Cell Lines

The amount of drugs delivered using the HVJ-E vector varies depending on the cell line tested.[40] We used A549 cells and B16 cells to evaluate the delivery of PPIX via HVJ-E. The cells were plated into a 96-well plate (2×10^4 cells/well). Three hours later, the medium was removed and replaced with 100 μL of HVJ-PPIX suspension (0 to 200 μM; dissolved in FBS-free DMEM). After 2 h, the supernatant was removed, the cells were washed and lysed, and the intracellular uptake of PPIX was quantified as described for the PPIX uptake assay.

2.8 Evaluation of the Photocytotoxic Activity of PDT

The B16 cells were inoculated into a 96-well Microtest™ Optilux™ plate (1.25×10^4 cells/well) and incubated for 48 h. The medium was removed and replaced with 100 μL of 5-ALA solution (0 to 50 μM; dissolved in FBS-free DMEM) or HVJ-PPIX solution (0 to 6.25 μM; dissolved in FBS-free DMEM). After 2 h, the cells were washed and re-suspended in 100 μL of FBS-free DMEM. Cells were then subjected to laser irradiation (50 mW/cm^2) for 0, 30, 60, or 180 s. After laser irradiation, the culture medium was replaced with a complete growth medium. After 24 h, the Cell Counting Reagent SF kit (Nacalai Tesque, Japan), based on a water-soluble tetrazolium compound, was used to determine the effect of PDT on cell survival. The microplate reader was used to measure the absorbance at a wavelength of 450 nm. All experiments were performed in triplicate.

2.9 Statistical Analyses

All data were expressed as the mean $\pm$ standard derivation. Statistical significance (defined as *P* values of <0.01) was evaluated using an unpaired Student's *t*-test (two-tailed).

3 Results

3.1 Photosensitizer Uptake by A549 Cells

The relationship between the photosensitizer administered and the intracellular fluorescence intensity is presented in Fig. 1. The amount of PPIX that was taken up by the cells reached a plateau at 2 h (data not shown). Fluorescence intensity with HVJ-PPIX was three times higher than that with the PPIX solution, and 20 times higher than that with the 5-ALA solution. Thus, uptake of PPIX was significantly higher with the HVJ-PPIX solution than with the other solutions.

3.2 Fluorescence Imaging of Intracellular PPIX

Figure 2 shows the results of fluorescence microscopy. PPIX was located eccentrically in the cytoplasm when the cells were exposed to 5-ALA. However, PPIX was ubiquitously distributed in the cell when HVJ-PPIX was used.
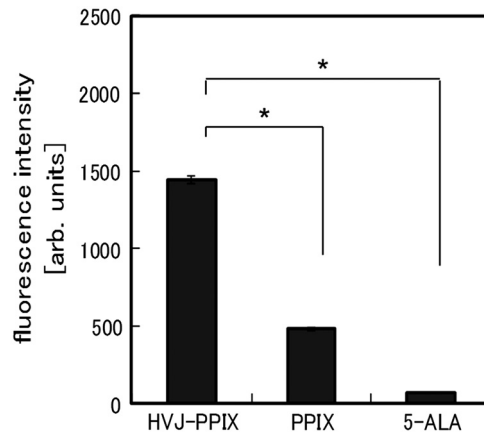

Fig. 1 Intracellular fluorescence intensity reflecting the intracellular uptake of 5-aminolaevulinic acid (5-ALA), protoporphyrin IX (PPIX), and hemagglutinating virus of Japan envelope-protoporphyrin IX (HVJ-PPIX) solutions ($*P < 0.01$).
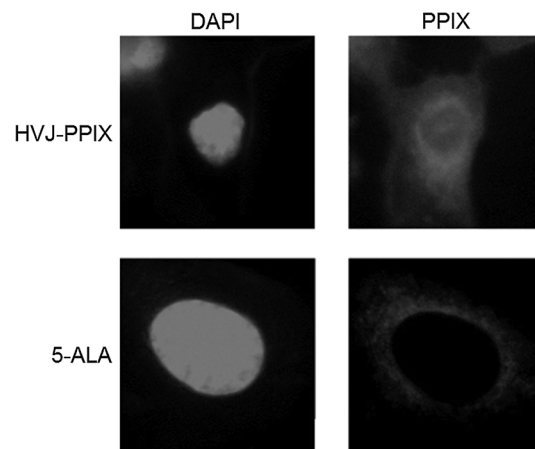

Fig. 2 Fluorescence imaging of protoporphyrin IX (PPIX) uptake in A549 cells. The excitation wavelength was 405 nm, and fluorescence emission was detected at 650 nm. The cell nuclei were stained with 4′,6-Diamidino-2-phenylindole hydrochloride (DAPI).

3.3 Evaluation of the Difference of PPIX Uptake between Cells

The relationship between the initial concentration of HVJ-PPIX in the culture medium and the fluorescence intensity from intracellular PPIX is shown in Fig. 3. At HVJ-PPIX suspension concentrations of >50 μM, PPIX uptake was higher in the B16 cells than in the A549 cells.

3.4 Evaluation of Photocytotoxic Activity of PDT

The cytotoxic effect of PDT was evaluated in A549 and B16 cells. The number of untreated cells was set at 100%, and the cell survival rate in the treatment groups was calculated relative to the untreated cells. Comparing Figs. 4 and 5, or Figs. 6 and 7, the HVJ-PPIX suspension showed a greater cytotoxicity after PDT than the 5-ALA solution.

4 Discussion

PDT is one of the least invasive cancer therapies, but the accumulation of photosensitizers in tumor cells is inefficient. One
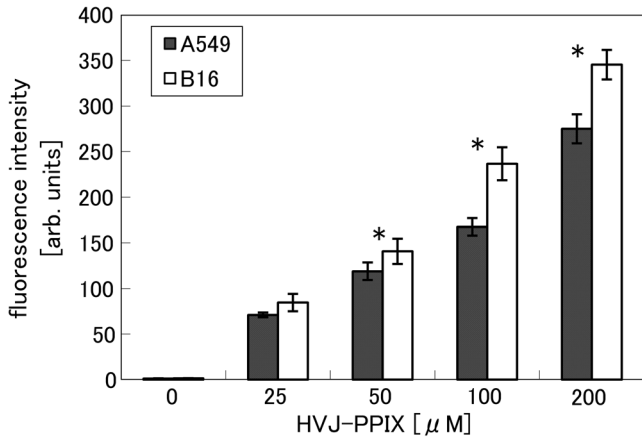
Fig. 3 Comparison of the fluorescence intensity of protoporphyrin IX (PPIX) taken up by cells with different initial concentrations of hemagglutinating virus of Japan envelope-protoporphyrin IX (HVJ-PPIX) (∗$P < 0.01$).
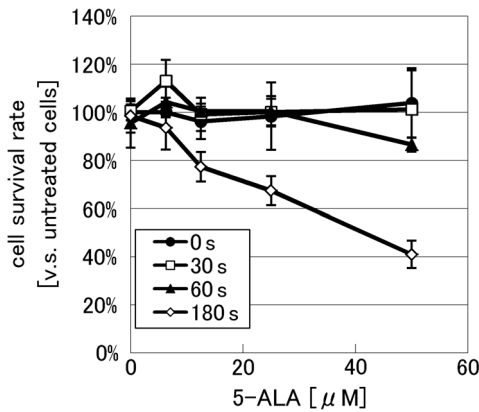

Fig. 4 Photocytotoxic effect of photodynamic therapy (PDT) with 5-aminolaevulinic acid (5-ALA) in the A549 cells. The symbols show irradiation time.
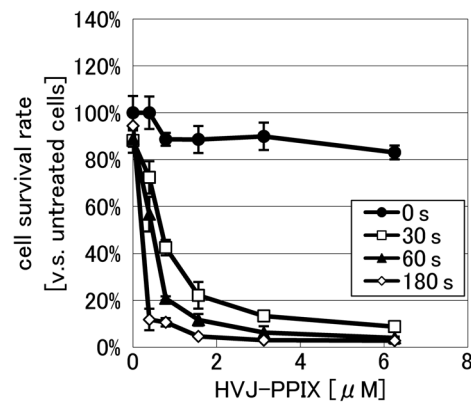

Fig. 5 Photocytotoxic effect of photodynamic therapy (PDT) with hemagglutinating virus of Japan envelope-protoporphyrin IX (HVJ-PPIX) for the A549 cells. The symbols show irradiation time.

Fig. 6 Photocytotoxic effect of photodynamic therapy (PDT) with 5-aminolaevulinic acid (5-ALA) in the B16 cells. The symbols show irradiation time.

Fig. 7 Photocytotoxic effect of photodynamic therapy (PDT) with hemagglutinating virus of Japan envelope-protoporphyrin IX (HVJ-PPIX) for the B16 cells. The symbols show irradiation time.

HVJ-E are very similar to that of native HVJ. Two glycoproteins, fusion (F) protein and hemagglutinin-neuraminidase (HN) protein, are present on the viral envelope, and these proteins are important for membrane fusion.[21,41] It is likely that efficient uptake of PDT photosensitizing compounds occurs using this vector through the fusion of the vector membrane with the tumor cell membrane.

In the current PDT method, 5-ALA was used. PPIX is formed from 5-ALA in the mitochondria.[42] We developed a novel method for the direct transportation of PPIX in tumor cells using HVJ-E, and showed that HVJ-PPIX could be used to inject more photosensitizers into a cell than 5-ALA. It is unlikely that the improvement in the injection efficiency of HVJ-PPIX relative to PPIX was only due to differences in the compounds. HVJ-E increases the injection efficiency by supporting cell adhesion and membrane fusion using surface proteins. The amount of PPIX induced by HVJ-PPIX was 20 times higher than that induced by 5-ALA solution (Fig. 1), which yields about two to five times better results compared with other vectors in earlier studies.[43–46]

The distribution of PPIX in the cell was observed using fluorescence microscopy (Fig. 2). PPIX was located eccentrically in the cytoplasm when the cells were exposed to 5-ALA. 5-ALA is metabolized along a pathway in the mitochondria into PPIX.

way to overcome this limitation is to use a tumor-targeted DDS. In our study, we tested the novel DDS vector HVJ-E for the delivery of photoactive compounds used in PDT. HVJ-E is expected to be a novel DDS vector. Because HVJ-E is constructed from the inactivated Sendai virus, the components of
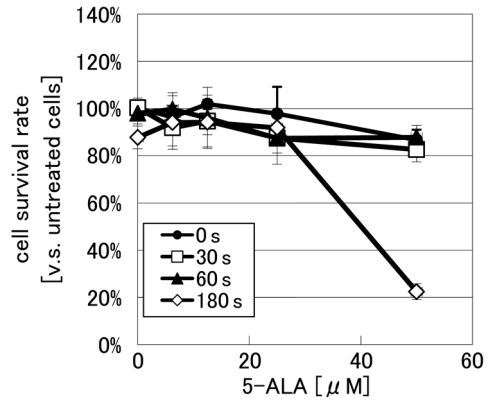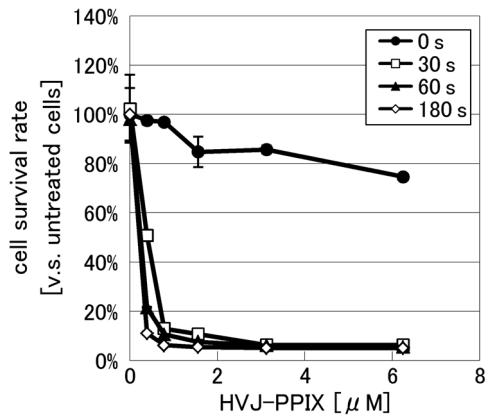
Thus ALA-PPIX is localized in mitochondria.[47] On the other hand, PPIX was ubiquitously distributed in the cell by applying HVJ-PPIX. The PDT ability was maintained. HVJ-E transfers drugs into cells by cell fusion. The membrane fusion pathway can spare the endosomal and lysosomal degradation.[25] In our study, it was considered that PPIX would also take up using the membrane fusion pathway.

In addition, PPIX-induced HVJ-PPIX was observed in the nucleus. Because HVJ-E is often used in gene transfer,[48–50] it is very likely that HVJ-E allows PPIX to move into the nucleus by a similar mechanism. PDT results in reactive oxygen species (ROS) and damage to cellular DNA. Thus, the use of HVJ-PPIX has an advantage over PPIX induced by 5-ALA.[28]

The amounts of PPIX taken up by B16 and A549 cells were measured (Fig. 3), and we determined that B16 cells took up significantly about 20% to 40% more PPIX than A549 cells. Of course, the growth rate was different between these cells. Although the uptake of PPIX can affect cell growth, our experiments lasted for only 2 h in serum-free medium, which eliminated the effect of changes on the cell growth rate.[51]

HVJ-E binding to the cell surface is mediated by an acetyl-type sialic acid recognized by the HN protein on the vector.[52] HN-depleted HVJ has low hemagglutinating activity and the infection activity was suppressed.[53] On the other hand, the transportation by HVJ-E is not affected by inhibitors of endocytosis.[21,22,54] HVJ-E vector—mediated membrane fusion was also observed by electron microscopy.[21,55] These results indicate that the PPIX can be directly delivered to the cytoplasm. Because membrane fusion is needed to the derivation, the receptor of A549 and B16 would take an interest in the amount of intracellular PPIX. The membrane fusion pathway has a higher potential to increase uptake efficiency than does endocytosis because the endosome aggregates PPIX and decreases the efficiency of absorption of light.[56,57] Furthermore the retention time is shortened because PPIX in coated vesicles is dissolved by the lysosomes.[58]

The photocytotoxicity of HVJ-PPIX (Figs. 5 and 7) was evaluated in comparison with that induced by 5-ALA (Figs. 4 and 6). High cytotoxicity was observed both in A549 and B16 cell lines. The cytotoxicity for B16 cells (Fig. 7) was twice as large as that for A549 cells (Fig. 5), which corresponded to the measured uptake results. The photocytotoxicity of B16 cells was higher than that of A549 cells compared in the same intracellular PPIX concentration. Thus, we demonstrated that the use of HVJ-PPIX resulted in efficient photocytotoxicity considering the amount of PPIX uptake.

HVJ-E alone has demonstrated antitumor efficacy without the addition of any therapeutic agents.[28,59] When the concentration of the HVJ-PPIX suspension was greater than 6.25 μM, little cytotoxicity was observed without laser irradiation (Figs. 5 and 7). This suggests that HVJ-PPIX retains its antitumor effect. Evaluation of the photocytotoxic effects of HVJ-PPIX in the tumor-bearing mouse models should also be evaluated, along with the *in vivo* cytotoxicity of the carrier alone. These findings may be useful for the clinical application of phototherapy in cancer patients.

The *in vitro* experiments reported in this study did not allow us to evaluate the tumor selectivity of the HVJ-PPIX, but the *in vivo* selectivity has been demonstrated in previous research.[19–23] The accumulation of photosensitizing compounds *in vivo* is critical in the medical application of PDT.

5 Summary

In this study, HVJ-E was applied to PDT as a novel DDS carrier, and the efficacy of PDT using HVJ-PPIX was evaluated *in vitro*. As a result, the uptake quantities of the photosensitizers increased to a greater extent with HVJ-PPIX than previously reported compounds. Future studies should evaluate the drug kinetics, the amount of accumulation in the tumor, and the photocytotoxic effect of PDT with HVJ-PPIX using *in vivo* tumor-bearing animal models.

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