Summary of Doctoral Dissertation

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Title of Doctoral Dissertation

Photodynamic Therapy for Cancer using Mitochondrial Drug Delivery System
（ミトコンドリア薬物送達システムを用いた癌光線力学療法の検証）

A non-invasive and specific targeting for cancer therapy is a necessity to manifest in order to minimize the harmful effect on the non-malignant cells. During the past century, photodynamic therapy (PDT) has been actively developed as a non-invasive approach to effectively eradicate the cancer cells with minimal effect on healthy cells. The PDT effect is derived from an energy transfer reaction between light as a source of energy to molecular oxygen, mediated by a light-activated molecule, known as the photosensitizer. The dynamic interaction among these three major components in PDT produces a lethal level of reactive oxygen species (ROS), mainly singlet oxygen. The selectivity of this therapy could be achieved by the specific accumulation of the non-toxic photosensitizer in the tumor region, accompanied by the precise delivery of light in the corresponding area. The singlet oxygen has a highly reactive characteristic that can readily react with several vital molecules in the biological system, resulting in the molecule dysfunction. This interaction may also lead to irreversible oxidative damage and further provoke a lethal effect for the cells. However, the harmful effects of singlet oxygen are restricted by their short lifetime and inadequate diffusion capacity. Therefore, the specific delivery of photosensitizer, mainly in organelle-level, could be a promising strategy to obtain the maximum benefits of this therapy. Moreover, as the important organelle that holds both the vital and lethal functions, mitochondria are identified to be an attractive target for optimizing the PDT outcomes.

In the current condition, the existing photosensitizers often manifest disadvantageous features for a practical PDT application. One of them is a non-specific accumulation either in the cellular or at the subcellular level. The other drawback is the inadequate ability of most photosensitizers in absorbing near-infrared (NIR) light. The application of NIR light in PDT, especially in the optical window of biological tissues, is profoundly beneficial because NIR light has an excellent penetration ability toward tissue consisting of water and biomolecules. These problems restrict the use of such compounds in clinical applications. Therefore, the development of a novel PDT system that can fulfill the requirements of the selective organelle accumulation in combination with the long-wavelength light activation is inevitable.

The main objective of this research was to construct a novel mitochondrial targeting PDT system with the long-wavelength light activation process. The π-electron conjugation with two triarylamine moieties linked with triple bond was introduced into the porphyrin core to produce a novel porphyrin-type photosensitizer, namely rTPA (the original compound synthesized by Yuta Takano, Ph.D., an associate professor at Research Institute for Electronic Science, Hokkaido University). The effectivity of this π-elongation was indicated by the robust light absorption capacity at around 700 nm ($\varepsilon = 6.2 \times 10^4$ M^{-1} cm^{-1} at 704 nm in DMSO), while negligible absorption was observed for the basic porphyrin compound. The rTPA was incorporated into the MITO-Porter system using the hydration method followed by surface modification with stearylated octaarginine (R8) to target mitochondria. Before the addition of the R8 to the liposomes, which consisted of 1,2-dioleoyl-sn-glycerol-3-phosphatidylethanolamine (DOPE) and sphingomyelin (SM), negatively charged nanoparticles were

obtained (rTPA-LPs). After R8 modification, the particles became positive (rTPA-MITO-Porter) with a diameter of 157 ± 7 nm and zeta potential of 32 ± 3, which indicates the attachment of R8 on the exterior of the liposomes. The rTPA was also incorporated into the non-mitochondrial targeting liposomes by merely replacing the DOPE with the non-hydrogenated egg phosphatidylcholine (EPC) (rTPA-EPC:SM-R8).

The singlet oxygen sensor green (SOSG) assay was employed to evaluate the singlet oxygen generation ability of the rTPA in a liposomal formulation. The rTPA in the nanocarrier system was mixed with the SOSG solution, and the change in fluorescence intensities of the SOSG was then recorded during the photoirradiation process using a Xenon lamp with an optical filter (700 ± 6 nm, 20 mW/cm^2). The same concentration of meso-tetra(4-sulfonatophenyl) phorphine, a commercially available porphyrin-based singlet oxygen photosensitizer, was used as a positive control with irradiation at 430 nm (20 mW/cm^2). As a result, rTPA generates singlet oxygen in a comparable manner with the pristine porphyrin molecule. Furthermore, the singlet oxygen level increases linearly with longer irradiation time, indicating the stability of the rTPA compound during the irradiation process. This result confirms the significant ability of rTPA in generating singlet oxygen under NIR light irradiation. Moreover, the rTPA-MITO-Porter also can effectively provoke the production of singlet oxygen inside the mitochondrial compartment of HeLa cells, recognized by Si-DMA, a specific probe for detecting singlet oxygen level in mitochondria of living cells.

The quantification of cellular uptake efficiency of the rTPA-nanocarriers was conducted by using fluorescence activating cell sorting (FACS) analysis. In this evaluation, the nanocarrier was labeled by N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD). The cellular uptake significantly improved by approximately 3.5 folds in the presence of R8, both the case of rTPA-MITO-Porter and rTPA-EPC:SM-R8 in comparison to the rTPA-LPs. It indicates the significant functions of R8 as a cell-penetrating peptide for improving the cellular uptake efficiency of the liposomes. From the intracellular trafficking analysis using confocal laser scanning microscopy (CLSM), it was found that the rTPA-MITO-Porter dominantly accumulated in the mitochondrial compartment of HeLa cells, while most of the rTPA-EPC:SM-R8 particles concentrated outside mitochondria. Pearson's correlation coefficient further verified this result with the value of 0.27 ± 0.06 (n = 12) and 0.11 ± 0.05 (n = 12) for the rTPA-MITO-Porter and the rTPA-EPC:SM-R8, respectively.

The significance of mitochondrial delivery of the rTPA was evaluated by analyzing the PDT cytotoxicity of the rTPA-MITO-Porter and the rTPA-EPC:SM-R8 against HeLa cells. This comparison is due to the difference in the mitochondrial accumulation level, as mentioned above. In the absence of irradiation, both the rTPA-nanocarriers showed negligible toxicity. In contrast, after 3 minutes irradiation of Xenon lamp (700 ± 6 nm, 68.5 mW/cm^2), only the rTPA-MITO-Porter could induce significant toxicity indicated by decreasing in the absorbance of WST-1 reagent. The effect of mitochondrial accumulation and irradiation process on the PDT outcomes was further investigated by using two-way ANOVA analysis. It revealed that a combination of mitochondrial accumulation and irradiation is required to produce a notable toxic effect. Besides, the dose-dependent PDT cytotoxicity was obtained with the minimum concentration for eliminating 50% of the cell population (EC_{50}), approximately 0.16 ± 0.02 μM.

The effectiveness of the rTPA-MITO-Porter was further evaluated using human squamous cell carcinoma of the tongue, namely SAS cells. The rTPA-MITO-Porter with 700 nm irradiation efficiently eradicated SAS cells with an EC_{50} of 0.41 ± 0.18 μM, while more than 85% of the cell population survived in the absence of irradiation. Furthermore, the results from the direct CLSM observation suggested that the PDT process of the rTPA-MITO-Porter could effectively stimulate the apoptosis cell death. The activation of the apoptosis pathway may be resulted through the increased levels of ROS inside the mitochondrial compartment, leading to the activation of the mitochondrial permeability transition pore.

The antitumor activity was evaluated using Balb/c Scl-nu/nu mice inoculated subcutaneously with SAS cells on the right flank. All animal experiments were carried out following the protocols reviewed

and approved by the Institutional Animal Care and Research Advisory Committee at the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan (Registration Number: 16-0015). A slight modification on the rTPA-MITO-Porter formulation, particularly on the helper lipid composition and the total lipids' concentration, was made without altering the mitochondrial targeting ability and the photo-induced cytotoxic capacity.

After the tumor volume reached 50 mm^3 or six days after tumor inoculation, the mice randomized into five groups and separately treated by intratumoral administration of HEPES buffer containing 290 mM glucose pH 7.4 (HBG) with light irradiation (+ L), empty MITO-Porter + L, free rTPA + L, rTPA-MITO-Porter, and rTPA-MITO-Porter + L. The rTPA dose was fixed at 8.2 μg/mouse for all treatments containing rTPA. The light irradiation process was performed using a Xenon lamp with the optical filter that produces 700 ± 6 nm light for 20 minutes, 12h after the administration of the solution. The tumor growth was monitored every two days until the endpoint of the experiment (20 days after tumor cells inoculation). The relative tumor volume was calculated by comparing the tumor volume with its corresponding initial tumor volume prior to treatment.

The remarkable inhibition of the tumor growth was obtained after a single PDT treatment of the rTPA-MITO-Porter in comparison to the other control groups. There was also no significant alteration on the bodyweight of the mice during the treatment, implying the promising *in vivo* cell-killing ability of this system with a high safety profile. Furthermore, the depolarization on the mitochondrial membrane was observed after the PDT process of the rTPA-MITO-Porter, as indicated by the significantly diminish of the TMRM fluorescence signal. This result suggests that the PDT process of the rTPA-MITO-Porter effectively induced depolarization on the mitochondrial membrane. This result also further confirms that the photochemical reaction was localized inside the mitochondrial compartment of tumors.

Finally, the findings presented in this research serve to verify the considerable functions of the MITO-Porter system as the mitochondrial selective drug delivery technology in potentiating the PDT outcomes as well as the importance of mitochondria as the predominant subcellular target for PDT. Moreover, this novel mitochondrial targeting PDT system demonstrates a promising feature for treating the superficial-type cancer cells through the photochemical reaction process.