Comparative photodynamic therapy cytotoxicity of mannose-conjugated chlorin and talaporfin sodium in cultured human and rat cells

Yo Shinoda1, Tsutomu Takahashi1, Jiro Akimoto2, Megumi Ichikawa2, Hiromi Yamazaki3, Atsushi Narumi3, Shigenobu Yano4 and Yasuyuki Fujiwara1

1Department of Environmental Health, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
2Department of Neurosurgery, Tokyo Medical University, 6-7-1 Nishi-Shinjuku, Shinjuku, Tokyo 160-0023, Japan
3Graduate School of Organic Materials Science, Yamagata University, Jonan 4-3-16, Yonezawa 992-8510, Japan
4Graduate School of Material Science, Nara Institute of Science and Technology, 8916-5, Takayama-cho, Ikoma, Nara 630-0192, Japan

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ABSTRACT — Photodynamic therapy (PDT) is a Food and Drug Administration authorized method for cancer treatment, which uses photosensitizer and laser photo-irradiation to generate reactive oxygen species to induce cell death in tumors. Photosensitizers have been progressively developed, from first to third generation, with improvements in cell specificity, reduced side effects and toxicity, increased sensitivity for irradiation and reduced persistence of photosensitizer in healthy cells. These improvements have been achieved by basic comparative experiments between current and novel photosensitizers using cell lines; however, photosensitizers should be carefully evaluated because they may have cell type specificity. In the present study, we compared a third-generation photosensitizer, β-mannose-conjugated chlorin (β-M-chlorin), with the second generation, talaporfin sodium (NPe6), using seven different rat and human cell lines and a neuronal/glial primary culture prepared from rat embryos. NPe6 was more effective than β-M-chlorin in human-derived cell lines, and β-M-chlorin was more effective than NPe6 in rat primary cultures and rat-derived cell lines, except for the rat pheochromocytoma cell line, PC12. These differences of phototoxicity in different cell types are not because of differences in photosensitivity between the photosensitizers, but rather are associated with different distribution and accumulation rates in the different cell types. These data suggest that evaluation of photosensitizers for PDT should be carried out using as large a variety of cell types as possible because each photosensitizer may have cell type specificity.

Key words: Photodynamic therapy (PDT), Photosensitizer, β-Mannose-conjugated chlorin (β-M-chlorin), Talaporfin sodium (NPe6), Malignant brain tumor

INTRODUCTION

Photodynamic therapy (PDT) is an established minimally invasive treatment for cancer (Dolmans et al., 2003; Juarranz et al., 2008; Savoia et al., 2015; Colcombe et al., 2016). PDT uses a nontoxic photosensitizer that is intravenously injected and accumulates in cancer cells, and a harmless visible laser to elicit the photosensitizer to produce reactive oxygen species (ROS) to destroy tumors (Dougherty et al., 1998; Sibata et al., 2000; Dolmans et al., 2003). Photosensitizers have been progressively developed from the first-generation clinical photosensitizer, porfimer sodium (Photofoin), which is useful for early lung cancer and superficial esophageal cancer, but has undesirable effects such as the requirement for long-term (4 to 6 weeks) shielding from light to reduce phototoxicity in the skin (Manyak et al., 1988; Triesscheijn et al., 2006; Juarranz et al., 2008). The second generation of photosensitizers, such as talaporfin sodium (mono-L-aspartyl-chlorin-e6, NPe6), requires a shorter period of light shielding, produces less skin toxicity compared with Photofoin and requires a longer wavelength to induce ROS (Nelson et
al., 1987; Horimatsu et al., 2012). Now a third generation of photosensitizers has been developed with reduced side effects and increased specificity and effectiveness (Shan, 2004; O’Connor et al., 2009; Senge, 2012); however, the evaluation of new photosensitizers and comparison with older ones should be carried out carefully. In the present study, we synthesized β-M-chlorin as a third-generation photosensitizer derived from the core structure of chlorophyll (chlorin) in which the four meso positions were substituted with 4-D-β-mannopyranosylthio-2,3,5,6-tetrafluorophenyl groups. Then we compared the actions of β-M-chlorin with those of the second-generation photosensitizer, NPe6, in several cell lines from different species and in a rat primary culture.

MATERIALS AND METHODS

Synthesis β-M-chlorin

5,10,15,20-Tetrakis[4-(β-D-mannopyranosylthio)-2,3,5,6-tetrafluorophenyl]-2,3-[methano(N,N-methyl)imidomethane]chlorin (β-M-chlorin) was synthesized according to the similar procedures described in the literature (Narumi et al., 2016) except that 1-thioacetyl-2,3,4,6-tetra-O-acetyl-β-D-mannopyranose was used as a starting material. UV-vis (c = 5.00 μM, DMSO, path length = 1 cm, 25°C): λ/nm (ε × 10⁻³ / M·cm⁻¹) = 412 (189), 505 (17.2), 531 (5.54), 598 (5.54), 652 (44.0).

Cell culture

Human glioblastoma T98G cells (Riken Cell Bank, Ibaraki, Japan) were cultured as described previously (Miki et al., 2014). Briefly, cells were seeded at 1 × 10⁴ cells/well in collagen-coated 96-well plates (Iwaki, Tokyo, Japan) and cultured in RPMI1640 medium (Nissui, Tokyo, Japan) supplemented 10% fetal bovine serum (FBS: Nichirei Bioscience, Tokyo, Japan) and incubated at 37°C in a 5% CO₂ atmosphere for 24 hr. Two other human glioblastoma cell lines, A172 and U251 (Riken Cell Bank), were cultured in non-coated cell culture plastic 96-well plates in minimal essential medium (Nissui) supplemented with 10% FBS, and rat glioblastoma C6 cells (Riken Cell Bank) were cultured in non-coated cell culture plastic 96-well plates in RPMI1640 medium supplemented with 10% FBS. Human meningioma HKB-MM cells (Riken Cell Bank) were cultured on non-coated cell culture plastic 96-well plates in Ham’s F12 medium (Nissui) supplemented with 15% FBS, and rat meningioma KMY-J cells (Riken Cell Bank) were cultured on non-coated cell culture plastic 96-well plates in minimal essential medium (Nissui) supplemented with 10% FBS.

Rat pheochromocytoma PC12 cells (Riken Cell Bank) were cultured in collagen coated 96-well plates in DMEM supplemented with 10% FBS and 10% horse serum (Sigma-Aldrich, Tokyo, Japan). Primary cultures of rat cortical neurons and glia were prepared as described previously with partial modification (Nakajima et al., 2016). Embryonic day 18 Wistar rat pups were deeply anaesthetized, decapitated and the brains were removed into ice-cold HEPES-buffered salt solution (Life and Technologies, Tokyo, Japan). Cortical tissues were digested with papain (Sigma-Aldrich), cells harvested and plated in 0.04% poly-ethyleneimine (PEI: Sigma-Aldrich) coated wells of 96-well plates or in a 100 mm Petri dish (Thermo Scientific) and cultured for 8 days. Cortical astrocytes were purified by trypsinization and subsequent passage of primary cultured cells in DMEM supplemented with 10% FBS. All experimental protocols were evaluated and approved by the Regulations for Animal Research at Tokyo University of Pharmacy and Life Sciences, and were carried out in accordance with the approved protocols.

PDT treatment

Cultured cells were treated with appropriate concentrations (0-62.5 μM) of NPe6 (Meiji Seika Pharma, Tokyo, Japan) or β-M-chlorin in fresh medium for 4 hr. Cells were washed with PBS and incubated for 1 hr in fresh medium (or conditioned medium for cortical neurons). The cells were subjected to laser irradiation (wave length: 664 nm, laser power 3.4 mW/cm², total dose of laser irradiation: 0-1 J/cm² [time of irradiation: 0-304 s]) using a semi-conductor laser irradiator ZH-L5011HJP (Meiji Seika Pharma).

Cell viability assay

Twenty-four hours after PDT treatment, cell viability was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, Cell Counting Kit-8 solution was mixed with culture medium to a final concentration of 10% and the mixture was applied to PDT treated cells for 1 hr at 37°C. After incubation, absorbance was measured at 450 nm using a Varioskan Flash microplate reader (Thermo Scientific). Phase contrast imaging was performed using a DMi1 inverted microscopy (Leica Microsystems, Wetzlar, Germany).

Photosensitizer transport assay

For fluorescence microscopy, T98G cells were cultured in 8-chamber glass bottomed dishes (Iwaki) coated with collagen type-I (Nitta gelatin, Osaka, Japan), and KMY-J cells were cultured on 8-chamber glass bottomed dishes coated with 0.04% PEI. Cells were cultured for...
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24 hr before the transport assay. Fresh medium containing 25 μM NPe6 or β-M-chlorin was applied to the culture and incubated for 1 to 4 hr. Photosensitizer-treated cells were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer for 30 min at room temperature. Fluorescence of NPe6 and β-M-chlorin was obtained using an Eclipse Ti-U inverted microscope (Nikon, Tokyo, Japan) equipped with a filter cube (Ex. 340-380 nm, DM 400 nm, Em. 672-716 nm) and a CMOS Zyla5.5 camera (Andor technology, Belfast, UK). Fluorescence data were collected and processed by NIS-elements (Nikon) and Photoshop (Adobe Systems, San Jose, CA, USA). Fluorescence intensities were measured and calculated by ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

If not stated otherwise, data are expressed as the mean ± S.E.M. Differences between multiple data sets were assessed using one-way ANOVA with post hoc Tukey-Kramer test. All the data were collected and analyzed using a double-blind approach.

RESULTS

Cell specificity of NPe6 and β-M-chlorin as PDT photosensitizers

We first compared the effect of NPe6 and β-M-chlorin as PDT photosensitizers on cell viability in human glioblastoma T98G cells. T98G cells were treated with NPe6 or β-M-chlorin for 4 hr, and were then irradiated by a 664 nm laser (1 J/cm²) (Fig. 1A). Neither NPe6- nor β-M-chlorin-treated cells without irradiation showed any significant cell death; however, laser-irradiated cells showed significant cell death after both NPe6 and β-M-chlorin treatment in a dose-dependent manner (Figs. 1A and 1B). The photosensitization effect of NPe6 was also stronger than that of β-M-chlorin. Two other human glioblastoma cell lines, U251 (Fig. 1C and Supplemental Fig. S1) and A172 (Fig. 1D and Supplemental Fig. S2), and human meningioma HKBMM cells (Figs. 1E and Supplemental Fig. S3) showed similar responses to that of T98G cells (Figs. 1A and 1B). These data suggest that NPe6 is a more effective photosensitizer compared with β-M-chlorin in the human-derived glioblastoma and meningioma cells tested.

Next we applied NPe6 and β-M-chlorin to rat-derived glioblastoma C6 cells using the same experimental procedure (Figs. 2A and 2B). Interestingly, in C6 cells, β-M-chlorin was more effective than NPe6. A similar effect can be seen in the other rat-derived meningioma cell line, KMY-J (Figs. 2C and Supplemental Fig. S4). We then performed PDT using NPe6 and β-M-chlorin on normal cells. Rat cortical neurons (CTX) and glia were prepared from embryonic rat brain and cultured. β-M-chlorin was also much more effective than NPe6 in these rat-derived normal cells (Figs. 2D, 2E, Supplemental Figs. S5 and S6). On the other hand, rat pheochromocytoma PC12 cells showed a significant PDT effect in response to NPe6 but not β-M-chlorin (Figs. 2F and Supplemental Fig. S7). These data suggest that NPe6 and β-M-chlorin have cell specificity for PDT, regardless of whether cells are human or rat derived.

Differences in cell specificity between NPe6 and β-M-chlorin may be associated with different accumulation rates and distribution

Next we investigated the accumulation rate of each photosensitizer in different cell types by fluorescence microscopy. Both NPe6 and β-M-chlorin fluoresce when excited by UV and emit infrared fluorescence. This fluorescence can be measured enabling the concentration of each photosensitizer to be estimated. NPe6 showed an endosome/lysosome-like accumulation pattern in T98G cells, but no or weak accumulation in KMY-J cells (Figs. 3A and 3B). Interestingly, however, β-M-chlorin shows a diffuse pattern in both cell lines (Figs. 3A and 3B). In T98G cells, normalized fluorescence intensities (normalized against the fluorescence intensity of 4 hr photosensitizer exposure) at each time point were not different between NPe6 and β-M-chlorin (Fig. 3C). In contrast to T98G cells, KMY-J cells showed significantly faster accumulation of β-M-chlorin compared to NPe6 at 1 to 3 hr (Fig. 3D). Absolute fluorescence values cannot be directly compared between NPe6 and β-M-chlorin because fluorescent intensity depends on both quantum yield and extinction coefficient; however, we measured absolute fluorescence values of NPe6 and β-M-chlorin in T98G cells (Fig. 3E). The absolute intensities of each photosensitizer in KMY-J cells are shown in Fig. 3F. Even though the absolute intensities of each photosensitizer are smaller in KMY-J cells compared with those in T98G cells, the reduction in value between T98G and KMY-J cells was larger for NPe6 compared to that for β-M-chlorin (Figs. 3E and 3F). These data suggest that the accumulation rates and distribution patterns of NPe6 and β-M-chlorin are cell-type dependent.

The photosensitivity of NPe6 and β-M-chlorin does not determine cell specificity

To investigate the reason for photosensitizer cell specificity, we examined the photosensitivity of NPe6 and β-M-
β-M-chlorin is less effective than NPe6 in cells of human origin. A, A representative image of T98G cells treated with NPe6 or β-M-chlorin, 24 hr after irradiation. T98G cells were treated with NPe6 or β-M-chlorin at each concentration shown (0 to 62.5 μM) for 4 hr and irradiated with 1 J/cm², using a 644 nm laser (+) or were not irradiated (-), and cultured for 24 hr. Scale bar = 200 μm. B-E, Cell viability of T98G (B), U251 (C), A172 (D) and HKBMM (E) cells. n = 9, *P < 0.05, **P < 0.01, statistical significance between irradiated group and each non-irradiation control at the same photosensitizer concentration; †P < 0.05, ‡P < 0.01, statistical significance between irradiated cells treated with NPe6 and β-M-chlorin at the same concentration, ANOVA with post hoc Tukey-Kramer test.
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Fig. 2. β-M-chlorin is more effective than NPe6 in the cells of rat origin tested, except for PC12 cells. A, A representative picture of C6 cells treated with NPe6 or β-M-chlorin, 24 hr after irradiation. Scale bar = 200 μm. B-F, Cell viability of C6 (B), KMY-J (C), rat cortical neurons (D), rat cortical glia (E) and PC12 (F) cells. n = 9, *P < 0.05, **P < 0.01, statistical significance between irradiated group and each non-irradiation control at the same photosensitizer concentration; ^P < 0.05, ^^P < 0.01, statistical significance between irradiated cells treated with NPe6 and β-M-chlorin at the same concentration, ANOVA with post hoc Tukey-Kramer test.
chlorin in specific cell-types by applying different irradiation levels to T98G- and KMY-J-treated cells. T98G cells were treated with 0 to 62.5 μM NPe6 or β-M-chlorin for 4 hr, then irradiated with a 664 nm laser for different durations to control laser fluence (0 to 1 J/cm²). Twenty-four hours after laser irradiation, cells were biochemically counted using a Cell Count Kit-8 and a microplate reader. For all irradiation fluences, NPe6 was much more efficient than β-M-chlorin (Figs. 4A, 4B, Supplemental Figs. S8 and S9). As shown in Fig. 2C, KMY-J cells treated with NPe6 and β-M-chlorin showed a significantly higher effect for β-M-chlorin with 1 J/cm² than with NPe6; however, at lower fluences, the effect of β-M-chlorin was less effective than NPe6 (Figs. 4C, 4D, Supplemental Figs. S10 and S11). These data suggest that the cell specificity of NPe6 and β-M-chlorin does not result from changes in their photosensitivity associated with cell type.

**DISCUSSION**

Effectiveness and toxicity, and cell-type specificity are important features of photosensitizers (Jori, 1996; Mehraban and Freeman, 2015). Therefore, evalua-

![Fig. 3. The distribution and accumulation of each photosensitizer in T98G and KMY-J cells. A, B, A representative fluorescence image of NPe6 and β-M-chlorin in T98G (A) and KMY-J (B) cells after 4 hr photosensitizer exposure. Note that NPe6 shows an endosome/lysosome-like accumulation pattern in T98G cells, but no or weak accumulation in KMY-J cells. β-M-chlorin shows a diffuse pattern in both cells. Scale bar = 10 μm. C, D, The relative fluorescence intensity of each photosensitizer in T98G (C) and KMY-J (D) cells during photosensitizer treatment for 4 hr. Fluorescence intensities were normalized against the intensity after treatment for 4 hr. E, F, The absolute fluorescent intensity of each photosensitizer in T98G (E) and KMY-J (F) cells after treatment for 4 hr.](image-url)
tion of photosensitizers should be carefully investigated. In the present study, we focused on the cell specificity of the photosensitizers, newly synthesized β-M-chlorin and clinically used NPe6. We found that these drugs do not always have the same photosensitizing effects in all cell types. We used seven different cell lines (glioblastoma, meningioma and pheochromocytoma) from rat and human, and a cortical neuron and glial primary culture from rat embryos for PDT experiments. NPe6 was much more effective than β-M-chlorin in human cell lines (Fig. 1), and β-M-chlorin was more effective than NPe6 in rat cell lines and in the CTX primary culture, except for PC12 cells (Fig. 2).

Cell type specificity of drug penetration and accumulation are thought to be associated with the composition of the cell surface (Sears and Perry, 2015). Specificity also depends on the components of the extracellular matrix, such as fibrous proteins (Ziemys et al., 2015), proteoglycans/glycoproteins (Schinkel, 1999) and glycosaminoglycans (Lilly and Parsons, 1990), which also determine physical features of the extracellular environment, such as drug permeability, diffusivity and stability. In addition, different cell types have different cell dynamics for exogenous molecules, such as endo- and exocytotic fea-
tures, inhalation and discharge, transportation, accumulation, distribution, degradation and modification properties (Devarajan et al., 2015). All these features of cells and their surroundings can affect the photodynamic effect of a photosensitizer. In the present study, we tried to determine the mechanism of cell type specificity for β-M-chlorin and NPe6. Some photosensitizers are known to accumulate in specific subcellular regions according to their physical properties (MacDonald et al., 1999; Leung et al., 2002; Hayashi et al., 2015), and these features are correlated with the strength of phototoxicity (Peng et al., 1996; Kessel et al., 1997; Hsieh et al., 2003). Therefore, we analyzed the distribution and accumulation rate of each photosensitizer by fluorescence imaging (Fig. 3). NPe6 is known to preferentially accumulate in endosomes/lysosomes in murine hepatoma Hepa-1c1c7 cells (Reiners et al., 2015). α-M-chlorin accumulates in mitochondria in the murine colorectal cancer cell line CT26 (Hayashi et al., 2015). Here, we used human T98G cells and rat KMY-J cells to evaluate the different distribution and accumulation rates of NPe6 and β-M-chlorin. The distribution of NPe6 was endosome/lysosome-like, especially in T98G cells, similar to previously reported observations (Reiners et al., 2002); however, β-M-chlorin was distributed in a diffuse pattern rather than in a mitochondrial pattern in both cell lines (Figs. 3A and 3B). Next, we measured the photosensitivity of NPe6 and β-M-chlorin associated with T98G cells and KMY-J cells (Fig. 4). Surprisingly, β-M-chlorin had a higher threshold of photooxidation compared with NPe6 in KMY-J cells, even though β-M-chlorin with 1 J/cm² fluorescence induced stronger cell death than NPe6 (Figs. 4C and 4D). Taken together, these data suggest that the different phototoxic effects of NPe6 and β-M-chlorin in T98G and KMY-J cells does not result from changes to their photosensitivity after cell uptake. In contrast, there are differences in the distribution pattern and accumulation rate of each photosensitizer depending on the cell type, which suggests that differences in subcellular distribution and/or accumulation rate may explain the different phototoxicity of NPe6 and β-M-chlorin in different cells.

Further investigation is required to clarify the correlation between photosensitizer features and phototoxicity in each cell type. However, the evaluation of photosensitizers should be carried out in as many different cell lines from different species as possible.

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

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