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Porphyrin Derivatives Mediated Sonodynamic Therapy on Malignant Gliomas in Vitro

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ABSTRACT

Due to its highly infiltrative property, malignant glioma is a cancer of poor prognosis despite multidisciplinary treatment strategies, such as aggressive surgery and chemoradiotherapy, unveiling a need for new therapeutic approaches to control migrated tumor cells. In our study, we investigated the efficacy of sonodynamic therapy (SDT) using porphyrin derivatives involving 5-aminolevulinic acid, protoporphyrin IX, and talaporfin sodium as sonosensitizers on glioma cells, in vitro. These substances have been known to accumulate in glioma cells and are expected to have cytotoxic effects by sonication. Our study demonstrated that the cytotoxicity of sonication on glioma cells was enhanced by each sonosensitizer and the efficacy of the SDT may depend on the degree of intracellular accumulation of sonosensitizer. Also, induction of apoptosis was suggested to be a major mechanism of cell death. Though further investigations will be necessary, our preliminary result showed a potential of SDT with sonosensitizers for glioma treatment.

16 **KEYWORDS:** 5-aminolevulinic acid, in vitro, malignant glioma, porphyrin derivatives,
17 protoporphyrin IX, sonodynamic therapy, talaporfin sodium, ultrasound

18 INTRODUCTION

19 Gliomas account for approximately 20 % of primary brain tumors in adults (Nakamura
20 et al. 2011). Regarding the malignant subtype of glioma, prognosis is very poor. Despite
21 intensive therapy, the actual 2-year survival rate is only 33.9 % (Chinot et al. 2014;
22 Gilbert et al. 2014). Though novel therapeutic trials have been recently carried out,
23 patients' survival has not been improved remarkably. Thus, it is necessary to develop a
24 different approach to treatment, enhancing conventional therapy. At present, the
25 standard treatment for malignant gliomas is maximal safe resection combined with
26 chemoradiation. Removal of the tumor is still the most important role in a therapeutic
27 strategy, but the boundary of the tumor is often unclear because glioma cells infiltrate
28 into adjacent normal brain tissue. Actually, 90 % of tumors relapse within 2 cm of the
29 primary site despite the use of chemoradiotherapy after gross total resection (Wilson
30 1992). These results suggest a key of successful treatment is to control these infiltrative
31 tumor cells.

32 Photodynamic therapy (PDT) is one of the expected treatments (Muragaki et al. 2013).

33 The mechanism of this therapy is that photochemically activated photosensitizers

34 generate reactive oxygen species (ROS), such as singlet oxygen and free radicals, which
35 lead to oxidative damage of target tumor cells. Among various photosensitizers,
36 porphyrin derivatives have been utilized in PDT for gliomas because these substances
37 are selectively accumulated in glioma cells, and they have been already applied for
38 clinical use in PDT and intraoperative fluorescence diagnosis (Stylli et al. 2006a; Stylli
39 et al. 2006b). Particularly, 5-Aminolevulinic acid (ALA) is widely used for
40 intraoperative fluorescence diagnosis. ALA, which is a natural amino acid, is the first
41 compound of the porphyrin biosynthetic pathway. In this pathway, protoporphyrin IX
42 (PpIX) is synthesized from ALA in mitochondria, and PpIX is suggested to be retained
43 in tumor cells when administrating exogenous ALA excessively. This is thought to be
44 due to metabolic dysfunction of PpIX in malignant tumor cell (Ishizuka et al. 2011).
45 Accumulated PpIX in a tumor cell acts as a strong photosensitizer. Furthermore,
46 talaporfin sodium (TS) is a second-generation photosensitizer that has recently been
47 approved in Japan for PDT to treat primary malignant brain tumors (Muragaki et al.
48 2013). Although PDT is an attractive treatment in glioma therapy, there is a great
49 problem of penetration depth of the light in brain tissue. To overcome this issue,

ultrasound has been expected as alternative energy source to excite the photosensitizers.

Rosenthal et al. have shown that various photosensitizers are excited by ultrasound as well (Rosenthal et al. 2004).

The aim of this study is to investigate the anti-tumor effects of SDT with porphyrin derivatives such as ALA, PpIX, and TS for gliomas in vitro.

MATERIAL AND METHODS

Reagents

ALA was purchased from COSMO BIO Co., Ltd (Koto, Tokyo, Japan). PpIX, Hoechst 33342 (bis Benzimide H 33342 trihydrochloride), and Propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TS was purchased from Meiji seika, Ltd. (Chuo, Tokyo, Japan). Calcein AM/Ethidium homodimer (EthD-1) costaining kit (Live/Dead Viability/Cytotoxicity Assay Kit[®]) was purchased from Life Technologies (Minato, Tokyo, Japan). Annexin V-FLUOS Staining Kit was purchased from Roche Applied Science (Minato, Tokyo, Japan).

66 *Cell culture*

67 Rat glioma cell line C6 and human glioblastoma cell line U87MG were obtained from
68 the American Type Culture Collection (Manassas, VA, USA). Cells were maintained as
69 monolayers in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis,
70 MO, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Life
71 Technologies, Minato, Tokyo, Japan), 100 U/mL of penicillin G, and 100 µg/mL of
72 streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified incubator
73 with 5 % CO₂ in air atmosphere. Unless stated otherwise, the conditions of the
74 incubation were as described above. Cells in the exponential growth phase after more
75 than 5 passages were used for all of the following experiments.

76

77 *Ultrasound exposure apparatus*

78 The ultrasound exposure apparatus was arranged as shown in Fig. 1. A
79 laboratory-assembled planar circular transducer (30 mm in diameter) with air backing
80 was submerged in a water bath (0.4×0.2×0.1 m³) filled with degassed water to avoid
81 attenuation of ultrasound wave, and facing upward with its acoustic surface parallel to

82 the water surface. A function generator (AFG3022, Tektronix, Minato, Tokyo, Japan)
83 was used to generate burst pulses of 1 MHz in center frequency and 100 Hz in pulse
84 repetition frequency. Burst pulses of 50 % in duty cycle contain 5,000 cycles in each
85 burst. The transducer was driven by the signal amplified using a power amplifier
86 (UOD-WB-1000, Tokin, Sendai, Miyagi, Japan). The spatial-average-temporal-average
87 intensity (I_{SATA}) of 0.16 W/cm² was used for all experiments, which was determined by
88 pressure calibration using an apparatus utilized for our previous study. (Yamaguchi et al.
89 2011) The calibration was carried out in a free water condition using a Polyvinylidene
90 difluoride membrane hydrophone (MHB-500, Onda, Sunnyvale, CA, USA). During
91 ultrasound exposure, the flat bottom of the well plate (MULTIWELL™ 6 well, BD
92 FALCON®, Bedford, MA, USA) was placed 5 mm from the surface of the transducer.
93 In this condition, the estimation of I_{SATA} that cells actually received is technically
94 difficult because strong reflections at the water surface generate a standing wave field
95 inside the well. An acoustic absorbing rubber plate is frequently used to prevent the
96 reflection by placing it on the water surface. However, it was not utilized in the
97 experiments to minimize disturbance in culture condition and the possibility of

contamination because cell incubation should be continued for up to 24 hours after the sonication. The wells measured 35 mm in diameter, 18 mm in depth, and were 1.62 mm thick. Furthermore, the height (volume) of solution in each well was stringently unified at 1.3 mm (4 mL/well) because the sound pressure transferred to the cells from the standing waves varied depending on the fluid surface height (data not shown). The water temperature in the bath was held at 37 °C to eliminate the temperature elevation of treated cells.

SDT treatment protocols

Cells were randomly divided into four treatment groups: control, sonosensitizer exposure, ultrasound exposure, and SDT (ultrasound exposure plus sonosensitizer exposure). Upon reaching 70 % confluence, cells were washed with phosphate-buffered saline (PBS; Nacalai tesque, Nakagyo, Kyoto, Japan) and collected using 0.05 % trypsin with 0.53 mM ethylenediaminetetraacetic acid. Approximately 3.0×10^5 cells/well were dispersed into the wells of each plate and subsequently incubated for 24 hours.

ALA and TS were stored at 4 °C in the dark, and dissolved in DMEM without phenol-red (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 200 and 30 µg/mL respectively. PpIX was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.005 g/mL and subsequently stored at -20 °C in the dark, and also dissolved in DMEM without phenol-red to a final concentration of 1.0 µg/mL. For the sonosensitizer and the SDT groups, the culture medium was replaced with an equivalent volume of sonosensitizer solution and incubated thereafter for 4 hours. For the other groups, the culture medium was replaced with fresh DMEM without sonosensitizer solution and phenol-red and subsequently incubated. Consecutively each well in the ultrasound and the SDT groups was subjected to ultrasound treatment at 1.0 MHz and 0.16 W/cm² for 60 seconds respectively.

124

Fluorescence intensity per cell after co-incubation with each sonosensitizer

The fluorescence intensity per cell after co-incubation with each sonosensitizer was analyzed by flow cytometry to verify differences in the uptake of the sonosensitizer in different cell lines. Following co-incubation with each sonosensitizer, cells were

129 harvested by trypsin and analyzed by a flow cytometer (Attune[®] Acoustic Focusing
130 Cytometer, Life Technologies, Minato, Tokyo, Japan) using excitation and emission
131 wavelengths of 488 and 690/50 nm, respectively.

132

133 *Cytotoxicity of SDT*

134 Following ultrasound exposure, cells were re-incubated for 24 hours. A Calcein
135 AM/EthD-1 costaining kit was used with Hoechst 33342 following incubation to assess
136 cell viability. After washing the cells with 1 mL of PBS, 100 µL of PBS containing 2
137 µM of Calcein AM and 4 µM of EthD-1, and 0.2 µL of Hoechst 33342 were added to
138 each well and the cells were incubated for 30 minutes at room temperature in the dark.
139 Cells were observed using a fluorescent microscope (IX71, U-LH100HGAP0,
140 OLYMPUS, Shinjuku, Tokyo, Japan). The polyanionic dye Calcein AM is well retained
141 within live cells, producing an intense uniform green fluorescence. EthD-1 enters cells
142 with damaged membranes and binds to nucleic acids, thereby producing a bright red
143 fluorescence in dead cells. Hoechst 33342 is a popular cell-permeation nuclear
144 counterstain that emits blue fluorescence when bound to double-stranded

145 deoxyribonucleic acid (DNA), regardless of cell survival. The average numbers of green,
146 red, and blue fluorescent cells were calculated in five random microscopic fields with at
147 least 1000 cells/field for each treatment group. Experiments were repeated
148 independently three times. Notably, exfoliated floating cells at 24 hours after treatment
149 were defined as dead.

150 The number of dead cells was calculated as [*number of Hoechst 33342 positive cells in*
151 *control group* — *number of Hoechst 33342 positive cells in each treatment group*].

152 Thus, compensated dead cells were estimated as [*number of EthD-1 positive cells in*
153 *each treatment group* + *number of dead cells in each treatment group*].

154 The survival rate (%) of the tumor cells was calculated as [*100* — *number of*
155 *compensated dead cells in each treatment group* / *number of Hoechst 33342 positive*
156 *cells in control group* × 100].

157

158 *Mechanism of ALA mediated SDT cytotoxicity on C6 cells*

159 Treated C6 cells were stained with Annexin V-FLUOS and PI to assess the cell
160 damage mechanism following re-incubation for 6 hours. After washing once with 1 mL

of PBS, 100 μ L of the incubation buffer (10 mM Hepes, pH7.4, 140 mM NaCl, 5 mM CaCl_2) containing 2 μ L of Annexin V-FITC and 2 μ L of PI (50 μ g/mL) was added to each well. Cells were then incubated for 15 minutes at room temperature in the dark. The cells were trypsinized, and then analyzed using a flow cytometer with an excitation wavelength of 488 nm and emission wavelength of 530/30 nm for Annexin V-FLUOS and 574/26 nm for PI respectively. Phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane in live cells; however, it is translocated from the inner to the outer leaflet of the plasma membrane in the acute phase of apoptosis, while Annexin V-FLUOS has a high affinity for PS exposed on the outer leaflet and produces green fluorescence. PI is impermeant to live and apoptotic cells; however, it binds tightly to the nucleic acids in dead cells, producing red fluorescence.

Statistical analysis

Experimental data was analyzed statistically with Statcel 3 software (OMS publishing Inc, Tokorozawa, Saitama, Japan). All values were presented as means $\pm$ standard deviation of triplicate experiments. The comparisons among these groups were assessed

by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test and p values < 0.05 were considered to be statistically significant.

RESULTS

Cytotoxicity of SDT with each sonosensitizer on C6 cells

The survival rate was evaluated by counting the number of live and dead cells with a fluorescent microscope. The number of exfoliated cells was prominent in the SDT group (Fig. 2). The cytotoxicity of the ultrasound was enhanced by each sonosensitizer on C6 cells, while neither each sonosensitizer alone nor ultrasound alone caused any cytotoxicity. The survival rate of C6 cells treated by ALA mediated SDT decreased from 85.1 ± 6.8 % to 69.1 ± 10.8 % (Fig. 3a), as well as for PpIX mediated SDT (from 100.0 ± 11.7 % to 78.9 ± 1.0 %, Fig. 3b) and for TS mediated SDT (from 100.0 ± 11.7 % to 59.8 ± 8.1 %, Fig. 3c). Furthermore, for all sonosensitizers, the survival rates of the SDT groups were lower than those from any of the other groups.

Mechanism of ALA mediated SDT cytotoxicity on C6 cells

Flow cytometry analysis demonstrated marked changes in the cell profile following ultrasound exposure on C6 cells (Fig. 4). The results of flow cytometry analysis were the following: cells in the lower-left quadrant (Annexin-V-/PI-) represent living cells, those in the lower-right quadrant (Annexin-V+/PI-) represent early apoptotic cells, and those in the upper-right quadrant (Annexin-V+/PI+) represent late apoptotic cells or necrotic cells. The early apoptotic cell rate in both ultrasound alone and SDT group were more than twice as high as that in the other groups, respectively (ultrasound alone group: Figure 4c 14.2 % and SDT group: Figure 4d 20.4 % vs. Control group: Figure 4a 5.1 % and ALA alone group: Figure 4d 1.5 %). In addition, late apoptotic and necrotic cell rates in the SDT group were the highest among all groups (Figure 4d).

Comparison of the cytotoxicity of SDT between C6 and U87MG cells

The cytotoxic effect of SDT on U87MG cells (Figure 3d-f) was smaller than that on C6 cells despite the fact that it was applied under the same conditions as for C6 in each sonosensitizer administered (Figure 3a-c). Figure 5 shows the results of the difference in uptake of sonosensitizer between these two cell lines in flow cytometry analysis. For

either sonosensitizer, the fluorescence intensity per cell of U87MG cells (Figure 5d-f) was lower than that of C6 cells (Figure 5a-c).

DISCUSSION

Although various therapeutic strategies for malignant gliomas have been tried clinically, satisfactory results have not been obtained yet (Chinot et al. 2014; Miyatake et al. 2014; Thomas et al. 2014). Japanese national survey reported that 1-, 2-, and 3-years survival rates of patients with malignant gliomas was approximately 60.3 %, 25.4 %, 15.9 %, respectively (Report of brain tumor registry of Japan (2001-2004) 13th edition 2014). Therefore, new methods of treatment should be developed as a breakthrough of this situation.

Since the 80's, PDT has been explored as a novel option to treat malignant gliomas because this therapy is of low toxicity for normal brain tissue by tumor-selectivity of photosensitizers (Stylli et al. 2006a; Stylli et al. 2006b). However, the most important problem of PDT is the limited penetration depth of the light in brain tissue. It was reported that the effective depth of laser beam penetration in brain tissue is

225 approximately 0.75–1.5 cm (Muragaki et al. 2013). This issue was recognized from the
226 beginning of the development of PDT. To overcome this issue, basic experiments were
227 conducted to confirm whether ultrasound could be used as an alternative to the light for
228 excitation of photosensitizers (Rosenthal et al. 2004; Chen et al. 2014). Further studies
229 have shown the cytotoxic effect of SDT, which was combined ultrasound and
230 conventional photosensitizers, for various cancers (Canaparo et al. 2006; Song et al.
231 2011; Yamaguchi et al. 2011; Yumita et al. 2011). Studies regarding ALA and PpIX
232 mediated SDT have also provided encouraging results (Wang et al. 2010; Song et al.
233 2011; Li et al. 2012; Lv et al. 2012; Su et al. 2014), however, knowledge of ALA
234 mediated SDT for malignant gliomas is limited yet (Ohmura et al. 2011; Jeong et al.
235 2012).

236 Ultrasound exposure to biological tissues is categorized into two effects: thermal and
237 non-thermal. Acoustic energy absorbed by a biological tissue produces a temperature
238 elevation and the thermal effect is utilized for hyperthermia. But heat injury sometimes
239 causes crucial problem in treatment of intracranial lesions. Consequently, our concept of
240 this study is that the principle of SDT for brain tumor should be based on non-thermal

241 effect.

242 A non-thermal effect is caused by various mechanisms of mechanical and chemical
243 phenomena. Acoustic radiation force produces tissue motion and acoustic streaming,
244 which increase cell membrane permeability and resulting drug delivery efficiency.

245 Cavitation is an important mechanism of the non-thermal effect and is categorized into
246 non-inertial and inertial cavitation. Non-inertial cavitation is caused by microbubble
247 oscillation under applied pressure and mainly produces mechanical effects, such as
248 microstreaming, excess temperature elevation, and acoustic radiation force. Inertial
249 cavitation occurs under a condition with higher intensity and longer duration than those
250 in the non-inertial condition. Bubble expansion and subsequent rapid adiabatic
251 contraction by inertia of the surrounding liquid causes a temperature rise inside the
252 bubble up to several thousands of degrees, in theory, generating free radicals by
253 pyrolysis of H_2O molecules. Generation of the free radicals is essential to induce SDT
254 effects, a series of chemical reactions of a sonosensitizer such as porphyrin derivatives
255 that was originally used as light activated chemicals. Porphyrin derivatives themselves
256 have no anti-tumor effects, and are less cytotoxic for normal tissues; however, inertial

257 cavitation can be followed by energy transfer to oxygen to generate ROS in the
258 presence of porphyrin derivatives, (Canaparo et al. 2006) and they are expected to
259 provide an anti-tumor effect without temperature rising.

260 Apoptosis is suggested as the mechanism of cell death by PDT. Apoptosis can be
261 induced by low intensity ultrasound exposure even below the threshold for temperature
262 elevation of the culture medium (Lagneaux et al. 2002; Feng et al. 2010; Cheng et al.
263 2013). In addition, Zhang et al. showed that low intensity ultrasound exposure can
264 induce apoptosis related to caspase-3, Bcl-2, and survivin in C6 cells (Zhang et al.
265 2012). In our study, flow cytometry analysis confirmed that ultrasound exposure
266 induced cell apoptosis at 0.16 W/cm^2 , even in the absence of a sonosensitizer. On the
267 other hand, cell survival analysis using immunofluorescences did not indicate the
268 presence of apoptosis in the ultrasound alone group. This discrepancy may be caused by
269 tumor-doubling time because measurement was performed 24 hours after treatment.

270 However, an important consideration is that survival rate of SDT group is still
271 suppressed. The sonication conditions are not fully determined, although they may have
272 been well reproduced between experiments, and will require further research.

273 Our findings also demonstrated a relationship between sonodynamic efficacy and the
274 relative fluorescence intensity of sonosensitizer per cell, referring to the degree of
275 intracellular accumulation of sonosensitizer per cell. We confirmed that the cytotoxicity
276 of SDT was distinct between C6 and U87MG cells, and the efficacy of SDT could
277 depend on the degree of intracellular accumulation of sonosensitizer for each cell line.
278 Yumita et al. also reported that the efficacy of SDT closely correlate with the
279 concentration of sonosensitizer in medium (Yumita et al. 2010). Moreover, results of
280 clinical research showed that PpIX accumulates in tumor cells in a histopathological
281 grade-specific manner after administration of ALA for intraoperative fluorescence
282 diagnosis of malignant gliomas (Ishizuka et al. 2011). This data may support that the
283 peak of the fluorescence intensity of the photo-/sonosensitizer is distinct among
284 different cell lines. Therefore, therapeutic strategies to avoid the regulatory feedback in
285 heme biosynthesis for accumulation of PpIX in tumor cells should be considered to
286 obtain an efficient anti-tumor effect of ALA mediated SDT. For example, Xu et al.
287 reported the efficacy of SDT for the glioma stem-like cells was improved by the
288 inhibition of ATP-binding cassette sub-family G member 2 (ABCG2) transporter

function, which regulates cellular accumulation of porphyrin derivatives, with a specific inhibitor (Xu et al. 2013).

When considering the development of SDT as clinical application, if the sonication could be given through the skull, SDT will be an ideal noninvasive treatment for malignant brain tumors. This may be possible in the near future given the research conducted nowadays on brain ultrasound imaging and therapy. Because magnetic resonance imaging guided focused ultrasound surgery (ExAblate[®] Neuro; InSightec, Haifa, Israel) has already been applied in global clinical trial to treat neurological disorders. This device induces focused tissue coagulation via ultrasound exposure generating the thermal effect through intact skull, which has been attracted as an alternative to deep brain stimulation (Lipsman et al. 2014). Clinical trials for transcranial focal ablation of deep-seated tumors, especially glioblastoma, have started (Ram et al. 2006; McDannold et al. 2010). However, there might be a limit to control infiltrative tumor cells by “thermal” effect when considering the injurious effect upon adjacent normal brain tissues. Hence, SDT based on “non-thermal” effect should be established for treatment of deep-seated brain tumors.

305

306 **CONCLUSIONS**

307 In this study, SDT with porphyrin derivatives such as ALA, PpIX, and TS
308 demonstrated the cytotoxic effect on glioma cells via apoptosis. Although further
309 investigation will be necessary, the “non-thermal” SDT has the potential to be a new
310 therapeutic modality for malignant gliomas.

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421

422

423 **FIGURE LEGENDS**

424 Figure 1

425 Schematic diagram of experimental ultrasound irradiation apparatus set up.

426

427 Figure 2

428 Calcein AM (upper line), Ethidium homodimer (EthD-1) (middle line), and Hoechst

429 33342 (lower line) were stained on C6 cells 24 hours after treatment. Control represents

430 cells without any treatments, 5-Aminolevulinic acid (ALA) alone represents cells

431 cultured with 200 $\mu\text{g/mL}$ of ALA for 4 hours, Ultrasound (US) alone represents cells

432 treated only with ultrasound at 1.0 MHz and 0.16 W/cm^2 for 60 seconds, and

433 Sonodynamic therapy (SDT) represents cells treated by ultrasound as described above

434 in the presence of 200 $\mu\text{g/mL}$ of ALA. A significant number of exfoliated cells were

435 observed in the SDT group.

436

437 *Figure 3*

Survival rates of C6 cells and U87MG cells after treatment (a, d: 5-Aminolevulinic acid (ALA) mediated sonodynamic therapy (SDT), b, e: Protoporphyrin IX (PpIX) mediated SDT, c, f: Talaporfin sodium (TS) mediated SDT, upper line: C6 cells, lower line: U87MG cells, n=3). Data are presented as the mean $\pm$ standard deviation and statistical analysis was performed using one-way analyzed of variance (ANOVA) followed by the Tukey-Kramer test. * shows a significant difference ($p<0.05$) between groups. ALA alone; cells cultured with 200 $\mu\text{g/mL}$ of ALA for 4 hours, PpIX alone; cells cultured with 1.0 $\mu\text{g/mL}$ of PpIX for 4 hours, TS alone; cells cultured with 30 $\mu\text{g/mL}$ of TS for 4 hours, Ultrasound (US) alone; cells treated only with ultrasound at 1.0 MHz and 0.16 W/cm^2 for 60 seconds, and SDT; cells treated by US as described above in the presence of each sonosensitizer.

Figure 4

Apoptosis and necrosis rates on C6 cells were analyzed via flow cytometry with Annexin V and Propidium iodide (PI) costaining to assess the mechanism of cell damage in 5-Aminolevulinic acid (ALA) mediated sonodynamic therapy (SDT) (a:

454 control group, b: ALA alone group, c: ultrasound alone group, d: SDT group). The
455 results were interpreted in the following fashion: cells in the lower-left quadrant
456 (Annexin-V-/PI-) represent living cells, those in the lower-right quadrant
457 (Annexin-V+/PI-) represent early apoptotic cells, and those in the upper-right quadrant
458 (Annexin-V+/PI+) represent late apoptotic or necrotic cells.

459

460 Figure 5

461 Relative fluorescence intensities of accumulated sonosensitizer in C6 and U87MG
462 cells were analyzed via flow cytometry. For each sonosensitizer, the fluorescence
463 intensity per cell of the U87MG cells was lower than that of the C6 cells. (a, d:
464 5-Aminolevulinic acid (ALA), b, e: Protoporphyrin IX (PpIX), c, f: Talaporfin sodium
465 (TS), upper line: C6 cells, lower line: U87MG cells)

Figure 1

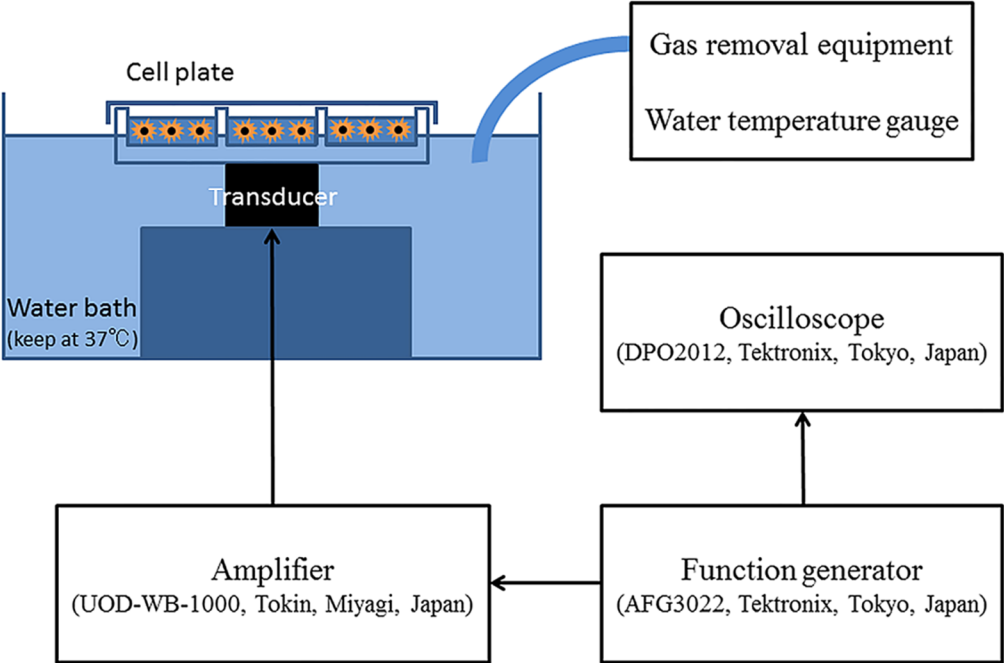


Figure 2

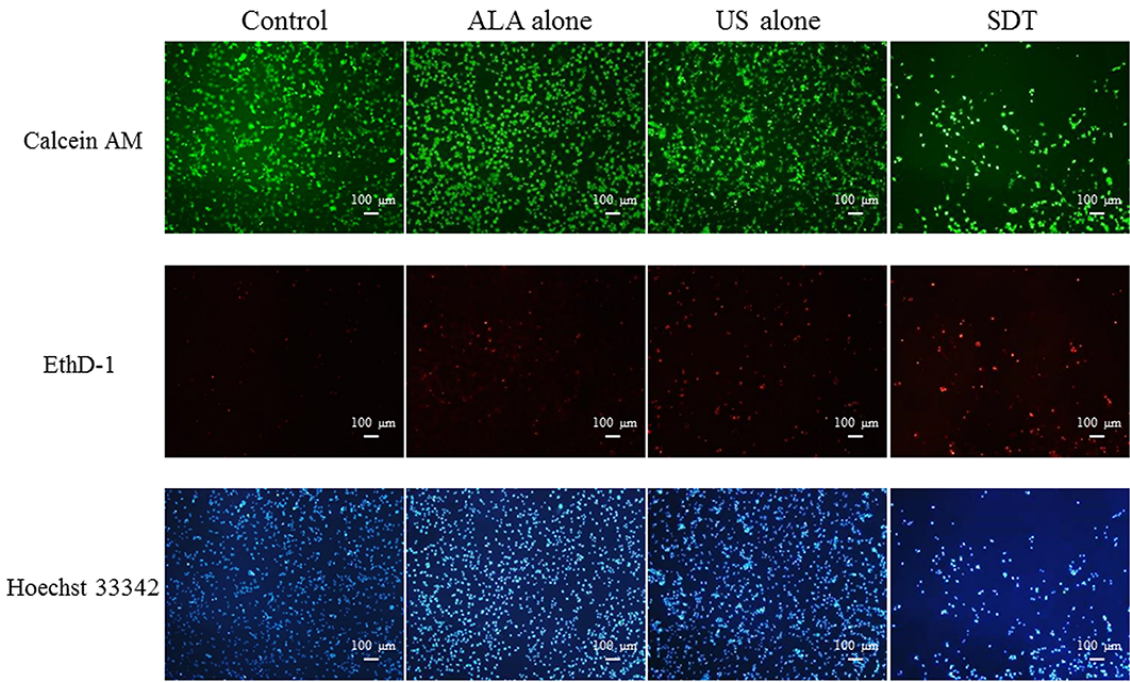


Figure 3

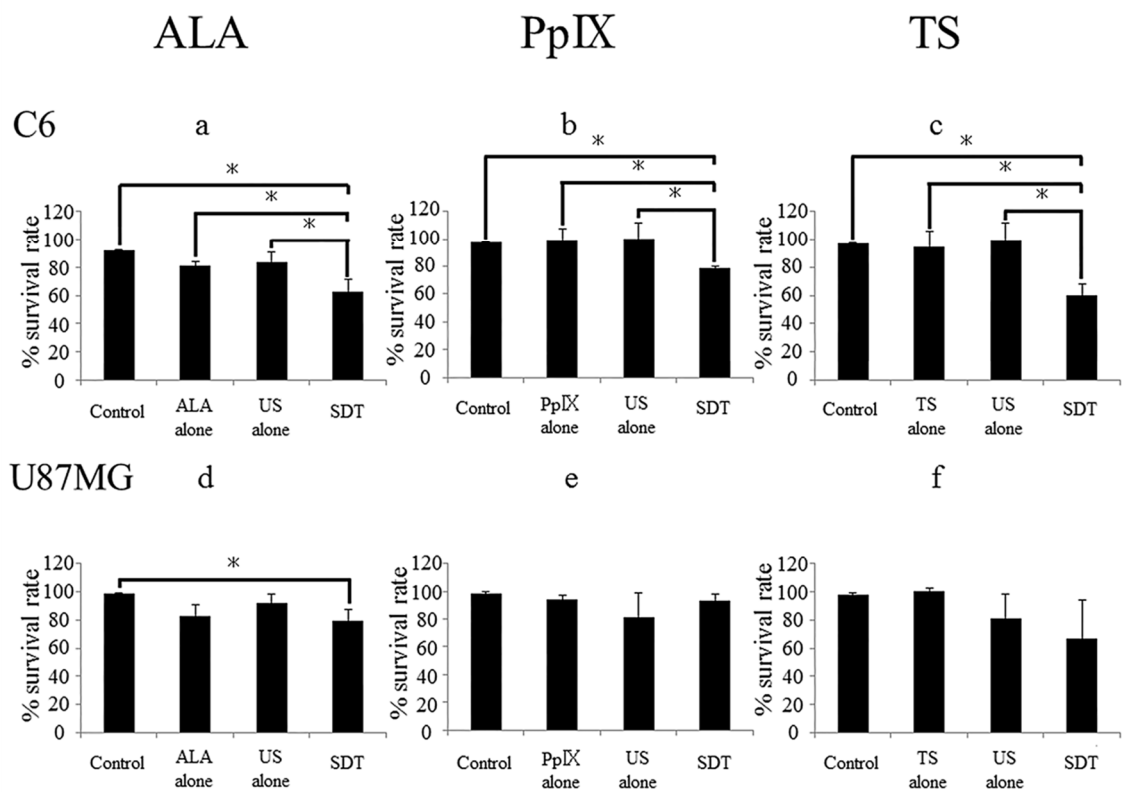


Figure 4

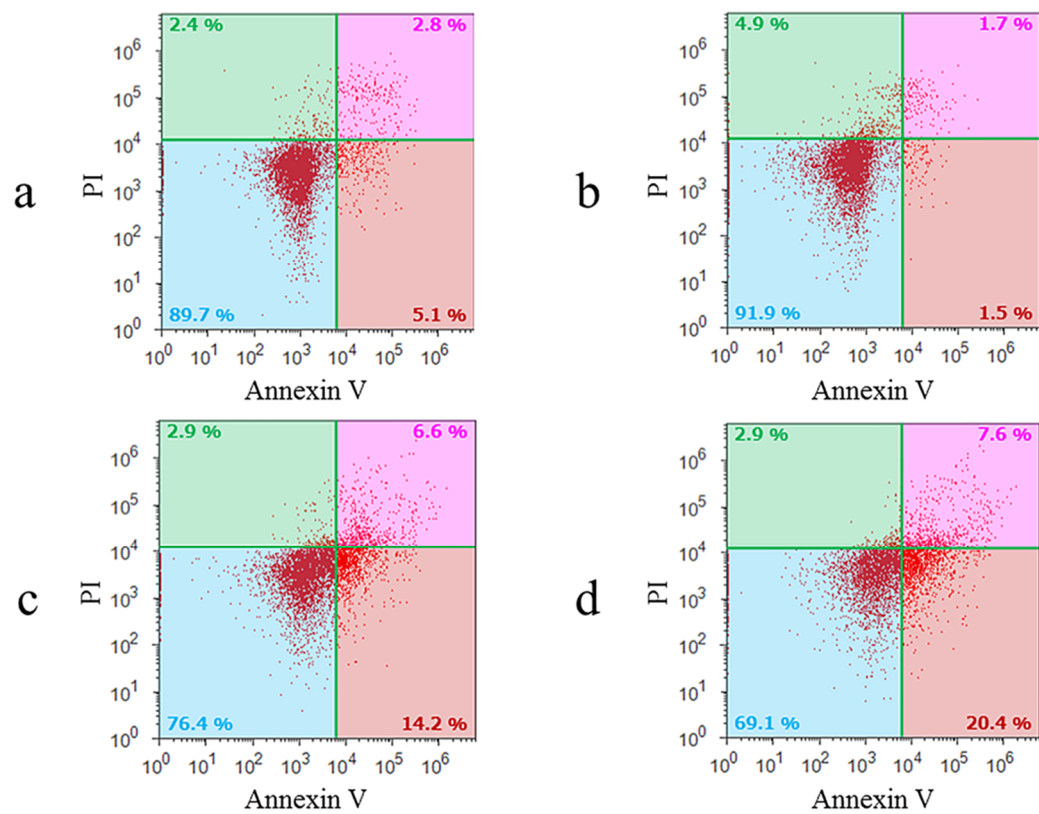


Figure 5

