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Sonodynamically Induced Cell Damage Using Rose Bengal Derivative

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Abstract. *Aim: The ultrasonically induced effect of a tumor accumulative derivative of rose bengal (RB) on isolated tumor cells was investigated to clarify whether the RB derivative (RBD) maintains the sonosensitizing ability of RB. Materials and Methods: Sarcoma 180 cells were suspended in air-saturated phosphate-buffered saline and were exposed to ultrasound in standing wave mode for up to 60 s in the presence and absence of RBD or RB. The viability of the cells was determined by the ability to exclude trypan blue. Results: The ultrasonically induced cell-damaging rate with 100 μM RBD was one order of magnitude higher than that with the same concentration of RB. This increase was significantly inhibited by the active oxygen scavengers histidine, tryptophan and N-acetyl-L-cysteine. Conclusion: Chemical modification of RB to RBD for tumor accumulation significantly increased the sonodynamically induced antitumor effect of RB.*

Photodynamic therapy (PDT) is known as a non- or less-invasive tumor treatment (1, 2). The key components of PDT are a photosensitizer and laser light. The light activates a photosensitizer to react with oxygen, and it forms singlet oxygen that kills tumor cells. However, this therapy can only be used to treat superficial regions because of the poor penetration of light into tissue.

Similar to laser light, ultrasound can enhance the bioeffect of some chemicals (3, 4) and can even activate its sensitizers through the collapse of cavitation bubbles (5). Furthermore, it has a unique feature of penetrating deeply into tissue. The thermal bioeffect of ultrasound alone has already been used clinically for treating tumors in high-intensity focused ultrasound treatment (6-8). It was found that some photosensitizers such as hematoporphyrin, protoporphyrin, a gallium-deutroporphyrin complex, ATX-70, and a chlorine derivative, ATX-S10, also showed an ultrasonically induced antitumor effect on tumor cells (9-15), based on which, sonodynamic therapy (SDT) was proposed.

From the effect of active oxygen scavengers on the ultrasonically induced *in vitro* cell damage, we hypothesized that active oxygen generated by ultrasonically activated porphyrins was the most important mediator for the porphyrin-enhanced ultrasonically induced cell damage (10, 11, 13, 15). Results of *in vivo* animal experiments strongly suggested the potential effectiveness of SDT with such porphyrins (13, 16, 17).

Some xanthene photosensitizers have been found not only to be ultrasonically activated (18) but also to reduce both *in vitro* and *in vivo* cavitation thresholds, the acoustic intensity needed to induce cavitation, by more than one order of magnitude (19-21). Therefore, their potential as sonosensitizers for SDT may be even higher than that of porphyrins. Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein disodium salt, RB, Figure 1) is one such xanthene photosensitizer and is known to produce singlet oxygen with a quantum yield of nearly 100% by visible light exposure (21). Furthermore, RB is reported to induce plasma membrane damage on illumination at 514 nm (22) and it is also reported to induce *in vitro* cell damage when activated with ultrasound (23). Although RB has high potential as a sensitizer for SDT, it cannot be used to treat solid tumors as is. The reason the sensitizing effects of RB were shown in only *in vitro* and not *in vivo* experiments with solid tumors is that RB cannot accumulate in tumor tissue. When RB is injected into a living body, it is excreted immediately into the bile after accumulation in the liver (24). We synthesized a tumor-accumulative RB derivative (RBD, Figure 1) potentially useful for SDT of solid tumors (25).

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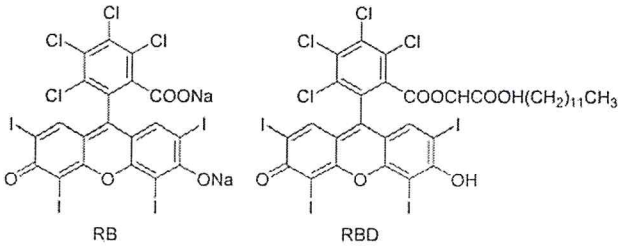

Figure 1. *Structure of rose bengal (RB) and rose bengal derivative (RBD).*

It was therefore of interest to clarify whether the obtained RBD maintains the sonosensitizing ability of RB. We report on the ultrasonically induced cytotoxic effects of RBD on isolated tumor cells.

Materials and Methods

Chemicals. We purchased RB, histidine (His), superoxide dismutase (SOD), mannitol (Man), tryptophan (Trp), *N*-acetyl-L-cysteine (NAC), and Dulbecco's phosphate-buffered saline (PBS, pH 7.4) from Sigma Chemical Company (St. Louis, MO, USA). We prepared RBD by using the method reported in a previous paper (25). All the other reagents were commercial products of analytical grade.

Tumor cells. Sarcoma 180 cells were supplied by Meiji Seika Kaisha (Tokyo, Japan). The cell lines were passaged weekly through male ICR mice in the form of ascites. Cells were harvested from the peritoneal cavity of a tumor-bearing animal 7 to 10 days after inoculation. The experimental animals were treated according to the guidelines proposed by the Science Council of Japan.

Insonation apparatus. The experimental set-up for the insonation is shown in Figure 2. This was very similar to that used in the previous studies (23, 26, 27). The ultrasound transducer used a piezoelectric ceramic disk 24 mm in diameter and was driven at its resonant frequency of 1.92 MHz. Although the insonation experiments were performed in standing wave mode, the acoustic output intensity from the transducer was calibrated against the voltage applied to the transducer in progressive wave mode to prevent difficulty in estimating acoustic intensity in the standing wave mode. The intensity measured in the progressive wave mode was used to specify the intensity in the insonation experiments. While real *in situ* intensity in standing wave mode can be higher by an order of magnitude, both intensities in progressive and standing wave modes at the same voltage were at least approximately proportional to each other.

The transducer and the lower part of a flat-bottomed glass container were submerged in degassed water at room temperature. The temperature rise in 3 ml air-saturated water in the container during 60 s insonation at the highest intensity used in the series of experiments was found to be less than 1°C. Tumor cells harvested from mice were suspended in air-saturated PBS. The cell suspension was stored on ice until used within a few hours in the experiments.

Evaluation of cell damage. The viability of the isolated cells was determined by staining them with trypan blue. A 1 ml aliquot was taken from the cell suspension and mixed with 1 ml of 0.5% trypan blue solution. The integrity of the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope. The integrity was checked immediately before each series of exposures, and cell suspensions with integrity above 99% were used. The number of intact cells immediately before exposure was regarded as the standard for determining the integrity after each exposure. A 3 ml portion of the cell suspension was transferred to the flat-bottomed container and insonated. Each result presented is the mean with standard deviation (SD) of three experiments.

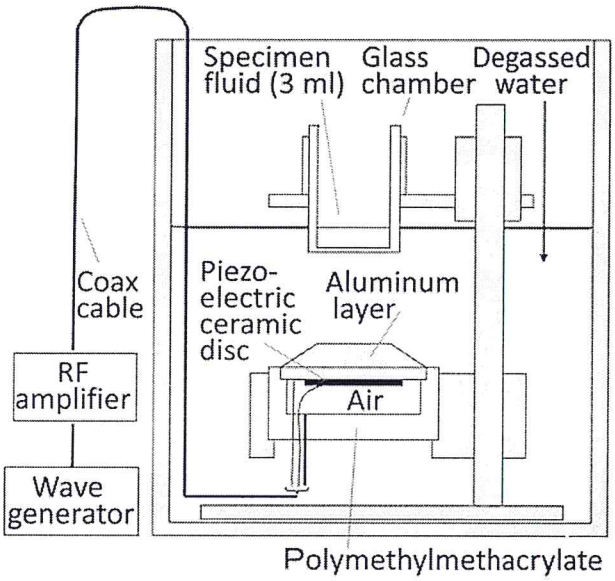

Figure 2. *Insonation apparatus set-up.*

Results

Cell damage. The unstained fractions of the isolated sarcoma 180 cells in the air-saturated suspensions, in the presence of 0 and 100 μM RBD after a fixed exposure time at an ultrasonic intensity of 8.3 W/cm^2, are plotted *versus* duration in Figure 3. The results with 100 μM RB, starting material, are also plotted *versus* duration. The unstained fractions plotted on a logarithmic scale decreased with exposure time primarily in a linear manner. Using RBD increased the ultrasonically induced cell-damaging rate 40-fold at a concentration of 100 μM and the unstained fraction was reduced to approximately 1% after the 60 s exposure. No cell damage was observed with RBD alone. The cell-damaging rate was one order of magnitude higher than that with same concentration of RB.

The unstained fractions in the presence of 0, 50 and 100 μM RBD after a 30 s exposure at an ultrasonic intensity of 0, 1.9, 4.5, and 8.3 W/cm^2 are plotted in Figure 4. Regardless of the presence of RBD, the intensity threshold for ultrasonically induced cell damage was observed to be around 2 W/cm^2.

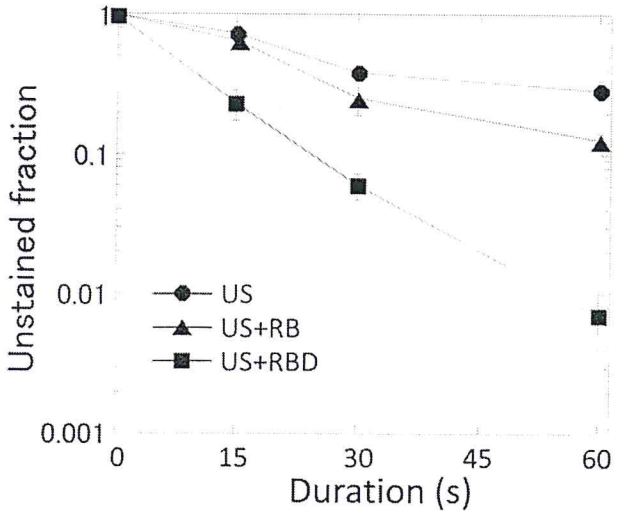

Figure 3. *Effect of ultrasound (US) with and without 100 μM RB or RBD on isolated sarcoma 180 cells.*

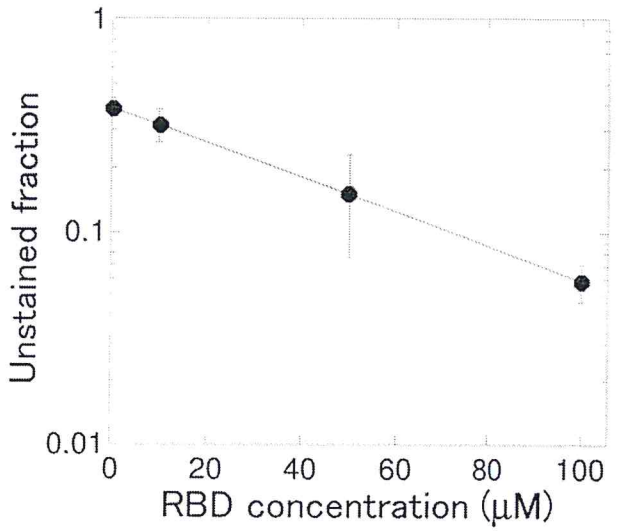

Figure 5. *Unstained fraction of isolated sarcoma 180 cells after 30 s exposure as a function of RBD concentration.*

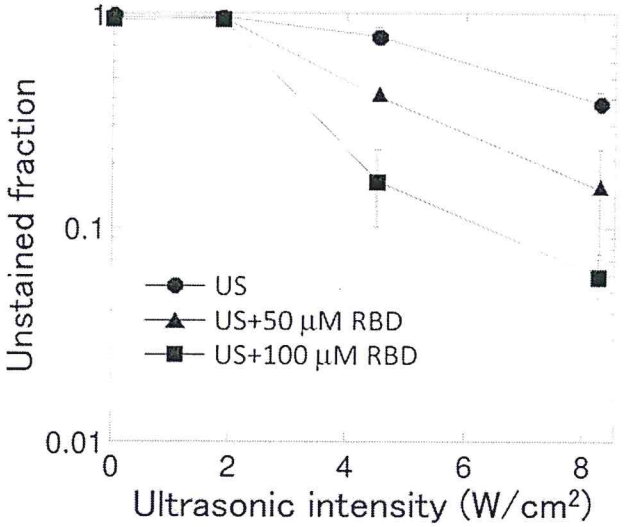

Figure 4. *Unstained fraction of isolated sarcoma 180 cells after 30 s exposure as a function of ultrasound (US) intensity. Cells were treated with US alone, 50 μM RBD plus US, and 100 μM RBD plus US.*

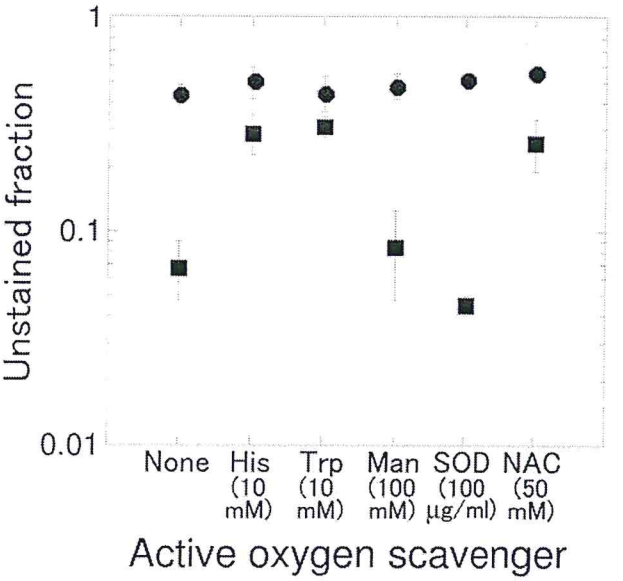

Figure 6. *Effect of active oxygen scavengers on cell damage in the presence (squares) and absence of 100 μM RBD (circles). Unstained fractions after 30 s exposure are plotted.*

The unstained fractions in the presence of RBD after the 30 s exposure at an ultrasonic intensity of 8.3 W/cm^2, are plotted for RBD concentrations of 0, 10, 50, and 100 μM in Figure 5. The unstained fractions plotted on a logarithmic scale decreased with RBD concentration primarily in a linear manner.

Effect of active oxygen scavengers. The effect of active oxygen scavengers on the ultrasonically induced *in vitro* cell damage with and without 100 μM RBD was tested. The unstained fractions, in the presence and absence of 10 mM His, 100 μg/ml SOD, 100 mM Man, 10 mM Trp, and 50 mM NAC are compared in Figure 6. The ultrasonically induced cell damage enhanced by RBD was significantly reduced by His, Trp, and NAC, but was not reduced by using either SOD or Man. On the other hand, the cell damage by ultrasound alone was not reduced by any of these scavengers.

Morphological observation. Cellular morphology with polarization micrograph after trypan blue staining is shown in Figure 7. Sarcoma 180 cells treated with RBD and ultrasound

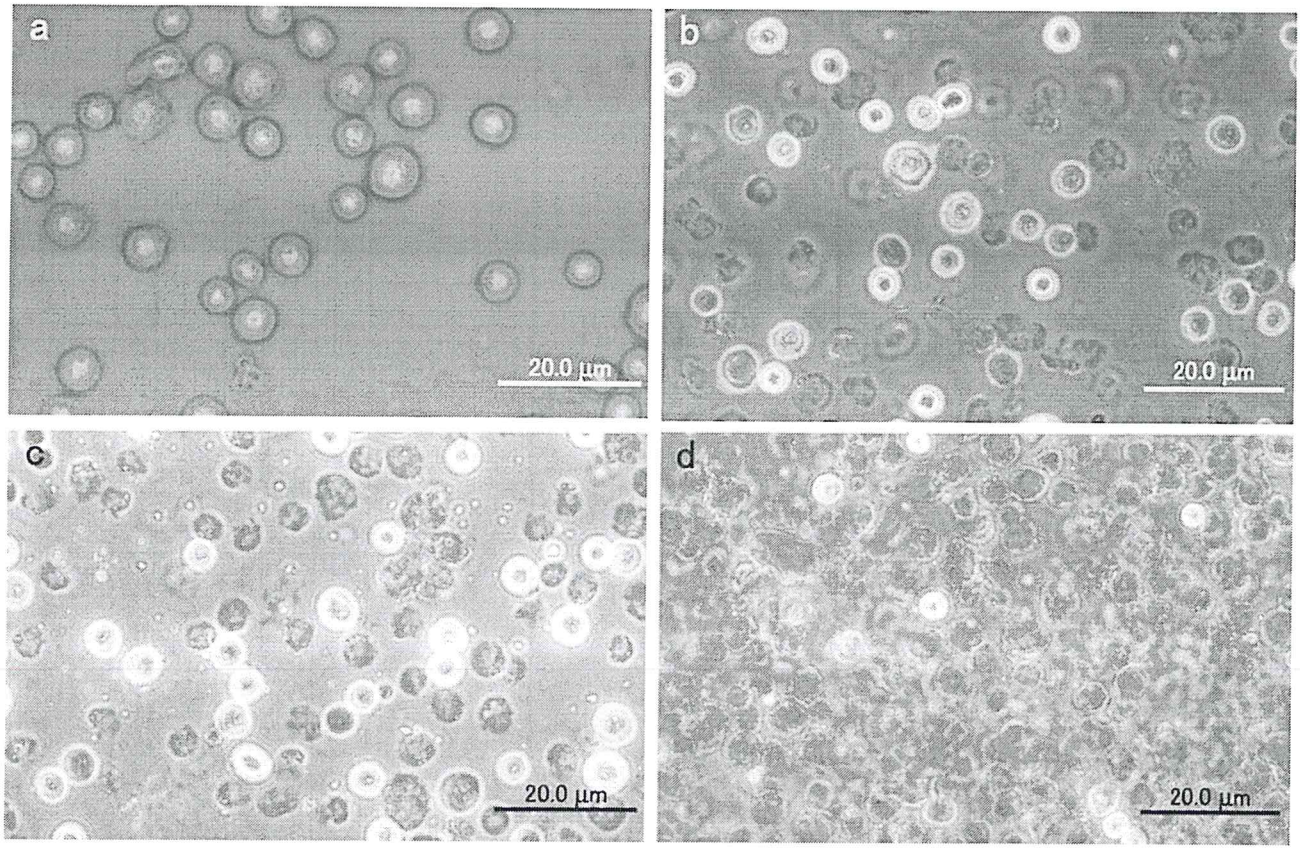

Figure 7. *Cellular morphology with polarization micrograph after trypan blue staining. a: Control; b: ultrasound (US) alone; c: RB plus US; and d: RBD plus US.*

clearly showed membrane destruction and cell lysis and aggregation, while slight morphological change was observed in the cells exposed to ultrasound alone. The morphology of cells treated with RB and ultrasound showed a tendency similar to those exposed to ultrasound in terms of cell destruction. However, the results for cell lysis and aggregation showed an average tendency between those treated with RBD plus ultrasound and those exposed to ultrasound alone.

Discussion

Ultrasonically induced *in vitro* cell damage was significantly increased by using RBD. The effect on cell damage with 100 µM RBD was one order of magnitude higher than that with the same concentration of RB after a 60 s ultrasound exposure (Figure 3). The molar concentration of RBD required for a similar enhancement factor was a half concentration of the RB required (Figure 3 and 5).

The cell damage increased with ultrasonic intensity above the threshold (Figure 4). This intensity dependence is typical for a phenomenon arising from acoustic cavitation. A threshold reduction in the presence of RBD was not observed, this was probably because this series of experiments was came out in standing wave mode rather than in progressive wave mode. The cavitation threshold in standing wave mode is known to be low, even without the use of chemical agents such as RB and RBD.

The enhancement by RBD was significantly inhibited by the active oxygen scavengers His, Trp, and NAC. The scavenger His is known to react with singlet oxygen (28) and possibly reacts with hydroxyl radical, and Trp is also reported as a scavenger of singlet oxygen and superoxide radical. Furthermore, NAC has been used as an antioxidant. It is reported to react with hydrochlorous acid and hydroxyl radical, and also reacts with H_2O_2 slowly (29). Thus, the significant reduction by His, Trp, and NAC suggests that the enhancement resulted from the ultrasonic generation of active oxygen by RBD. This result may further suggest that the increase in the ultrasonically induced cytotoxic effect by RBD was induced sonochemically. A concentration of Man of 10 mM, which is one-tenth of the concentration used in the experiments above, has been verified under the same

acoustic conditions to be high enough to inhibit iodine release from potassium iodine solution mediated by hydroxyl radicals (10, 30). Thus, the fact that enhancement in ultrasonically induced cell damage by RBD was not significantly affected by the presence of 100 mM Man but was significantly reduced by the presence of His may imply that ultrasonically generated singlet oxygen, not hydroxyl radicals, is an important mediator of the enhancement by RBD. Since SOD showed no significant effect either, superoxide radical may also be less important than singlet oxygen as the mediators. The same hypothesis of singlet oxygen as the mediator has also been proposed for ultrasonically induced cell damage increased by porphyrins, anthracyclines, and RB (10, 11, 15, 23, 26, 27).

In morphological observation, sarcoma 180 cells treated with RBD and ultrasound showed cell lysis and aggregation. Ultrasound exposure induces aggregation of the cells at a position of a node or an anti-node in standing wave mode. Moreover, a significant amount of cell aggregation was observed with RBD and ultrasound. Zheng *et al.* reported that the lipophilicity of photosensitizer was related to uptake and efficacy (31). RBD is a tumor-accumulative derivative which becomes lipophilic by the addition of an alkyl group to RB (25). It is considered that in RBD, the tumor-accumulative property increases, thus the affinity to tumor cells also becomes higher. The high affinity of RBD to the cells is expected to produce the cell lysis and aggregation, and, furthermore, we considered that this affinity causes the significant effect of RBD on cell damage.

In conclusion, the increase of ultrasonically induced *in vitro* cell damage was demonstrated with RBD. The ultrasonically induced cell-damaging rate with RBD was one order of magnitude higher than that with same concentration of RB. Chemical modification of RB to RBD for tumor accumulation significantly increased the sonodynamically induced antitumor effect. The enhancement effect by RBD was suppressed by His, Trp, and NAC but not by Man or SOD. A hypothesis can be suggested that the *in vitro* cell damage enhancement was mediated primarily *via* active oxygen, most likely singlet oxygen, generated by ultrasonically activated RBD. Thus, RBD may be potentially useful as a tumor-selective sensitizer for ultrasound therapy. Further *in vitro* and *in vivo* experiments are currently underway to find a way to treat cancer by using the synergistic effect of RBD and ultrasound.

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