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Sonodynamic therapy using 5-aminolevulinic acid enhances the efficacy of bleomycin

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ABSTRACT

Sonodynamic therapy (SDT) kills tumor cells through the synergistic effects of ultrasound and a sonosensitizer agent. We examined whether 5-aminolevulinic acid (5-ALA)-based SDT at 1 or 3 MHz could enhance the cytotoxicity of bleomycin (BLM) toward mouse mammary tumor cells both *in vitro* and *in vivo*. At 1 MHz, cell viability in the 5-ALA-based SDT group at 1, 2, and 3 W/cm^2 was 34.30%, 50.90%, and 60.16%, respectively. Cell viability in the 5-ALA-based SDT + BLM group at 1, 2, and 3 W/cm^2 was 0.09%, 0.32%, and 0.17%, respectively. In contrast, at 3 MHz, 5-ALA-based SDT + BLM did not show pronounced cytotoxicity. In the *in vivo* study, 5-ALA-based SDT + BLM was significantly more cytotoxic than 5-ALA-based SDT at 1 MHz and 3 MHz. These findings suggest that the mechanism of tumor shrinkage induced by 5-ALA-based SDT + BLM might involve not only direct cell killing, but also vascular shutdown. Thus, we show here that 5-ALA-based SDT enhances the efficacy of BLM both *in vitro* and *in vivo*.

1. Introduction

In photodynamic therapy (PDT), excited photosensitizers (PSs) contribute to the generation of reactive oxygen species (ROS), resulting in oxidative damage to intracellular macromolecules and ultimately cell death [1,2]. The therapeutic effects of PDT might be attributable to direct cytotoxicity, vascular damage, and immunological responses [1,3]. However, the depth to which the light used in PDT can penetrate the tissue is limited [4]; for example, penetration is only 3–8 mm for wavelengths of 630–800 nm [5]. Thus, it is difficult to treat deep-seated tumors using PDT.

For deep-seated tumors, sonodynamic therapy (SDT), which can penetrate deeper than PDT, has emerged as an alternative. SDT kills tumor cells through the synergistic effects of ultrasound (US) and a sonosensitizer agent. US at 1 MHz is absorbed primarily by tissue at a depth of 3–5 cm, while US at 3 MHz is recommended for more superficial lesions [6]. PSs such as porphyrins, chlorins, and phthalocyanines used in clinical PDT have been extensively studied for use in SDT [7]. These sensitizers give rise to either the singlet oxygen or hydroxyl radicals in the presence of an acoustic field, and these agents then mediate the cytotoxic effects observed in SDT. The prodrug 5-aminolevulinic acid (5-ALA) is a protoporphyrin IX (PpIX) precursor. Many studies have demonstrated the generation of ROS by 5-ALA induced-PpIX when exposed to US, and 5-ALA-based SDT might be a potential therapeutic modality for the management of malignant tumors. However, these results showed that 5-ALA-based SDT has limited effectiveness against malignant tumors [8,9].

On the other hand, SDT could be useful in enhancing the efficacy of the current treatments for malignant tumors. For example, bleomycin (BLM) is a water-soluble antibiotic used as a chemotherapeutic agent. However, the plasma membrane limits BLM uptake; therefore, a number of different methods have been used to permeabilize the cell membrane, including treatment with lysophosphatidylcholine [10], streptolysin-O [11], and electroporation [12,13]. However, these treatments only increased cellular uptake by 2- to 4-fold [10]. In this case, the sonosensitizer-derived radicals generated by SDT could destabilize the cell membrane, thereby rendering the cell more susceptible to US-enhanced drug transport into the cell [7].

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We reported that aluminum phthalocyanine disulfonate (AlPcS2a)-based SDT enhanced the efficacy of BLM [14]. However, there are no reports whether SDT based on other PSs enhances the efficacy of BLM. In this study, we examined whether 5-ALA-based SDT at 1 or 3 MHz could enhance the cytotoxicity of BLM toward mouse mammary tumors both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Ethics statement

Animal use and procedures were approved by the Animal Research Committee of Tottori University (project number: 14-T-26). The study was conducted in accordance with the Institute of Laboratory Animal Resources guidelines for the use of experimental animals.

2.2. Cell line and culture conditions

EMT-6 mouse mammary tumor cells (supplied by Dr. Shin-ichiro Masunaga, Kyoto University, Japan) were maintained as an adherent monolayer culture in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and PSN (5 mg/mL penicillin, 5 mg/mL streptomycin, and 10 mg/mL neomycin; Invitrogen), and were incubated in 5% CO_2 at 37 °C.

The cells were harvested from near-confluent cultures by brief exposure to a solution containing 0.25% trypsin and 1 mmol/L EDTA·4Na solution with phenol red (Invitrogen). Trypsinization was stopped using RPMI 1640 containing 10% fetal bovine serum. The cells were centrifuged and re-suspended in RPMI 1640. Trypan blue staining was used to assess cell viability.

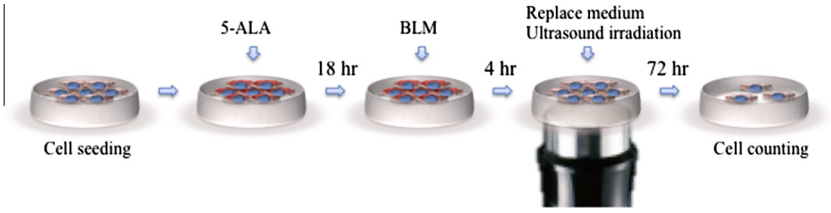

Fig. 1. *In vitro* experimental schemes. The gap between the culture dish and the probe was filled with echo gel.

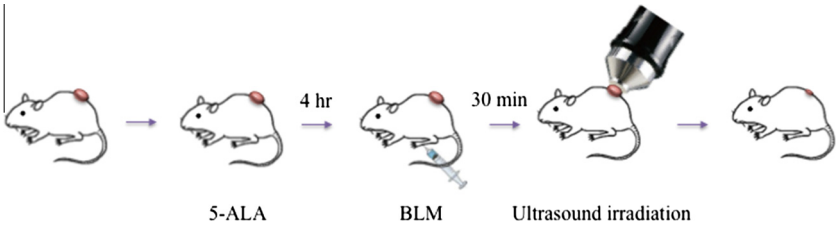

Fig. 2. *In vivo* experimental schemes. The gap between the tumor and the probe was filled with echo gel.

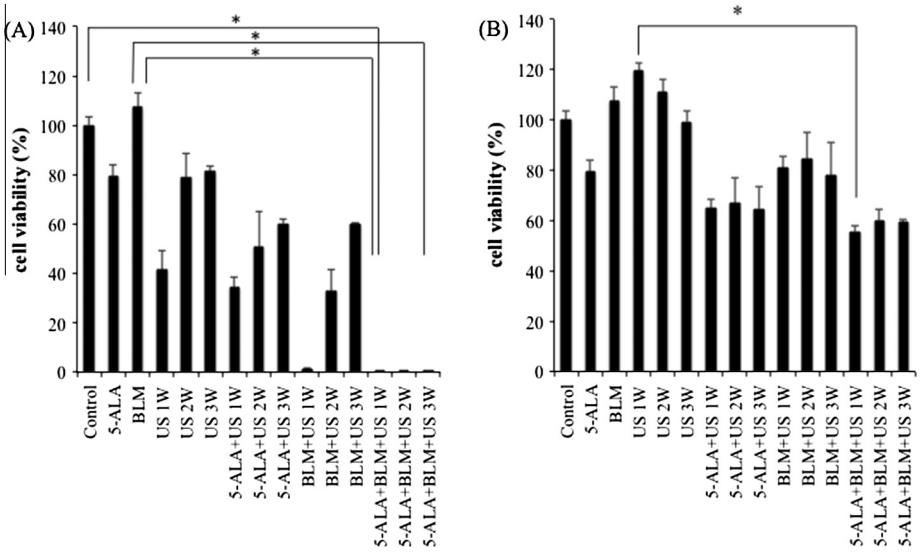

Fig. 3. Ultrasound (US) intensity-dependent cytotoxicity induced by sonodynamic therapy with bleomycin (BLM). EMT-6 cells were incubated with 1 mM 5-ALA for 18 h followed by 4 h in 5 μg/mL BLM. After washing with fresh medium, the cells were exposed to US at 1 MHz (A) or 3 MHz (B) frequency, an intensity of 0, 1, 2, or 3 W/cm^2, and a duty cycle of 20% for 60 s. Subsequently, the cells were re-incubated at 37 °C for 72 h in the dark. Following incubation, cells were harvested and prepared for cell counting. (*$p < 0.05$). Error bars show the standard deviation ($n = 3$).