Ultrasonics Sonochemistry 28 (2016) 161–168

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Bleomycin enhances the efficacy of sonodynamic therapy using aluminum phthalocyanine disulfonate

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 12 July 2015
Accepted 14 July 2015
Available online 14 July 2015

Keywords:
Bleomycin
Disulfonated aluminum phthalocyanine
Sonodynamic therapy
Ultrasound

ABSTRACT

Sonodynamic therapy (SDT), or ultrasound combined with sonosensitization, is a promising approach because it is noninvasive and penetrates deeper than light does in photodynamic therapy. We examined whether bleomycin (BLM) could improve the efficacy of SDT. We performed an *in vitro* study using Colon-26 cells, which are derived from mouse colon cancer. SDT with BLM was significantly more cytotoxic than SDT alone both *in vitro* and *in vivo*. We also observed an ultrasound intensity-dependent cytotoxic effect of SDT with BLM. These findings suggest that SDT with BLM might provide a novel noninvasive treatment for deep-seated tumors.

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 tissue is limited [4], as penetration is only 3–8 mm for wavelengths of 630–800 nm [5]. Accordingly, it is difficult to treat deep-seated tumors with PDT.

Sonodynamic therapy (SDT) kills tumor cells through the synergistic effects of ultrasound (US) and a sonosensitizer agent, and it can penetrate deeper than PDT. US with a frequency of 1 MHz is absorbed primarily by tissue at a depth of 3–5 cm, while a frequency of 3 MHz is recommended for more superficial lesions [6]. PSs such as porphyrins, chlorins, and phthalocyanines have been extensively studied for use in SDT [7], as has aluminum phthalocyanine disulfonate ($AlPcS_{2a}$), which has been shown to accumulate in the inner leaflet of endocytic vesicles [8].

Bleomycin (BLM) is a water-soluble antibiotic used as a chemotherapeutic agent. However, the plasma membrane limits BLM uptake; accordingly, a number of different methods have been used to permeabilize the cell membrane, including treatment with lysophosphatidylcholine [9] and streptolysin-O [10], and electroporation [11,12]. However, these treatments only increased cellular uptake by 2- to 4-fold [9]. However, SDT might induce the generation of sonosensitizer-derived radicals, which may lead to destabilization of the cell membrane, thereby rendering the cell more susceptible to US-enhanced drug transport into the cell [7].

In this study, we examined whether BLM could enhance the cytotoxicity of $AlPcS_{2a}$-based SDT for 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:

Abbreviations: $AlPcS_{2a}$, disulfonated aluminum phthalocyanine; BLM, bleomycin; PCI, photochemical internalization; PDT, photodynamic therapy; PSs, photosensitizers; ROS, reactive oxygen species; SDT, sonodynamic therapy; US, ultrasound.

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http://dx.doi.org/10.1016/j.ultsonch.2015.07.013
1350-4177/

13-T-1). The study is in accordance with the Institute of Laboratory Animal Resources guidelines for the use of experimental animals.

2.2. Cell line and culture conditions

Colon-26 cells (murine colon cancer cells; RIKEN cell bank, Tsukuba, 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 incubated in 5% CO_2 at 37 °C.

The cells were harvested from near-confluent cultures with 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.

2.3. Chemicals

$AlPcS_{2a}$ was provided by Frontier Scientific, Inc. (Logan, UT), and a stock solution of 500 μg/mL in phosphate-buffered saline was kept at 4 °C. BLM (PubChem CID: 5360373; Bleo, Nippon Kayaku, Tokyo, Japan) was dissolved in 5 mL 0.9% NaCl and adjusted to 1 mg/mL.

2.4. BLM toxicity

Colon-26 cells were incubated with BLM (0, 1, 5, and 10 μg/mL) for 4 h. After being washed with fresh medium, the cells were re-incubated at 37 °C for 72 h in the dark. Following incubation, the cells were harvested and prepared for counting.

2.5. US exposure

To expose tumor cells to US *in vitro*, culture dishes were placed above a probe, and the gap between the culture dish and the probe was filled with echo gel (ITO Physiotherapy & Rehabilitation, Tokyo, Japan; Fig. 1A). We used a 3 MHz US generator (UST-770, ITO Physiotherapy & Rehabilitation) with a focus US transducer that was a single, 35 mm-diameter circular disk. The US generator offers duty cycles (i.e., the proportion of the pulse period in which US is generated) of 5%, 10%, 20%, 50%, and 100%.

2.6. $AlPcS_{2a}$ dose-dependent cytotoxicity induced by SDT with BLM

We seeded 5×10^4 Colon-26 cells into 35 mm-diameter Petri dishes (Nunc, Ltd., Roskilde, Denmark) containing 2 mL of cultivation medium for 24 h. The dishes were then divided into four groups: (1) no treatment (control group); (2) 1, 5, and 10 μg/mL $AlPcS_{2a}$ alone ($AlPcS_{2a}$ group); (3) sonication with 0, 1, 5, and 10 μg/mL $AlPcS_{2a}$ ($AlPcS_{2a}$ + US [SDT] group); and (4) sonication with 0, 1, 5, and 10 μg/mL $AlPcS_{2a}$ and 5 μg/mL BLM (SDT + BLM group). During *in vitro* work with $AlPcS_{2a}$, the cells were protected from light.

Colon-26 cells were incubated with $AlPcS_{2a}$ for 18 h followed by 4 h in BLM. After washing in fresh medium, the cells were exposed to US (except for groups 1 and 2). Tumors were sonicated with a 3 MHz frequency, a 3 W/cm^2 power intensity, and a 20% duty cycle 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 counting.

2.7. US duty cycle and intensity-dependent cytotoxicity induced by SDT with BLM

Cells (5×10^4) were seeded as described above. Afterward, the dishes were divided into five groups: (1) no treatment (control group); (2) sonication without $AlPcS_{2a}$ (US group); (3) sonication with 10 μg/mL $AlPcS_{2a}$ (SDT group); (4) sonication with 5 μg/mL BLM (BLM + US group); and (5) sonication with 10 μg/mL $AlPcS_{2a}$ and 5 μg/mL BLM (SDT + BLM group).

The cells were then incubated with $AlPcS_{2a}$ followed by BLM, as described above. The cells were then exposed to US (except for group 1). Tumors were sonicated with a 3 MHz frequency, a 1, 2, and 3 W/cm^2 power intensity, and a 10% or 20% duty cycle 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.

2.8. Irradiation time-dependent cytotoxicity induced by SDT with BLM

Cells (5×10^4) were seeded as described above. The cells were then incubated with 10 μg/mL $AlPcS_{2a}$ for 18 h followed by 5 μg/mL BLM for 4 h in the dark. The cells were exposed to US with a 3 MHz frequency, 3 W/cm^2 power intensity, and 20% duty cycle for 0, 5, 10, 30, and 60 s. Subsequently, the cells were re-incubated at 37 °C for 72 h in the dark. Cells were then harvested and prepared for cell counts. Untreated tumor cells were used as a control.

2.9. Cell viability

Cellular cytotoxicity was determined using Muse™ Count & Viability Kit (EMD Millipore Co., Billerica, MA) according to manufacturer instructions. Cells were trypsinized 72 h after sonication and co-incubated with Muse™ Count & Viability Reagent at room temperature for 5 min in the dark. Single-cell suspensions were loaded onto the instrument, and cell viability was measured (100 × average of test group/average of control group).

2.10. Morphological changes in Colon-26 cells

Cells (5×10^4) were seeded as described above. The dishes were then divided into five groups: (1) no treatment (control group); (2) sonication without $AlPcS_{2a}$ (US group); (3) sonication with 10 μg/mL $AlPcS_{2a}$ (SDT group); (4) sonication with 5 μg/mL BLM (BLM + US group); and (5) sonication with 10 μg/mL $AlPcS_{2a}$ and 5 μg/mL BLM (SDT + BLM group). Subsequently, the cells were re-incubated at 37 °C for 5 h in the dark. Following incubation, cells were harvested and prepared for cell assessment.

The cells were stained with 1 mM bisbenzimidazole (Hoechst dye 33342) for 15 min at room temperature. Nuclear morphology was examined using an Olympus BX51 microscope (Olympus, Tokyo, Japan) 5 h after sonication.

2.11. Annexin V apoptosis test

Cells (5×10^4) were seeded as described above. Afterward, the dishes were divided into seven groups: (1) no treatment (control group); (2) 10 μg/mL $AlPcS_{2a}$ ($AlPcS_{2a}$ group); (3) 5 μg/mL BLM (BLM group); (4) sonication at 3 MHz frequency with a 3 W/cm^2 power intensity and a 20% duty cycle for 60 s (US group); (5) sonication with 10 μg/mL $AlPcS_{2a}$ (SDT group); (6) sonication with 5 μg/mL BLM (BLM + US group); and (7) sonication with 10 μg/mL $AlPcS_{2a}$ and 5 μg/mL BLM (SDT + BLM group). The cells were then re-incubated at 37 °C for 5 h in the dark and harvested and prepared for cell assessment.