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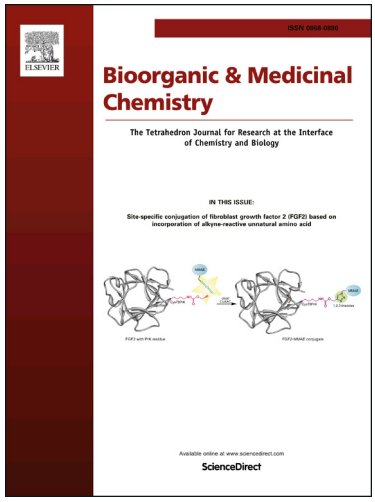

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Ultrasound-dependent cytoplasmic internalization of a peptide-sonosensitizer conjugate

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ABSTRACT

A method to induce cytoplasmic peptide delivery, using ultrasound, was demonstrated using a molecular conjugate of a cell-penetrating peptide (CPP), a functional peptide, and a sonosensitizer. As a model of such molecular conjugates, TatBim-RB, consisting of the Tat CPP, the Bim apoptosis inducing peptide, and the sonosensitizer rose bengal was synthesized. CPPs have been widely used for intracellular delivery of various cargos; however, CPP-fused molecules tend to become entrapped in endosomes, as was observed for TatBim-RB molecules applied to cells. To promote escape of the entrapped TatBim-RB molecules, cells were irradiated with ultrasound, which successfully induced endosomal escape and cytoplasmic dispersion of TatBim-RB, and subsequently apoptosis. Our results suggest that this peptide-sonosensitizer conjugate strategy may facilitate numerous kinds of medicinal chemistry studies, and furthermore, this specific conjugate may exhibit potential as a novel therapeutic agent for the promotion of apoptosis.

1. Introduction

Recently, the fusion of peptides and proteins to cell-penetrating peptides (CPPs) to facilitate their transport into cells has been widely used for therapeutic and biological purposes.[1,2] However, this strategy is often limited by the inefficient transfer of the CPP-fused peptides and proteins to the cytosol consequent to their endosomal entrapment.[3] One of the methods to overcome this problem is to use photosensitizers and light to mediate endosomal escape.[4,5] In this method, the light causes the entrapped photosensitizer to generate reactive oxygen species (ROS), which disrupts the surrounding endosomal membrane. For example, we previously designed a TatBim-Alexa molecule,[6] comprising a conjugate of Tat CPP from the HIV-1 transactivator of transcription (TAT) protein,[7] the BH3 domain derived from Bim apoptosis-inducing protein,[8,9] and the Alexa Fluor 546 dye as a photosensitizer. TatBim-Alexa molecules enter cells by the endocytic pathway, are entrapped in endosomes, and then escape from the endosomes and induce apoptosis by photoirradiation. Similar fusion molecules, such as TatU1A-Alexa and TatU1A-DY750 for photo-dependent cytoplasmic RNA delivery, were also reported.[10–13] However, the poor tissue penetration of light limits the application of these photosensitizing molecules. Alternatively, ultrasound (US) represents a promising substitute for light as an external stimulus because of its deeper penetrating property. The high tissue-penetrating ability has prompted extensive evaluations of US for medical purposes.[14,15] Recently, high-intensity, focused ultrasound has attracted attention because it can specifically irradiate a target tissue and provides a potential noninvasive therapeutic strategy.[16]

Sonosensitizers are known as molecules that generate ROS in a US-dependent manner. Sonosensitizers include inorganic materials (*e.g.* titanium dioxide)[17] and organic dyes (*e.g.* porphyrin derivatives and rose bengal).[18–20] In the current study, a sonosensitizer- and CPP-fused functional peptide was developed as a molecule exhibiting US-dependent intracellular function. As an example of this design, TatBim-RB, containing the Tat CPP, the Bim apoptosis-inducing peptide, and the sonosensitizer rose bengal was synthesized. The use of a sonosensitizer and US instead of a photosensitizer and light was attempted to facilitate endosomal escape of the CPP-fused functional peptide (Fig. 1).

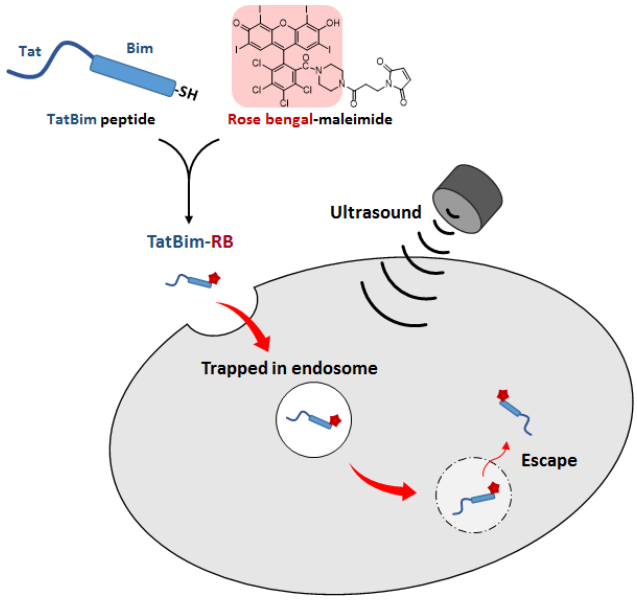

Figure 1. Conceptual diagram of US-dependent cytoplasmic internalization of TatBim-RB.

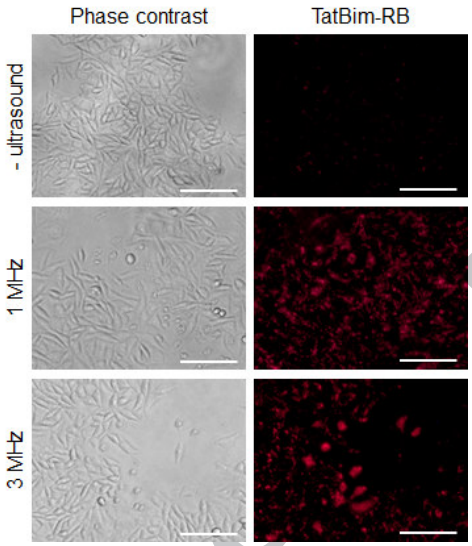

Figure 2. Phase contrast and TatBim-RB fluorescence images immediately after US irradiation. Cellular images in the middle of the irradiated area are shown. TatBim-RB was imaged by rose bengal fluorescence. CHO cells were treated with TatBim-RB and irradiated with 1 or 3 MHz pulsed US (30% duty cycle) at 0.5 W/cm^2 for 15 min. Scale bars indicate 100 μm.

2. Results and Discussion

2.1. US-dependent internalization of TatBim-RB

TatBim-RB was synthesized by reacting TatBim bearing a Cys residue at the C-terminus with rose bengal maleimide. After the purification, the majority of free rose bengal maleimide was removed from TatBim-RB (Fig. S1 in Supporting Information). To attempt US-dependent internalization of TatBim-RB, Chinese hamster ovary (CHO) cells were treated with 2 μM TatBim-RB followed by US as described in Materials and Methods (Fig. 2). Without US irradiation, low levels of TatBim-RB were detected in the cells with a dotted localization pattern, indicating that the TatBim-RB was entrapped in endocytotic compartments as previously shown using a similar Tat-fused molecule.[11] Tat-fused peptide and protein tends to enter cells by macropinocytosis.[21,22] After US irradiation, stronger and diffuse fluorescence of TatBim-RB was observed in the cells, indicating that TatBim-RB molecules had escaped from the endocytotic compartments and diffused into the cytosol. The increase of TatBim-RB fluorescence was probably due to the release from the concentration quenching and the pH increase upon relocalization from endosomes to the cytoplasm, as the rose bengal fluorescence ratio (pH 7.6/ pH 5.2) was 1.4. Both of 1 MHz and 3 MHz US induced cytoplasmic internalization of TatBim-RB by the cells. These frequencies are included in the common frequency range of ultrasonography, which has already been applied in the clinic. In general, the depth of penetration of US depends on the frequency.[23] For example, the penetration property of 10 MHz is 3.5-fold higher than that of 20 MHz. Hence, we chose a lower frequency (1 MHz) for the following experiments. Maximum tissue penetration ability of 1 MHz US was reported to be about 80 cm.[24]

2.2. Detection of apoptosis after the treatment with TatBim-RB and US

Next, we evaluated apoptosis induced by TatBim-RB and US (Fig. 3). CHO cells were treated with TatBim-RB and irradiated with US. At 8 h after the irradiation, apoptotic cells were stained with NucView488. Apoptosis was detected in the cells treated with TatBim-RB followed by US but not in the cells treated with TatBim-RB only. Therefore, TatBim-RB at this concentration (2 μM) induced apoptosis US-dependently. TatBim-RB images indicated the US-dependent cytoplasmic dispersion of TatBim-RB, as observed in Figure 2, although the timing of the imaging differed, with the images in Figure 2 being obtained immediately after the irradiation whereas those in Figure 3 were obtained at 8 h after the irradiation. US did not induce apoptosis in the absence of TatBim-RB or in the presence of TatBim peptide without conjugated rose bengal. In addition, we investigated the effects of US intensity toward the cellular internalization of TatBim-RB (Fig. S2 in Supporting Information). As expected, US at stronger irradiation induced a higher efficiency of apoptosis. Direct cytotoxic effect of TatBim-RB/US-induced ROS, independent of Bim activity, was estimated by trypan blue staining, which suggested that this effect was not apparently shown (Fig. S3 in Supporting Information). Furthermore, as a previous report showed that cell viability was decreased by US irradiation in the presence of 100 μM rose bengal but not by US in the presence of 10 μM rose bengal,[25] we expected that the Bim-independent damage was not likely to be apparent in our experimental conditions using 2 μM TatBim-RB but may appear in conditions using higher TatBim-RB concentrations.

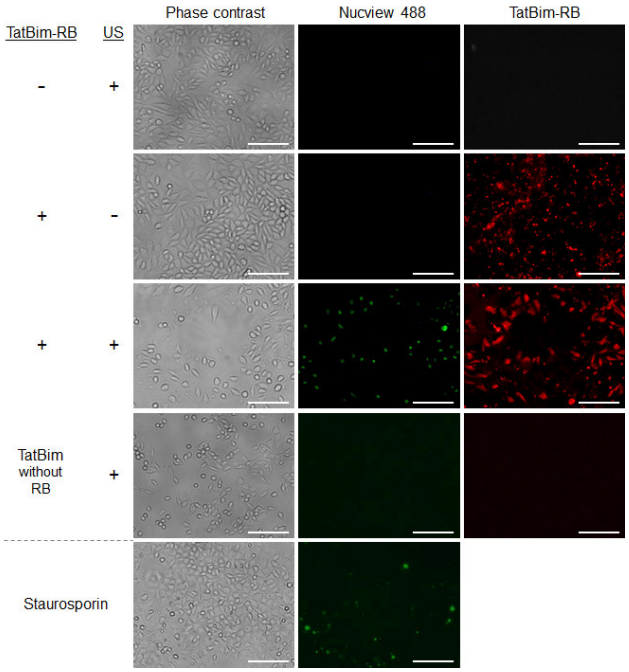

Figure 3. Apoptosis induction in CHO cells. Cellular images were captured at 8 h after US irradiation (1 MHz, 0.5 W/cm^2, duty cycle 30%, 15 min). Apoptosis was detected using a NucView 488 Caspase-3 Assay Kit. To avoid photochemical damage of the cells by rose bengal excitation, TatBim-RB images were obtained after the apoptosis imaging. Scale bars indicate 100 μm.

2.3. Mechanism of US-independent TatBim-RB endosomal escape

A fundamental question related to this study is the means by which US induces the endosomal escape of TatBim-RB. A minimal answer may be that US induces ROS generation in the presence of TatBim-RB and the endosomal membrane might be disrupted by the attack of ROS generated from the endosomally entrapped TatBim-RB. We confirmed the US-dependent generation of ROS in the presence of rose bengal using 2', 7'-dichlorodihydrofluorescein (H_2DCF) (Fig. 4a); additionally, a previous report has also shown the same phenomenon using 1,3-diphenylisobenzofuran.[25] US-induced ROS was hardly generated in the presence of TatBim peptide (data not shown). In a photochemical internalization strategy using photosensitizers and light, photoinduced singlet oxygen ($^1O_2$) has been reported as a main trigger for endosomal escape[4,26]. Therefore, we evaluated US-induced $^1O_2$ generation using singlet oxygen sensor green (SOSG) (Molecular Probes, Eugene, OR, USA); however, rose bengal-dependent $^1O_2$ generation was barely detected under US irradiation (Fig. S4 in Supporting Information). This indicated that US with rose bengal induced the generation of ROS that were reactive with H_2DCF, although the generated ROS were mainly of types other than $^1O_2$.

It should be noted that US-induced ROS generation was dependent on the materials used for cell culture. US-induced ROS generation in a polyethylene 96-well culture plate was much less than that on a glass-bottomed dish (Fig. 4). The difference of the US-induced ROS generation between these substrates appeared to be due to differences of the reflection coefficients of the water-polyethylene (7.7%) and water-glass (80%) interfaces, which were estimated using an established equation.[27] This difference of ROS generation was coordinate with the results of the cellular TatBim-RB internalization experiments (Fig. S5 in Supporting Information). Specifically, US-dependent TatBim-RB internalization and apoptosis were not observed in a polyethylene 96-well culture plate whereas both were observed when a glass-bottomed dish was used and the cell-culture solution was applied only on the glass region.

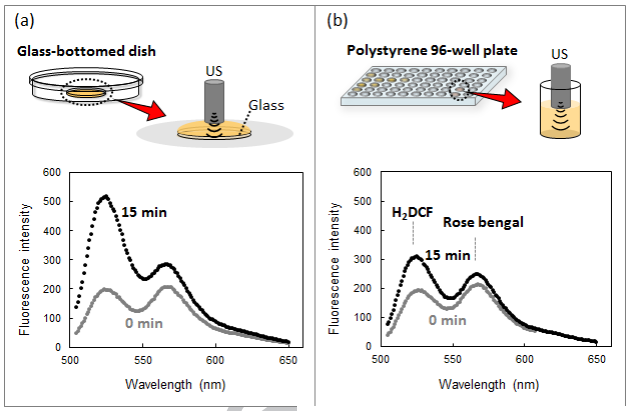

Figure 4. US-induced generation of ROS in the presence of rose bengal. ROS was detected using the fluorescent ROS indicator H_2DCF on a glass-bottomed dish **(a)** and in a 96-well plate **(b)**.

2.4. Contribution of sonoluminescence to US-induced ROS generation

Rose bengal has been shown to act as a sonosensitizer;[25] however, its sonosensitizing mechanism has not been clarified. One possible explanation is that sonoluminescence[19,28] generated by the US energy might cause excitation of rose bengal and initiate a photochemical process resulting in ROS generation. To address this possibility, sonoluminescence was measured and its intensity was compared to the light intensity necessary for photochemical internalization of TatBim-RB by cells. In this measurement, the rose bengal concentration and US conditions were similar to the previous cellular experiments. Figure S6 in the Supporting Information shows that sonoluminescence could barely be detected, representing less than 1/1000 of the light conditions for a typical photochemical internalization, indicating that sonoluminescence does not constitute the main pathway for the US-dependent rose bengal excitation followed by ROS generation. This observation agrees with the results that singlet oxygen was also barely detected in US-irradiated rose bengal solution (Fig. S4 in the Supporting Information), because singlet oxygen would be generated if rose bengal is excited by light.[29]

3. Conclusions

This study aimed to develop an US-dependent cytosolic peptide delivery method. The US-sensitive molecule was designed such that a functional peptide of interest was conjugated with a CPP and a sonosensitizer. TatBim-RB was prepared as a conceptual model of the sonosensitizer- and CPP-fused functional peptide. TatBim-RB entered cells but was entrapped in endocytotic compartments; its escape from the compartments was subsequently facilitated by US irradiation. Thus, US treatment induced cytoplasmic TatBim-RB delivery and subsequent apoptosis. This peptide delivery method is novel, differing from the other primary US-dependent method, sonoporation, which utilizes nano- and microbubbles.[30] Sonosensitizers have been used in the study of sonodynamic therapy (SDT) for cancer treatments and are known to induce ROS-mediated cytotoxicity under US irradiation.[31] Our US irradiation conditions are not stronger than those of most SDT studies,[31] and much weaker than high-intensity focused

ultrasound (HIFU) treatments.[32] The present peptide-delivery method also utilized a sonosensitizer but is distinct from the simple cell disruption method. Furthermore, a previous report demonstrated that cell viability was decreased by US irradiation in the presence of 100 μM rose bengal but not affected by US in the presence of 10 μM rose bengal.[25] In the current study, the cells were treated with 2 μM TatBim-RB; thus, the direct cytotoxic effect of TatBim-RB/US-induced ROS, independent of Bim activity, was not high. Therefore, the present method is considered promising for *in vivo* biological studies, as it allows the delivery of functional peptides into target cells and may represent an effective alternative to conventional SDT.

4. Materials and methods

4.1. Preparation of TatBim-RB

To covalently attach rose bengal to peptides, rose bengal maleimide, in which the maleimide group can react with the thiol group of peptides, was synthesized as described.[26] The TatBim-C peptide (H-RKKRR QRRRE IWAQE LRRIG DEFNA YYARGC-NH2) was prepared by conventional Fmoc-based solid-phase peptide synthesis. TatBim-C containing a cysteine at the C-terminus was reacted with rose bengal-maleimide to generate TatBim-RB. The reaction mixture containing 10 mM HEPES-KOH (pH 7.6), 2 μM Tris (2-carboxyethyl) phosphine, 25 μM TatBim-C, and 25 μM rose bengal-maleimide was incubated at room temperature for 1 h. TatBim-RB was purified by reversed-phase HPLC (Symphonia C18 Column [4.6 × 150 mm, 5 μm particle diameter, Jasco, Tokyo, Japan]) eluted with 0.1% aqueous trifluoroacetic acid (A)/acetonitrile (B) gradient mixture (B: 0 min; 0%, 10 min; 40%, 30 min; 65%, 40 min; 100%) at a flow rate of 0.6 mL/min. The purified TatBim-RB was analyzed by SDS-PAGE. Rose bengal fluorescence in the gel was imaged using an FLA-9000 imager (Fujifilm, Tokyo, Japan) with λex= 532 nm.

4.2. US-dependent internalization of TatBim-RB

CHO cells were cultured at 37 °C under 5% CO_2 in Ham's F12 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 100 units/mL penicillin (Gibco, Gaithersburg, MD, USA), and 100 μg/mL streptomycin (Gibco). Confluent CHO cells (70–90%) were incubated in a 12-mm glass-bottomed dish (IWAKI, Japan) for 2 h at 37 °C with 2 μM TatBim-RB in T buffer (20 mM HEPES-KOH (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 13.8 mM glucose) under an atmosphere of 5% CO_2. After washing the cells twice with T buffer, the cells in T buffer were irradiated with unfocused US using a Sonitron2000V equipped with an US probe (ϕ 6 mm) (Nepa Gene, Ichikawa, Japan). The US probe was set so that the probe tip touched the surface of the cell-culture solution (Fig. 5). After the irradiation, intracellular internalization of TatBim-RB was visualized using an IX51 fluorescence microscope with a DP72 digital camera (Olympus, Japan).

4.3. Detection of apoptosis following treatment with TatBim-RB and US

CHO cells were treated with 2 μM TatBim-RB and irradiated with US, as described above. After US-irradiation followed by two washes with T buffer, the cells were incubated with Ham's F12/10% FBS medium at 37 °C for 8 h. Then, the buffer on the cells was removed and the cells were stained using a NucView 488 Caspase-3 assay kit (Thermo Fisher Scientific, Waltham, MA, USA) in T buffer at 37 °C for 30 min. After the staining, the cells were washed with T buffer and the apoptotic cells stained with NucView 488 were observed using an IX51 fluorescence microscope (λex = 470–490 nm).

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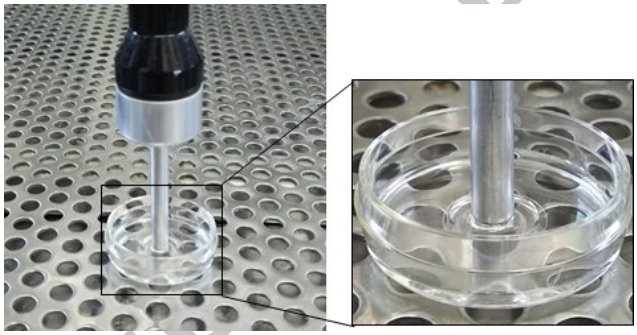

Figure 5. Setup for irradiation of the cells with US. The cells were cultured and adhered on the glass region of a glass-bottomed dish (glass diameter 12 mm). The cell-culture solution (200 μL) remained in contact only with the glass region by utilizing the surface tension. The US probe (ϕ 6-mm) of the Sonitron2000V apparatus was placed above the center of the dish. The probe tip was placed in contact with the surface of the cell-culture solution and the cells were irradiated.

4.4. Detection of ROS

Production of ROS was detected using 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Wako). H_2DCF-DA (10 mM) was first treated with NaOH (10 mM) for 30 min at room temperature to generate H_2DCF, which is a ROS indicator that can be rapidly oxidized to generate highly fluorescent 2′, 7′- dichlorodihydrofluorescein. H_2DCF (10 μM) and rose bengal (10 μM) were mixed in 100 μL T buffer and loaded on a MicroWell 96-well optical bottom plate (Nunc, Rochester, NY, USA) or on a glass (12-mm)-bottomed dish (IWAKI). The solution was irradiated with US (1 MHz, duty cycle 30%, 0.3 W/cm^2) using a Sonitron2000V equipped with an US probe (ϕ 6-mm). After irradiation, fluorescence spectra were measured at an excitation wavelength of 492 nm using an FP-6600 spectrofluorometer (Jasco).

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Graphical Abstract