

Ultrasound-dependent RNAi using TatU1A-rose bengal conjugate

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ABSTRACT

Tat-U1A-rose bengal conjugate (TatU1A-RB) was prepared as an ultrasound-sensitive RNA carrier molecule. This molecule consists of Tat cell-penetrating peptide, U1A RNA-binding protein, and rose bengal as a sonosensitizer. We demonstrated that TatU1A-RB delivered RNA via the endocytosis pathway, which was followed by ultrasound-dependent endosomal escape and cytosolic dispersion of the RNA. A short hairpin RNA (shRNA) delivered by TatU1A-RB mediated RNA interference (RNAi) ultrasound-dependently. Even by ultrasound irradiation through blood cells, RNAi could be induced with TatU1A-RB and the shRNA. This ultrasound-dependent cytosolic RNA delivery method will serve as the basis for a new approach to nucleic acid therapeutics.

Cell-penetrating peptide (CPPs)-based molecular delivery systems have been used for delivery of cargo molecules, including drugs, peptides/proteins, and nucleic acids into cells.^{1,2} However, CPP-fused cargo molecules tend to become entrapped in endosomes, which results in their inefficient transfer to cytosol.^{3,4} Photochemical internalization (PCI) using light and photosensitizers is one of the methods used to facilitate endosomal escape of the cargo molecules.⁵⁻⁷ In this method, the photosensitizer entrapped in the endosome along with the cargo generates reactive oxygen species (ROS) in response to photoirradiation, which destabilizes the surrounding endosomal membrane. For example, PCI can be mediated by TatU1A-PS, a fusion protein of Tat CPP⁸ and U1A RNA-binding protein⁹, bound to a photosensitizer, such as Alexa633 and DY750.¹⁰⁻¹² Complexes of TatU1A-PS and RNA enter cells via endocytosis, become trapped in endosomes, and escape from the endosomes by photoirradiation, which enables photo-dependent cytosolic delivery of short hairpin RNAs (shRNAs) and pre-miRNAs.^{13,14} However, the application of photo-dependent RNA delivery methods such as these is limited by the poor tissue penetration of light.

Thus, in this study, we investigated an RNA delivery method using ultrasound (US) instead of light. US is a promising alternative to light as an external stimulus because of its deeper penetrating property, which enables applications for medical purposes.¹⁵⁻¹⁷ US can induce ROS generation in the presence of sonosensitizers, such as titanium dioxide, porphyrin derivatives, and rose bengal (RB), which enables sonodynamic therapy for cancer treatment.¹⁸⁻²¹ Previously, we developed a method to

induce US-dependent apoptosis based on US-mediated cytosolic delivery of TatBim-RB composed of Tat CPP, proapoptotic Bim peptide, and sonosensitizer (RB).²² This method is not based on simple sonodynamic cell killing, but instead relies on a US-dependent endosomal escape mechanism probably related to US- and RB-mediated ROS generation. The US-dependent endosomal escape mechanism, referred to as sonochemical internalization, was initially demonstrated using the sonosensitizer AlPcS_{2a} and the anticancer agent bleomycin.^{23,24} Based on these findings, we here attempted to combine the above-described RNA carrier (Tat-U1A fusion protein) with the sonosensitizer RB for US-dependent cytosolic delivery of RNA (Fig. 1). We also tried to demonstrate US-dependent RNAi and higher US permeability through blood cells compared with light.

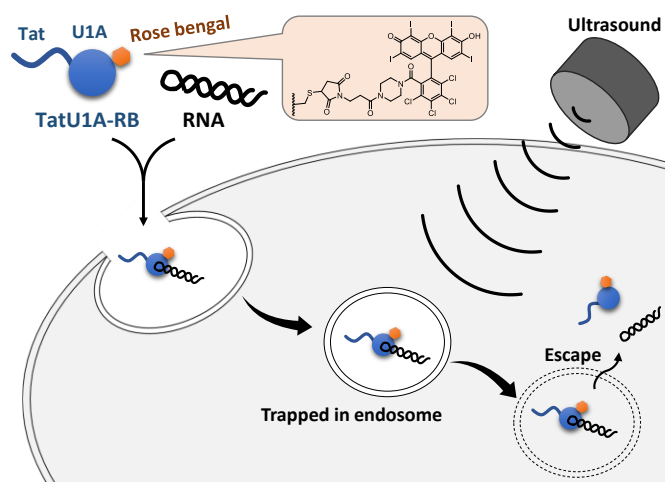


Figure 1. Conceptual diagram of US-dependent cytosolic RNA delivery.

Ultrasound-dependent cytosolic RNA delivery

We first demonstrated ultrasound-dependent cytosolic shRNA delivery with TatU1A-RB. The carrier protein TatU1A binds to a shRNA containing an U1A-binding sequence, and the TatU1A/shRNA complex is endocytosed and entrapped in an endosome.³ We assumed that TatU1A-RB/shRNA trapped in endosomes would escape from endosomes and disperse in the cytoplasm based on ROS production from the sonosensitizer RB by US irradiation. This was assumed because of the results of a previous study using TatBim-RB.²² We found that shRNA did not disperse into the cytoplasm at all after 0-5 min of US irradiation but did disperse in the cytoplasm in some of cells after 10 min irradiation, while it dispersed in the cytoplasm of many cells after 15 min irradiation (Fig. 2). Similar treatments without carrier or with TatU1A lacking the RB moiety could not induce US-dependent cytosolic shRNA release (Fig. S1).

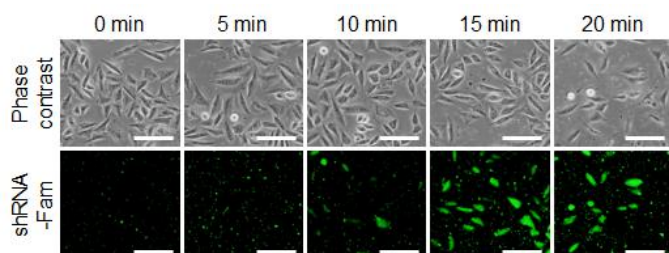


Figure 2. Phase contrast and shRNA-Fam (fluorescein) fluorescence images after US irradiation for 0-20 min. Cellular images in the middle of the irradiated area are shown. Chinese hamster ovary (CHO) cells were treated with TatU1A-RB and shRNA-Fam and irradiated with 1 MHz pulsed US (30% duty cycle) at 0.3 W/cm². Scale bars indicate 50 μ m.

Ultrasound-dependent EGFP knockdown

The RNAi efficiency was examined by evaluating EGFP knockdown in CHO-EGFP cells treated with TatU1A-RB and shGFP followed by US irradiation (Fig. 3). EGFP knockdown was not induced by US irradiation alone. In contrast, EGFP intensity was decreased to 50% in the cells treated with TatU1A-RB and shGFP and US-irradiated compared to non-treated cells. Without US irradiation, TatU1A-RB and shGFP did not significantly knock down EGFP, indicating that US induced shGFP-mediated RNAi. EGFP knockdown was dependent on shGFP because it was not

observed in the cells treated with TatU1A-RB alone or TatU1A-RB and shControl. These results (Fig. 3, in addition to Fig. 2) suggest that the US-dependent EGFP knockdown was due to US-mediated cytosolic delivery of shGFP.

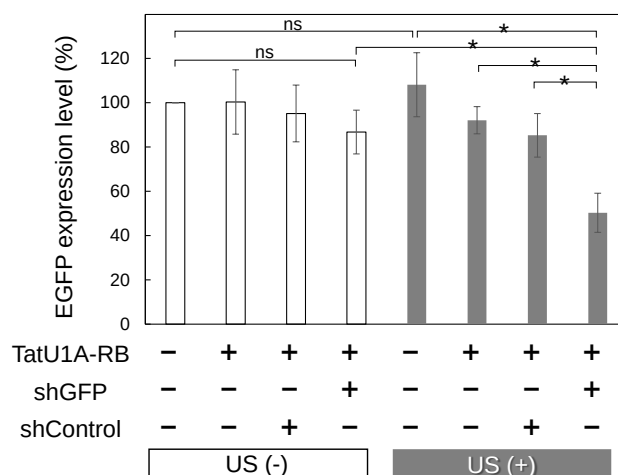


Figure 3. EGFP knockdown in the presence of TatU1A-RB/shGFP complexes. US irradiation was performed at 1 MHz, 0.3 mW/cm², 15 min, duty cycle 30%. Data represent the means $\pm$ SEM (n = 6; each of the analyzed areas included more than 100 cells). *P < 0.01, ns: not significant.

EGFP knockdown in the presence of blood cells

To demonstrate higher bio-permeability of US than light for inducing RNAi, we compared the US- and light-induced EGFP knockdown efficiencies using CHO-EGFP cells covered with blood cells. Hemoglobin contained in red blood cells absorbs visible light, especially below 600 nm, which is one of the reasons for the poor tissue penetration of light.²⁵ RNAi efficiencies were investigated by irradiating the TatU1A-RB/shGFP-treated CHO-EGFP cells with US or light through the blood cell layer. The difference between the efficiencies of light- and US-mediated RNAi is probably due to the difference between light- and US-mediated ROS generation efficiencies from RB.^{22,26} Light-dependent EGFP knockdown efficiency was quite high in the absence of blood cells, but in the presence of blood cells, EGFP knockdown was no longer detectable (Fig. 4). In contrast, US-dependent EGFP knockdown efficiency was not affected by the presence of blood cells. These results suggest that, as expected, light was absorbed by the blood cell layer, but US penetrated the blood cell layer and retained sufficient energy to cause the endosomal escape of TatU1A-RB/shGFP.

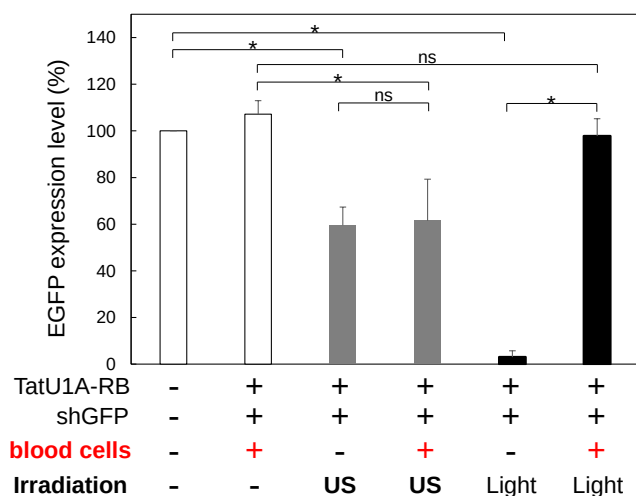
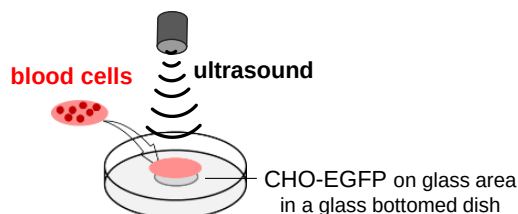


Figure 4. EGFP knockdown by light and ultrasound irradiation through blood cells. US irradiation was performed at 1 MHz, 0.5 mW/cm², 20 min, duty cycle 30%. Light irradiation was performed at 1 J/cm² (45 mW/cm² for 23 s) at 530–550 nm. Data represent the mean $\pm$ SEM (n = 3; each of the analyzed areas included more than 100 cells). *P < 0.01, ns: not significant.

Conclusion

In this study, we succeeded in US-dependent cytosolic RNA delivery. US-dependent RNAi did occur, although at a lower level than in the case of light-dependent RNAi. In addition, we demonstrated a higher permeability of US through blood cells compared to light.

Numerous efforts have been made to establish ultrasound-mediated delivery of nucleic acids, including siRNAs.^{27–30} Sonoporation method, which allows the intracellular delivery of nucleic acids through temporary pores on the cell membrane caused by acoustic cavitation, has been mainly used. This process can be enhanced using microbubbles. In principle, sonoporation is considered to be a method with low-specificity for delivery of extracellular molecules into cells. However, the present method is designed only for the delivery of RNA bound to the carrier. Since a sonosensitizer-based RNA delivery method is mechanistically different from sonoporation, they could be combined in the future.

This is the first report of US- and sonosensitizer-mediated RNAi via US-dependent cytosolic RNA delivery. Further improvement and development of the US-dependent RNAi methods based on this study will be promising for medical applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmcl.2022.128767>.

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