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Cancer treatment using an optically inert Rose Bengal derivative combined with pulsed focused ultrasound

Yoo-Shin Kim, Valentina Rubio, Jianjun Qi, Rongmin Xia, Zheng-Zheng Shi, Leif Peterson, Ching-Hsuan Tung *, Brian E. O'Neill **

Department of Radiology, The Methodist Hospital Research Institute, Weill Medical College of Cornell University, 6565 Fannin St., Houston, TX 77030, USA

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ABSTRACT

Pulsed high intensity focused ultrasound (HIFU) has been combined with a photo-insensitive Rose Bengal derivative (RB2) to provide a synergistic cytotoxicity requiring the presence of both ultrasonic cavitation and drug. *In vitro* tests have shown that a short treatment (less than 30 s) of pulsed HIFU with peak negative pressure >7 MPa (~27 W acoustic power at 1.4 MHz) destroys >95% of breast cancer cells MDA-MB-231 in suspension with >10 μM of the compound. Neither the pulsed HIFU nor the RB2 compound was found to have any significant impact on the viability of the cells when used alone. Introducing an antioxidant (N-acetylcysteine) reduced the effectiveness of the treatment. *In vivo* tests using these same cells growing as a xenograft in nu/nu mice were also done. An ultrasound contrast agent (Optison) and lower frequency (1.0 MHz) was used to help initiate cavitation at the tumor site. We were able to demonstrate tumor regression with cavitation alone, however, addition of RB2 compound injected i.v. yielded a substantial synergistic improvement.

1. Introduction

Ultrasound has been used successfully in medical imaging for many years because it is safe, real-time, portable, noninvasive, and relatively inexpensive. High intensity focused ultrasound (HIFU) has been developed for therapeutic use, including thermal ablation of tumor tissue, known as focused ultrasound surgery (FUS) [1]. Pulsed-mode high intensity focused ultrasound (pHIFU) is a means of achieving high peak powers but limited average powers, resulting in focal deposition of mechanical energy but reduced peak temperatures. Pulsed HIFU has been used for enhanced drug delivery applications for some years now [2–5]. At extremely high powers and short duty cycles, pHIFU causes violent cavitation in tissues, resulting in tissue lysis *in vivo*, sometimes known as histotripsy [6,7]. Alternatively, low frequency ultrasound has been tested in combination with various drugs. The synergistic effect of ultrasound and drugs on cells is known as sonodynamic therapy [8–20]. The cavitation resulting from the application of high power, low frequency ultrasound in fluids is known to produce free radicals. It is generally thought that this effect acting on particular compounds in solution can result in significantly enhanced cytotoxicity. This technique is often seen as the ultrasonic analog of the clinically tested photodynamic therapy, and many researchers have used the same photosensitizers for both therapies [10,12]. However, many of the problems associated with photodynamic therapy, including side effects resulting from exposure to ambient light, could be solved by using a compound that is activated by sound (a "sonosensitizer") but not by light. The attractive feature of this modality in cancer treatment emerges from the ability to locally activate a preloaded sonosensitizer on malignancy sites buried deeply in tissues by focusing the ultrasound energy.

In this study, we have introduced and tested a potent new sonosensitive but photo-insensitive compound derived from Rose Bengal (RB). It is known that fluorescence and photosensitivity are closely related properties [21–23]. We have previously confirmed that when a photosensitizer loses its fluorescent property, it also loses its photosensitivity [24]. RB is an excellent photosensitizer and its fluorescent property can be turned off by acetylation [21] or amidation. In addition, a recent study with RB derivatives has indicated that long aliphatic lipid chains could facilitate cell and tumor association to promote the photodynamic effect [25], and demonstrated a potential role for this compound as a sonodynamic agent [26]. Thus, RB derivatives were selected for this study, aiming to find a sonosensitizer without photosensitivity. The resulting derivative (RB2) was tested *in vitro* and *in vivo* in combination with cavitation driven by pHIFU. The *in vitro* work consisted of looking for a synergistic cytotoxicity between RB2 and pHIFU treatment of a breast cancer cell line. The *in vivo* studies were designed to test for synergistic anti-tumor effects when applied to a breast cancer xenograft model.

* Corresponding author. Tel.: +1 713 441 8682.
** Corresponding author. Tel.: +1 713 441 8695.
E-mail addresses: ctung@tmhs.org (C.-H. Tung), beoneill@tmhs.org (B.E. O'Neill).

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2. Materials and methods

2.1. Synthesis of RB1 and RB2

A readily available, small molecule photosensitizer, Rose Bengal (RB), was obtained from a commercial source (Sigma-Aldrich, St. Louis MO). To prepare RB1, Rose Bengal (200 mg, 0.2 mmol) was reacted with $Br(CH_2)_{12}CH_3$ (158 mg, 0.6 mmol) in DMF (2 ml) at 80 °C overnight. The solvent was removed by evaporation, and the residue was added to ethyl acetate, stirred overnight, filtered and washed with ether. The collected solid was further stirred overnight in water, filtered and washed with water to give deep purple powder, 153 mg (66.2% yield). ^{1}H-NMR (DMSO, 300 MHz) δ 8.17 (1H, br), 7.46 (2H, s), 3.92 (2H, t, J = 6.3 Hz), 1.23–1.03 (22H, m), 0.85 (3H, t, J = 7.2 Hz). MS (ESI) m/z 1155 (M^-). To prepare RB2, Rose Bengal (300 mg) was activated by HBTU (120 m, 0.3 mmol) in DMF (5 ml) for 2 h, then NH_2-$(CH_2)_{12}CH_3$ (300 mg, 1.5 mmol, 0.3 mmol) was added and stirred overnight at room temperature. The solvent was removed by evaporation. The residue was dissolved in dichloromethane (DCM), and then purified by a silica gel column using DCM as the elution solvent, yielding white solid product, 290 mg (85.0% yield). ^{1}H-NMR (DMSO, 300 MHz) δ 7.04 (2H, s), 3.12 (2H, t, J = 7.2 Hz), 1.40–1.00 (22H, m), 0.90 (3H, t, J = 6.9 Hz). MS (ESI) m/z 1154 (M^-). The final compounds' chemical structures are shown in Fig. 1A. For comparison, a set of commercially available photosensitizers and potential sonosensitizers, hematoporphyrin IX (HP), mesoporphyrin IX (MP), protoporphyrin IX (PP), and isohematoporphyrin IX (IP), were obtained from Frontier Scientific, Inc. (Logan, UT). The compounds were dissolved in DMSO (20 mM) and stored at 4 °C until further use. N-acetylcysteine (NAC) (Sigma-Aldrich.) was chosen as a reactive oxygen species (ROS) scavenger.

2.2. In vitro initial screening

Initial screening was designed to compare the efficacy of the prepared RB derivatives and literature reported sono- and photosensitizers mesoporphyrin IX (MP), hematoporphyrin IX (HP), protoporphyrin IX (PP) and isohematoporphyrin IX (IP) in treating MDA-MB-435S and MBA-MB-231 cells in combination with ultrasound. On the day of use, the compounds were diluted with PBS. Fifteen milliliter polystyrene vials (15 mm diameter) were used, filled with 12 ml of 1% agarose (Invitrogen Corporation, Carlsbad, CA, USA). The tubes were sterilized using the UV lamp in the hood for 15 minutes prior to introducing cells. MDA-MB-231 and MDA-MB435S cells were obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were grown at 37 °C and 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM, South Logan, UT) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (100 μg penicillin/ml and 100 μg streptomycin/ml (Invitrogen). The day prior to treatment, 10×10^6 cells were seeded in 10 cm tissue culture plates and incubated over night. The day of the treatment, the medium was removed and the cells were washed twice with cold PBS. The cells were incubated in serum free medium with different concentrations of the various compounds for 30 minutes prior to ultrasound treatment. Then the cells were suspended in the same medium (non-degassed, still with the compound) and 10^6 cells/ml were aliquoted into the tubes (around 3 ml per tube) and sent to ultrasound treatment. Following ultrasound treatment, the cells were spun (1000 rpm for 5 minutes) to remove the medium and re-suspended in fresh medium with 10% FBS and antibiotics.

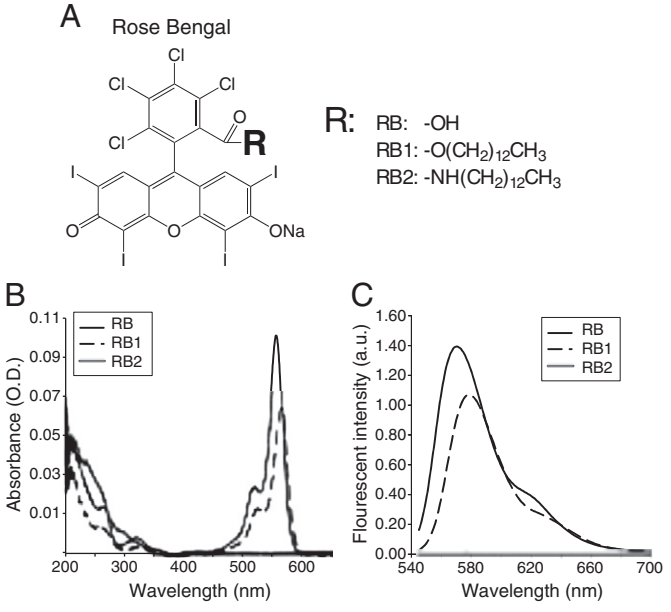

Fig. 1. Structure and UV spectra of RB, RB1 and RB2. A) RB and derivatives; B) UV absorption of 1.0 μM RB, RB1 and RB2 in MeOH; C) Fluorescence emission spectra of 1.0 μM RB, RB1 and RB2 in MeOH, λ_{ex} = 530 nm.

2.3. Pulsed HIFU treatment

Cells were HIFU treated while in suspension in the tubes as follows. A tank of degassed water was prepared to hold the tubes and provide coupling between the focused ultrasound transducer and the tube. The focused ultrasound was produced using a Philips Unison system (Philips Research North America, Briarcliff Manor, NY). The ultrasound transducer is an 8-ring annular array device, capable of producing up to 40 W total acoustic power at a 1.4 MHz center frequency. The −6 dB bandwidth of the device is from 0.9 to 1.7 MHz. The calibration experiments were carried out by the manufacturer using a HGL-0200 hydrophone (ONDA corp. Corp., Sunnyvale, CA) in a scanning tank containing degassed water at 25 °C. Positive and negative amplitudes at the focus for each driving voltage/frequency combination were determined by averaging over the 6 central peaks of a 10 peak tone burst. These voltages were converted to MPa using the calibration file of the hydrophone. Acoustic power was also calibrated at the manufacturer using at 1.4 MHz, and repeated by ourselves at a later date using a commercial radiation force balance (UPM-DT-10AV, Ohmic Instruments, Inc., Easton, MD). Our values at this frequency were approximately 17% lower than those given by the manufacturer. A hole at the center of the transducer was used to mount a 15 MHz broadband b-mode imaging transducer (Siemans Acuson Sequoia 512) for ultrasonic guidance and bubble monitoring. Bubble numbers were qualitatively assessed based on the b-mode backscatter intensity. The focal center of the system can be electronically located between 6.5 and 9.5 cm from the transducer, and the focal spot size is approximately 1.1 mm in diameter and 5 mm in length. The acoustic intensity at the focus is therefore on the order of 3000 W/cm^2. For convenience, a waveguide of degassed water was used to couple the transducer into the water bath. Each tube was prepared for treatment by filling to the brim with serum-free medium. Agarose gel filling the bottom of the tubes prevented cells from settling too far outside the focal zone or interacting directly with the plastic or glass of the tube where it might be heated in the far field. Any bubbles were removed from the brim and a film of 1 mil (25 μm thickness) polyethylene was placed on top and secured with an o-ring. This could be done without trapping any visible bubbles, however, the medium was not degassed, and is assumed to be have been saturated. When not being sonicated, tubes were covered with foil to prevent unwanted interactions with ambient light. The tubes were submerged in the tank directly under the window of the Philips system. The ultrasonic treatment parameters were set using the Philips control software, including ultrasound pressure, focal depth, frequency, duty cycle, and repetition rate. The values for peak