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

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Abstract

Pulsed high intensity focused ultrasound (HIFU) produced 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 over either cavitation or RB2 injection alone.

Keywords

high intensity ultrasound (HIFU); Rose Bengal; Cavitation; Optison; sonodynamic therapy

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*, known as histotripsy [6, 7]. Alternatively, low frequency ultrasound has been tested in combination with various drugs. The synergistic

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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, 22, 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 and 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.

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 mM) was reacted with $Br(CH_2)_{12}CH_3$ (158 mg, 0.6 mM) 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^--H). To prepare RB2, Rose Bengal (300 mg) was activated by HBTU (120 mg) in DMF (5 ml) for 2 h, then $NH_2(CH_2)_{12}CH_3$ (300 mg) 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). 1H-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^--H). 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, St. Louis MO) 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 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 % CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, South Logan, UT) supplemented with 10 % fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA) and antibiotics (100 μg penicillin/ml and 100 μg streptomycin/ml (Invitrogen Corporation, Carlsbad, CA)). 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.

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 0.9–1.7 MHz. The calibration experiments were carried out by the manufacturer using a HGL-0200 hydrophone (ONDA corp.) 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 prevents 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 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 negative pressures used here are the values given by the system, and depend upon the accuracy of the manufacturer's calibration (see above). Pressure was generally used rather than power as the acoustic parameter because it is more relevant to cavitation. In these studies, the ultrasound focal depth remained at 8.0 cm (4–5 mm below the acoustic window of the tube), center frequency 1.4 MHz, duty cycle 50 % and repetition rate 1 Hz. Treatment included a small rastering motion via mechanical translation of the transducer in a 1 mm square designed to normalize any possible alignment issues that might result in exclusion of some fraction of the beam energy by the mouth of the tube. Treatment times reported are total times for treatment of the tube; that is, combined time for all four points. The peak negative pressure was varied from 3.0 Mpa (5 W) up to 7.5 MPa (35 W) during different tests. Acoustic streaming was relied on to mix the cell suspension during treatments. Heating under these conditions is expected to be low but not insignificant; tests with a thermocouple indicated a temperature rise of 10 °C, not enough significantly impact cell viability, particularly since treatment was done at room temperature. The length of time the cells remained in the tubes, either waiting or during ultrasound treatment, was less than one hour. Following treatment, tubes were opened and the cells prepared for the viability assay. Control tubes went through this same process, however never received ultrasound. A minimum of 6 tubes were used in each group, and treatments of members of all groups were done in a single session.

2.4. Cell viability test

The cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. The MTS assay was performed using a commercially available kit (Promega, Madison, WI, USA). Briefly, after plating the cells (15,000 cells/well), 20 ul of MTS solution was added to each well. The plates were incubated at 37 °C and 5 % CO2 for 4 hours (time point, 5 hours after treatment). This procedure was repeated three hours (time point 8 hours) and a day later (time point 24 hours). The data displayed is based on the second measurement (8 hours) as this gave the most time for cells to re-attach, and results of the subsequent measurements appeared attributable to normal cell growth. The absorbance at 492 nm was measured using a FLUOstar OPTIMA microplate reader (BMG LABTECH Inc., Durham, NC). All the measurements were done in quadruplicate. The results are expressed in terms of cell viability by normalizing the measurements relative to control (untreated) cells. Trypan blue dye exclusion and direct observation under light microscopy was also used to evaluate the results. Immediately after treatment, the cells were diluted in 0.4 % of trypan blue solution (Invitrogen Corporation, Carlsbad, CA, USA), loaded in a hemocytometer, and counted under a microscope.

2.5. Optimization of RB2 treatment

A second set of studies focused on validation of the most promising sonosensitizer, RB2. Measurements of cell viability vs. ultrasound peak negative pressure, ultrasound treatment time, and RB2 dose were done. Other tests included the effects of adding a scavenger of reactive oxygen and bubble production on the process. For these studies, borosylicate glass tubes (11 ml, 13 mm diameter, filled with 9 ml 1 % agarose) and the MDA-MB-231 cell line were used. This reduced the variability compared to the polystyrene tubes, but an increase in ultrasound power (from 5 to 7 MPa) was needed to achieve the similar results (see Discussion). The previously described methods of loading the cells and compound, treating with pHIFU, and assaying viability were used. To study of the effect of reactive oxygen species (ROS), the ROS scavenger N-acetylcysteine (NAC) was pre-loaded (10 mM, 1 hr prior to treatment) and allowed to remain throughout the treatment phase.

2.6. Analysis and statistics

For each ultrasound treatment and drug combination, MTS assay mean results from the time point 4 hours after ultrasound treatment were used as data for further analysis. All trials were repeated five or more times. Independent tests included: variation of viability with ultrasound power and compound dose for the various compounds; variation with ultrasound treatment time; and impact of adding NAC. When relevant, the significance of varying different parameters was found using a linear model or ANOVA in *R* [27].

2.7. Animal preparation for *in vivo* studies

All animal procedures were approved by the Methodist Hospital Research Institute Animal Care and Use Committee and the United States Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office. The human breast cancer cell line MDA-MB-231 was obtained from ATCC (Rockville, MD, USA) and tested negative for human and rodent pathogens. The cells were maintained *in vitro* as above. Female nu/nu mice (Charles River Labs, Wilmington, MA) were inoculated by injecting 10^7 cells subcutaneously in each of the hind flanks. Once one or both tumors reached the target size range (100–250 mm^3), the animal was assigned to a treatment group. Treatment consisted of either the full combination therapy outlined below, or a partial treatment with one or more steps omitted. The full combination treatment is as follows. The tumor to be treated is intra-tumorally (i.t.) injected with up to 50 μl of Optison ultrasound contrast agent (GE Healthcare), after which the animal received 25 mg/kg (0.5 mg/20 g) dose of RB2 in a 200 μl volume tail vein injection. Fifteen minutes later, the animal is anesthetized with 3 % isoflurane and partially submerged using a specially designed fixture in a shallow bath of degassed water maintained at 36 °C by circulating warming water through a copper coil attached to a warm water pump (Gaymar Industries, Inc., Orchard Park, NY). Pulsed HIFU from the Unison system was coupled to the target tumor through the degassed water, aligning the axis of the treatment beam and tumor by guiding with b-mode ultrasound imaging (Siemans Sequoia) or laser pointer. The laser pointer was aligned by comparing the beam location to a burn produced by HIFU in a plastic target. Treatment consisted of no more than 9 points (less if the entire tumor was covered) in a 3×3 rastered grid pattern, treated consecutively with a dwell time of 30 s (30 pulses) at each location. The HIFU frequency was reduced to 1.0 MHz to promote cavitation and avoid heating, and the negative pressure was 7 MPa (39 W peak acoustic power). These acoustic parameters are a little different from those used in vitro, but were expected to assist in reproducing the in vitro microenvironment that led to the observed cytotoxicity (see Discussion). After receiving treatment, the mouse was placed back in its cage to recover, and monitored for signs of toxicity. If significant pain was suspected, an i.p. dose of buprenorphrine was used as analgesic.

2.8. Initial histology and toxicology

A selected number of animals were sacrificed within 24 hours of treatment to undergo blood tests for compound toxicity and tumor histopathology. The tumors were carefully extracted and fixed in 10 % formalin, then paraffin embedded. Sections were cut, stained with H & E, and examined for gross pathological changes, including necrosis, cell lysis, hemorrhage, and inflammation. Blood samples from selected animals were screened for toxicity at the highest dose of the compound (25 mg/kg). Before sacrificing, following general anesthesia the animal underwent a thoracotomy and the blood was drained from the left ventricle using a 27 Ga syringe. We looked for reduced blood cell counts as well as blood albumin, protein, urea, alanine, aspartate, and creatinine levels for assessment of marrow, liver, and kidney function. Histology on these organs, plus the heart and spleen, were used to assess for tissue damage or malfunction.

2.9. Tumor regression studies

Animals were divided into groups for tumor regression studies to test the effect of adding RB2 to the tumor environment in combination with ultrasound cavitation. To guarantee ultrasonic cavitation, the combination of Optison direct injection and pHIFU was used. All animals had bilateral tumors grown on either rear flank. In all cases, one tumor was treated with ultrasound and the other used as control. The main treatment group received both direct i.t. injection of Optison and i.v. injection of RB2, along with focused ultrasound treatment. Control tumors in this group received i.t. injection of Optison and i.v. injection of RB without ultrasound treatment. A second treatment group received Optison i.t. plus ultrasound on the treated side, Optison injection only on the control side. A third group received ultrasound only on the treated side and no treatment of any sort on the control. Each of these groups contained 5–7 animals. In all groups, ultrasound treatment consisted of up to 9 focal spots to cover the tumor on a square grid of 2 mm separation distance, with each focal spot receiving 30 pulses of 6 or 7 MPa peak negative pressure ultrasound (28 or 39 W total acoustic power) at 20 % duty cycle, 1 Hz repetition rate, and 1.0 MHz center frequency. Reducing the duty cycle from 20 % (compared to 50 % *in vitro*) was done to avoid thermal necrosis. The total energy thus deposited was less than 240 J (40 W × 20 % × 30 pulses) per site, and very comparable to the 200 J per site deposited by [28] (40 W × 5 % × 100 pulses), who measured a temperature rise of less than 5 °C. The Optison injected i.t. was approximately 20 % of the tumor volume, up to 50 μl. RB2 i.v. injection was 0.5 mg/mouse (approximately 25 mg/kg) in a 200 μl dose. The time between RB2 injection and pHIFU treatment was less than 20 minutes. For monitoring of tumoricidal effects, the individual size of the tumor was measured with calipers in two orthogonal diameters every day for one week following treatment. Tumor volume was calculated using the formula $V = ab^2/2$, where b is the minor axis [29].

2.10. Statistical methods

The combined and individual effects of ultrasound, Optison, and RB2 on tumor growth were analyzed using a generalized linear model (*geepack* in *R*, [30, 31]). The measured variable (tumor volume) was normalized relative to the initial tumor size on the treatment day. These degrees of freedom are then removed from the dataset. The normalized tumor volume was then transformed by taking the logarithm of the variable, resulting in a near-Gaussian distribution of the measured variable. The resulting data were analyzed with the following linear model:

$$\ln\left(\frac{V(t)}{V(0)}\right) = [a_0 + a_1 O + a_2 R + a_3 H + a_{13} OH + a_{123} OHR]t + b \quad (1)$$

where $V(t)$ is the measured volume, V_0 is the initial volume at treatment, t is time in days, R is the RB2 dose, O is the Optison dose, and H is the HIFU peak negative pressure. The coefficients a_1 through a_3 model the effect of an individual treatment on the tumor growth, a_{13} and a_{123} model the combination of two and three treatments (a_{12} is safely excluded because there is no expectation or evidence of an interaction between microbubbles and RB3 in the absence of ultrasound), and b is an overall intercept term that should, by definition, be close to zero. By including all of the individual treatments as well as the combination therapy in a single model, it is possible to directly compare the contribution of additive and synergistic effects on the tumor growth rate. For example, the growth of an untreated control ($O=R=H=0$) tumor is governed by $a0$ alone. The growth of a tumor treated with RB3 ($O=H=0$; $R>0$) is governed by a_0 and a_2R. If the treatment has no effect, the difference in growth to the control is small, that is, $a_2 \sim 0$ will be insignificant. A combination treatment depends on both additive and multiplicative (synergistic) terms. The full treatment ($O>0$;

H>0; *R>0*) will have a growth including all terms, and the effect of the combination is additive if $a_{123} \sim 0$. This model allows full characterization of the treatment combination with just 6 groups, that is, one experimental group for each growth term. In determining significance, measurements taken in the same animal were grouped, to take into account repeated measures over time as well as the bilateral tumors when reporting statistical significance.

3. Results and discussion

3.1. Optical properties of RB and its analogs

To modify photosensitivity, the acid group on RB was converted into a tridecyl ester or amide. As expected, the esterification of RB only caused moderately reduced absorbance and fluorescence, 66 % and 78 %, respectively (Fig. 1). In contrast, RB2, an amidated RB, lost its optical characteristics completely. Neither absorbance, nor fluorescence between 500–600 nm could be seen. RB and RB1 are red-orange color, while RB2 is white. The sonosensitivity of these three molecules and several reported sonosensitizers were then validated in cancer cell lines.

3.2. *In vitro* initial screening

The initial tests of the entire panel of compounds showed RB2 (20 μM) yielding very good cytotoxicity in combination with pHIFU at levels above 5 MPa. With ultrasound treatment, the cell viability relative to untreated control tubes dropped from 95 % down to 5 %. It was found that MP, RB1 and RB2, showed significant synergistic behavior in combination with HIFU at 5 MPa (Fig. 2). The other compounds (PP, IP, HP, RB) showed no significant increases in cytotoxicity with application of ultrasound. HP was found to be cytotoxic (>80 % cell death) to MDA-MB-435S cells at 5 μM or greater concentration. Trypan blue exclusion demonstrated identical behavior to the MTS assay when viability is defined in the same way (number of live cells compared to controls). The number of intact cells found following successful treatments was very low compared to controls, indicating that the cell killing by RB2/HIFU was caused by immediate lysis (Fig. 3) rather than apoptosis. Some variability in cell kill was likely related to differences in cavitation initiation. A positive link between bubble formation and cell kill was observed (Fig. 4). Although MP and RB2 showed similar effect with same amount of drug and pHIFU pressure, their optical properties are significantly different. MP is fluorescent and photosensitive, but RB2 is optically silent. Thus RB2 was selected for the following studies.

3.3. Optimization of RB2 treatment

Fig. 5 A and B show the changes in cell viability with the ultrasound parameters of peak negative pressure and treatment time. Higher pHIFU pressure and number of pulses killed more cells. These factors are very significant ($p < 10^{-12}$). Based on the data in Fig. 5 B, it is possible to fit an exponential attrition model,

$$Viability\ (t) = e^{-\alpha t} \tag{2}$$

where α is a parameter to be determined and *t* is the length of treatment in seconds. Fitting was done in the linearized form using the least squares routine in *R* [27]. The resulting parameter is $\alpha = 0.117 \pm 0.008\ s^{-1}$ with $R^2 = 0.73$. Although this fit is not as tight as one might hope, the trend is clear and unambiguous. Thus, with every pulse (at 1 Hz), 11 % of the remaining cells are killed, and thirty seconds is sufficient to kill over 96 % of the cells in solution. Provided treatment is longer than 30 seconds, it is possible to treat each tube as either viable (<10 % kill) or non-viable (>85 % kill), for easier analysis on the margins of

the cavitation threshold, where cavitation seems to either initiate or fail to initiate in any given tube. Most importantly, this cell kill effect is RB2 dose dependent (Fig. 5 C). A minimum of 10 μM is required to achieve >90 % of kill, but the dose effect for both drug and ultrasound is quite sharp. To confirm the correlation between sonotoxicity and reactive oxygen species (ROS), NAC was added to the medium. In the presence of NAC, following 7 MPa, 30 s ultrasound treatment with 20 μM RB2, about 50 % of tubes treated remained viable (Fig. 5 D). This may suggest a role for ROS induced by pHIFU treated RB2 in cell toxicity, possibly by a lipid peroxidation mechanism [10].

3.4. *In vivo* tumor regression

Prior to the regression experiments, potential systemic toxicity was tested after i.v. injection of the experimental dose of RB2 (25 mg/kg). Histology and blood tests all appeared normal, and the animals appeared to eat and gain weight normally. No toxicity could be detected at this dose. Successfully treated tumors typically had immediately evident lesions, presumably where the focal zone passed through the skin. With Optison microbubble (Opt) and RB2, this was accompanied by a rapid loss of tumor volume in the 24 hrs following pHIFU treatment (Fig. 6). Tumors extracted at that time showed various levels of increased necrosis with micro- or macro-hemorrhage. Results of the tumor regression study are given in Table 1 and illustrated in Fig. 7. The slopes of the linear fits are derived from the model coefficients assuming Optison, RB2 and HIFU values of 25 μl, 0.5 mg/20 g mouse, and 7 MPa, respectively. The respective p-values in Table 1 give the probability that the coefficient is zero, or alternatively, the probability that the tumor growth rate of that group can be explained without including the given term in the model. In particular, the a_{13} term is not significant, $p > 0.13$, implying that the tumor regression of the “Opt+HIFU” group might possibly be explained as the additive effects of “Opt” and “HIFU” alone. The effect of the “Opt+RB2+HIFU” is statistically significant, however, with $p < 0.023$, suggesting that additive effects alone are not sufficient to explain tumor regression in this group. Of the groups studied, only the treatments involving cavitation (“Opt+HIFU” and “Opt +RB2+HIFU”) demonstrated tumor regression, with the administration of RB2 significantly enhancing the effect.

Discussion

We were able to demonstrate a significant cytotoxic synergism between high intensity focused ultrasound and a new, non-toxic, optically inert compound (RB2) based on Rose Bengal. Neither the compound by itself nor the ultrasound by itself caused significant cell death, but in concert, they resulted in 90 % or more dead cells *in vitro*. Traditional light-sensitive sonodynamic compounds (porphyrins) were compared with the RB2. Only MP demonstrated a similar effect at nearly the same concentration. However, a photo-insensitive sonosensitizer should have a much wider therapeutic window than photosensitive ones, which may be activated by ambient lighting. RB2-based sonodynamic therapy should therefore avoid most of the current limitations of photodynamic therapy. The experimental data suggest that the cytotoxicity is based on cell lysis and necrosis rather than apoptosis, and the cell killing effect appears to have a very sharp threshold when varying compound dose or acoustic power. In any given tube, either the great majority of the cells are lysed, or they are viable, depending on whether or not cavitation was initiated in that tube. Cavitation initiation near the threshold appears to be somewhat stochastic in nature, but once cavitation is initiated, cell viability drops rapidly. Interpretation of this fact led us to recognize problems with the use of polystyrene tubes during the initial phase of the study and the subsequent adoption of borosilicate glass tubes. From our observations, cavitation initiation appeared more random in the polystyrene tubes, and the created bubbles tended to collect on the walls of the tube. This did not happen with the glass, however, higher power ultrasound

was required to initiate cavitation in general. This might suggest that microscopic voids existing in the plastic play a role in initiating cavitation. The hydrophobicity of the surface might also play a role by attracting surfactants. Either way, we observed that the cavitation threshold is dependent on the presence of the RB2 and other trace compounds, making assessment of the mechanism difficult. The fact that RB derivatives seem to reduce the cavitation threshold was also mentioned by [26]. It is unclear whether the primary responsibility for the cytotoxicity lies in the physical action of the bubbles themselves or in the generation of reactive species. Cavitation may lyse the cells directly or in concert with reactive species produced from the sonosensitizer, possibly, as suggested previously by Rosenthal [10], by lipid peroxidation. Numerous papers have suggested that the mitochondrial membranes are a major site of damage, eg., [16,19, 20] (although others [17,18] have also suggested the cytoskeleton). Clearly any reactive oxygens released from damage within the cell would enhance the effect. Given that the acoustic focal zone *in vitro* treats only a small fraction of the cell suspension, it might be argued that the chemical effects dominate. Also, the addition of the ROS scavenger NAC to the solution dampens the cytotoxic effect, and this suggests a potential role of reactive oxygen species. However, based on our observations, we cannot rule out the possibility that the NAC might also shift the cavitation threshold. The attrition model of Eq. 2 can also be interpreted in two ways: either it is related to the mixing time of the suspension (time required to cycle a significant fraction of the cells through the focal zone) or the rate of ROS production. It therefore appears to be very difficult, if not impossible, to completely separate these two mechanisms. The translation of this synergistic effect to an *in vivo* model is complicated by the difficulty of initiating cavitation *in vivo*. It should be noted that, under any conditions, the in vivo microenvironment is very different from the in vitro microenvironment, particularly, acoustic absorption is increased and the cavitation threshold is greatly increased. The changes made (reducing the frequency, shortening the duty cycle, injecting microbubbles and running at the highest peak power) are designed to reproduce to whatever extent possible the in vitro conditions. However, it is impossible at this time to state with certainty that the in vivo and in vitro results arise from the same mechanism. We used a microbubble injection (Optison) to assist in this. In a clinical setting, it would be preferential to insert the microbubbles i.v. along with the sonodynamic compound, however, the very different pharmacokinetics of the two agents would need to be addressed, as the microbubbles are known to clear rapidly through the lungs. Perhaps using tumor vascular targeted microbubbles would be a viable option. Our decision here was to avoid all such issues by injecting the microbubbles i.t., where we knew they would be trapped, while injecting the RB2 i.v. on the assumption that, as a relatively small molecule, it would have no problem leaking into the tumor region. Ultimately, an alternative to microbubble-based initiation might be to use a more powerful HIFU system than we currently have available. It is known that an order of magnitude higher acoustic power will lyse cells *in vivo* [32], and it may be that introducing a RB2-like sonodynamic agent can enhance or reduce the threshold for this effect. The use of cavitation *in vivo* also complicates the issue of mechanism, since cavitation is known to be accompanied by a significant additional rise in temperature. Despite the fact that we took steps to avoid this by using shortened duty cycle and limited pulse number, at least some of the anti-tumor effect may be attributed to thermal necrosis. In fact, a significant retardation of tumor growth was already observed when Optison was combined with pHIFU. However, the addition of RB2 is certainly beneficial and results in an enhanced effect. Tumors treated using Optison plus pHIFU grew at a pace that was 115 % below that of untreated control tumors; adding the RB2 slowed growth down further to 154 % less than controls. The histology results suggest a strong anti-tumor effect (Fig 6D). In contrast, without HIFU, RB2 alone had no effect (Fig 6C). Based on the growth curves of Fig. 7, RB2 alone likely increases the tumor growth, an effect that would be an obvious issue for future translation of this particular compound. Most of these *in vivo* pilot studies were done using a very narrow range of ultrasound parameters and compound doses that

largely mimicked the most successful *in vitro* design. There is plenty of room for further optimization. It is very possible that, at some lower acoustic power, or at different concentrations, the addition of RB2 might have an even larger relative impact on tumor regression. Also, although RB2 has shown promise as a sonosensitizer, the detailed mechanisms of sonosensitivity require further elucidation. Better understanding of the correlation between chemical structure and sonosensitivity would be critical for new sonosensitizer design; unfortunately, this area has not been well studied. A systematic comparison with known sonosensitizers might be a good direction to solidify the technology.

4. Conclusions

In summary, it is proven that photosensitivity is not an essential factor for sonotoxicity. The amidated RB analog is not photosensitive, but acts as a potent sonosensitizer. In conjunction with microbubbles and pHIFU, a synergistic anti-tumor effect can be achieved. HIFU has near mm precision and deep tissue penetrating capability. We have demonstrated that pHIFU could act as a drug activator, converting a non-toxic sonosensitizer into a cytotoxic agent, killing cells only in a well defined area. Potentially, this non-invasive technology will be extremely useful in treating otherwise hard-to-reach tumors.

Acknowledgments

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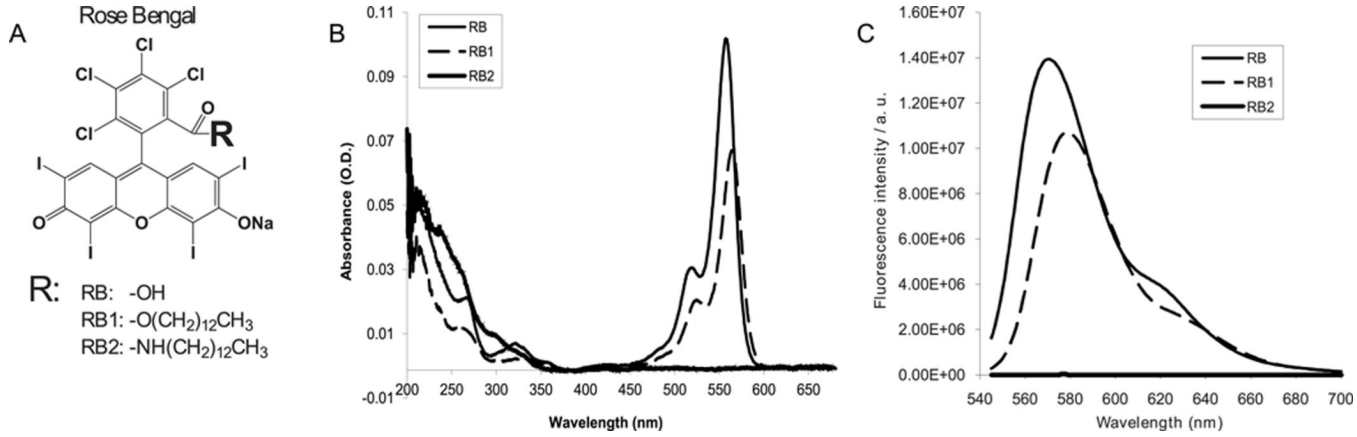

Figure 1.

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.

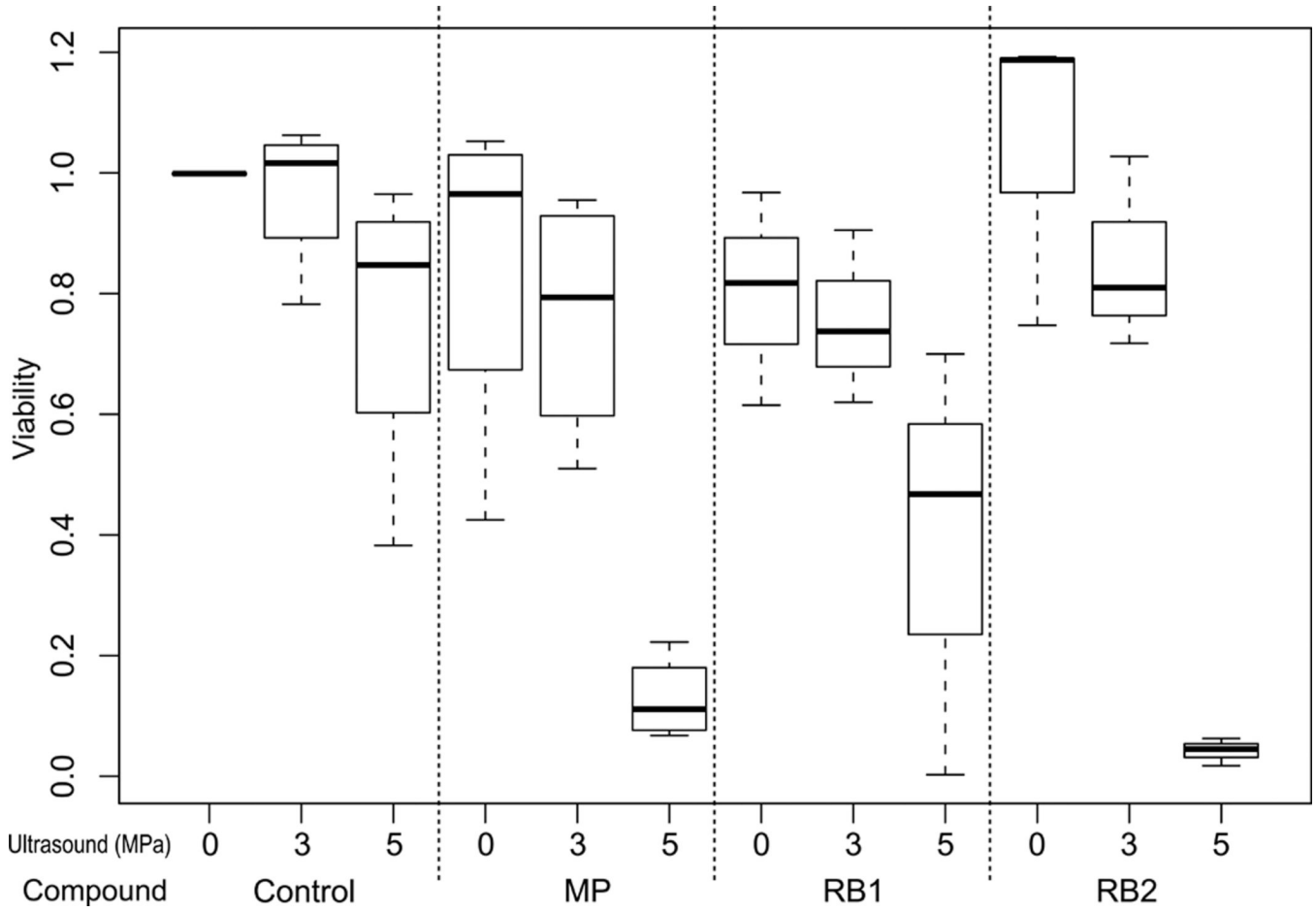

Figure 2.

Synergistic effect of pulsed HIFU and candidate sonosensitizers: mesoporphyrin (MP) and Rose Bengal derivatives, RB1 and RB2, demonstrated during initial screening in MDA-MB-435S cells. Only RB2 is not a photosensitizer. The compounds were all present at a concentration of 20 μM, and were treated with 1 MHz ultrasound at 5 MPa, 50 % duty cycle, 300 pulses at 1 Hz repetition rate. Similar results were obtained with MDA-MB231 cells (not shown).

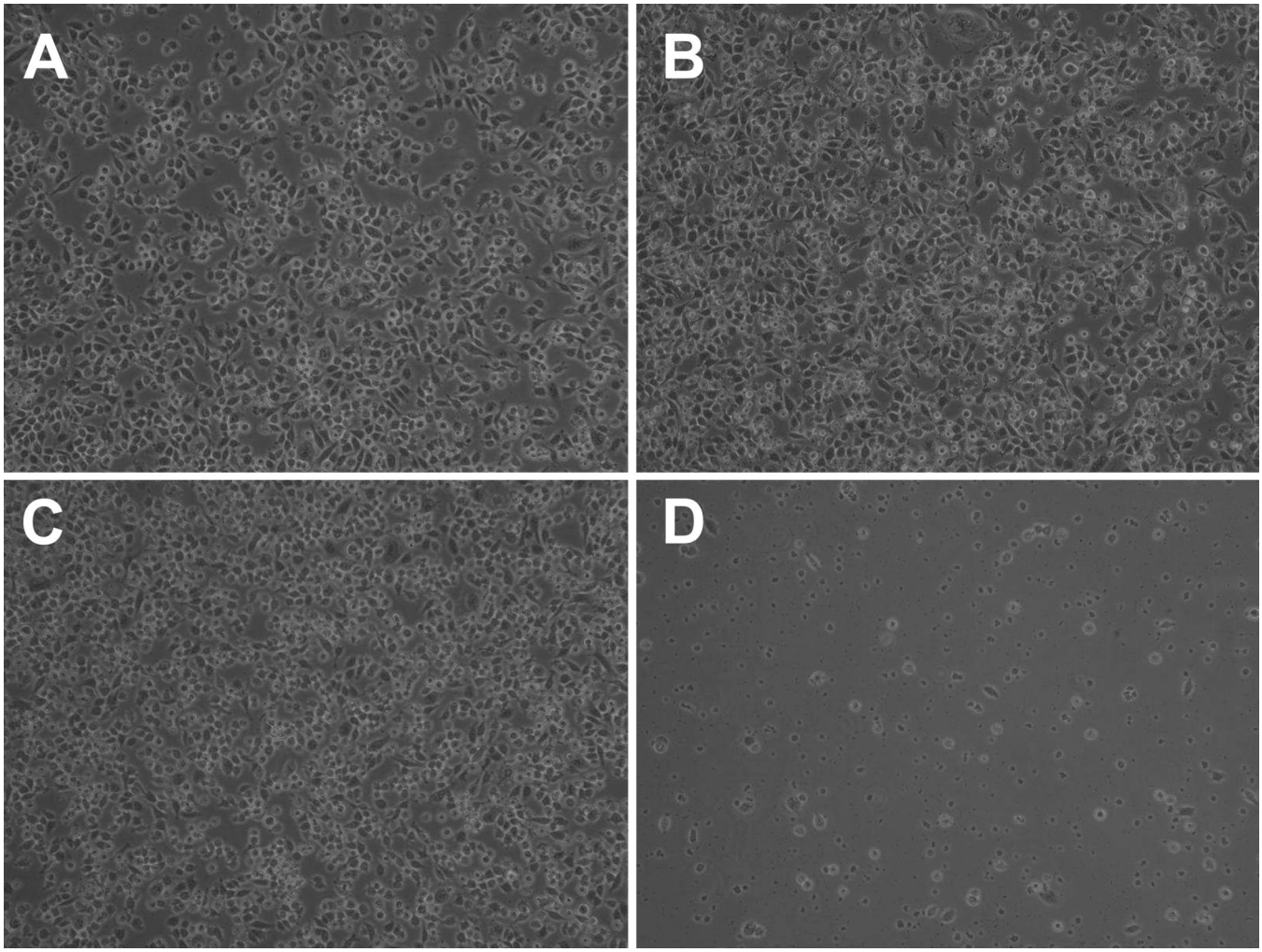

Figure 3.
MDA-MB231 cells following treatment (initial screening). A) Control; B) RB2 20 μM; C) pHIFU (5 MPa); D) RB2 (20 μM) plus pHIFU (5 MPa). The combination treatment results in near-complete cell lysis. The results for MDA-MB435S are similar (not shown).

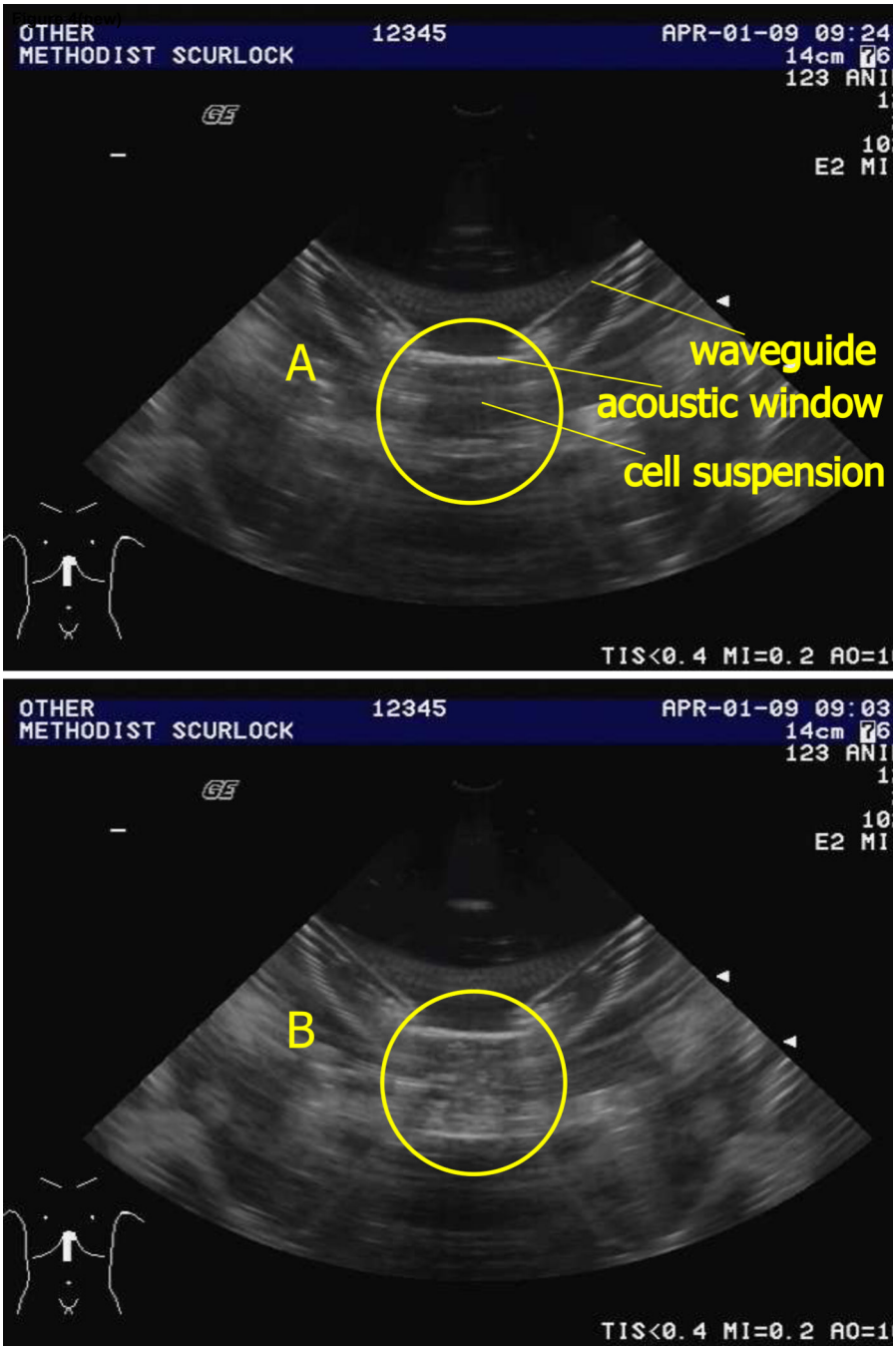

Figure 4.

Ultrasound imaging of acoustic cavitation produced during treatments in vitro. A) no visible bubbles formed B) many bubbles, typically associated with cytotoxic effects. The tube containing cell suspension is circled; the bottom of the tube is filled with agar gel. Ultrasound (pHIFU as well as imaging) originates at the top of the image and is coupled to the tube through a degassed water waveguide and acoustic window.

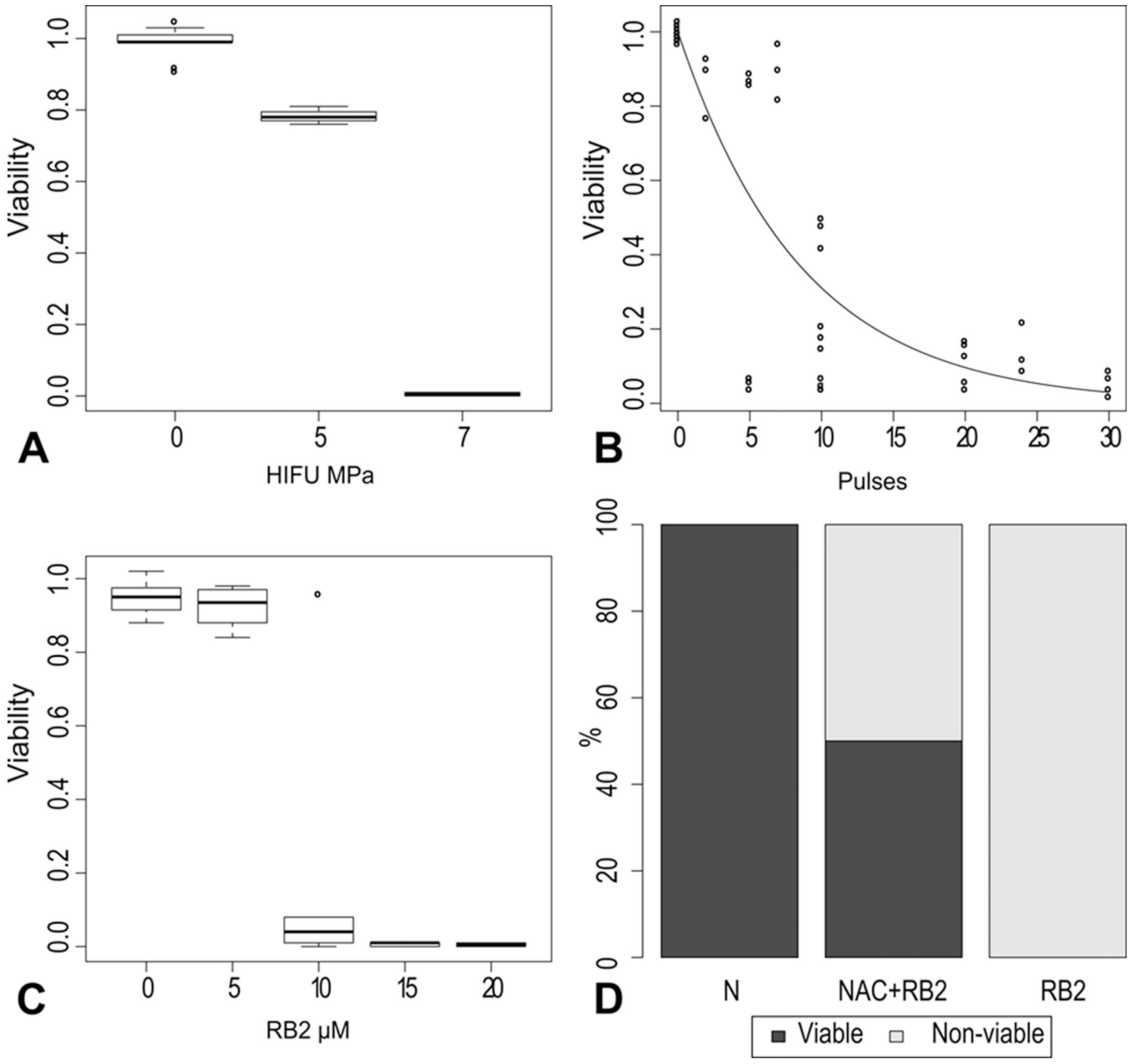

Figure 5.

A) Viability vs. ultrasound pressure, 20 μM RB2, 300 pulses in MDA-MB-231 cells (N>5). Pulsed HIFU was maintained at 50 % duty cycle, 1 Hz repetition rate, 1 MHz center frequency for these studies. B) Viability vs. number of pulses for 20 μM RB2, 7 MPa pHIFU. Curve is the data fit to an exponential attrition model: $e^{-\alpha t}$, where $\alpha = -0.117 \pm 0.008$ (see discussion). C) Viability vs. RB2 dose after 300 pulses of 7 MPa (35 W peak acoustic power) pHIFU. D) Percentage of tubes with predominantly (>85%) viable vs. non-viable (<10%) cells after 30 pulses treatment with 6 MPa pHIFU, with no added compound (N), with 20 μM RB2 added (RB2), and with addition of 15 mM antioxidant NAC plus 20 μM RB2 (NAC+RB2) 6 tubes were used in each group.

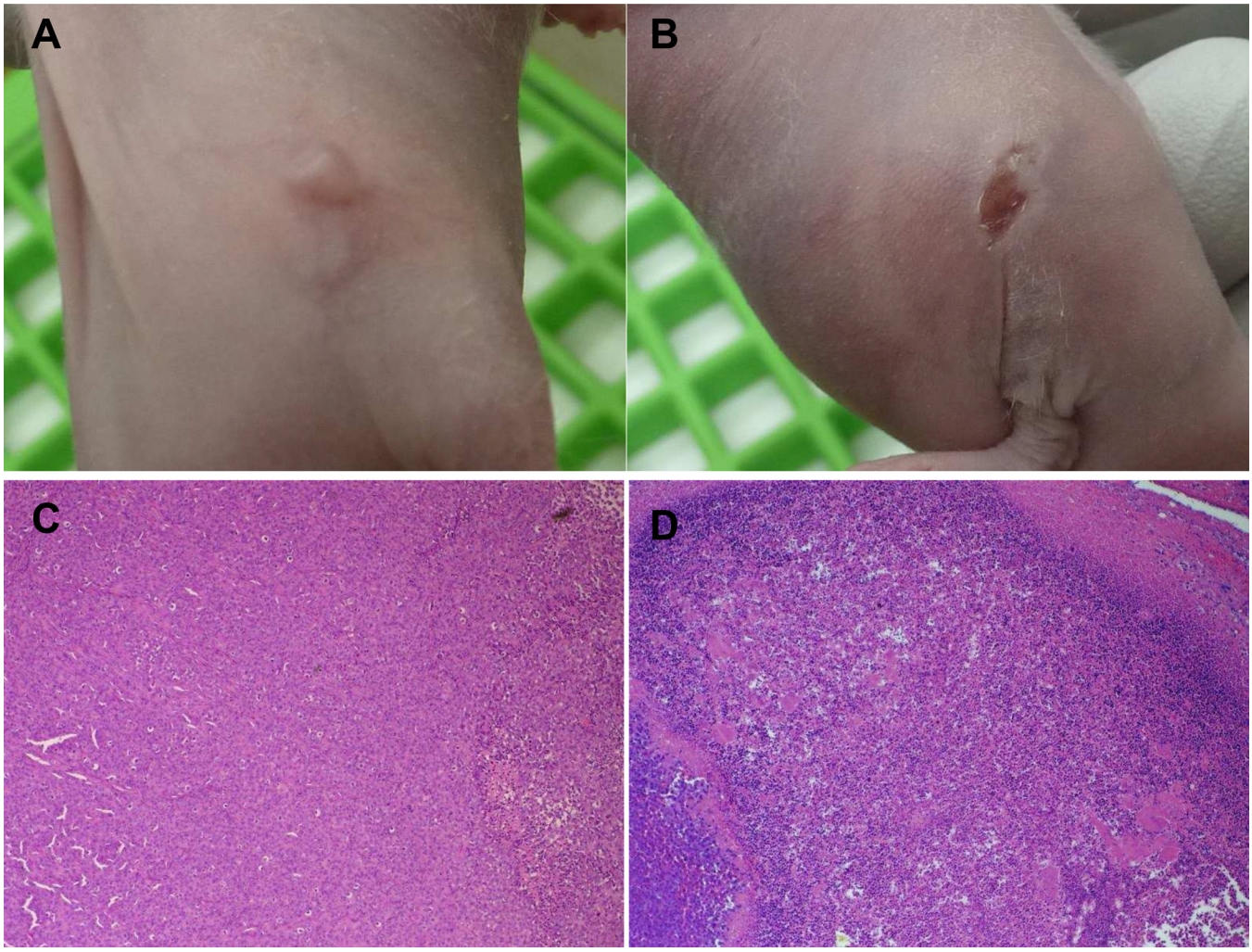

Figure 6.

Animal tumor growth and tumor histology. A) RB2 only control, B) tumor with HIFU, Optison and RB2, 24 hrs after treatment. C) histology of RB2 control tumor, D) in combination with HIFU, showing severe necrosis 24 hrs following treatment. Histology of HIFU plus Optison controls without RB2 demonstrated some necrosis, but with less frequency and consistency (not shown). Tumors here are from the same animal. Pulsed HIFU treatment was 1 MHz center frequency, 20 % duty cycle, 30 pulses at 1 Hz repetition rate.

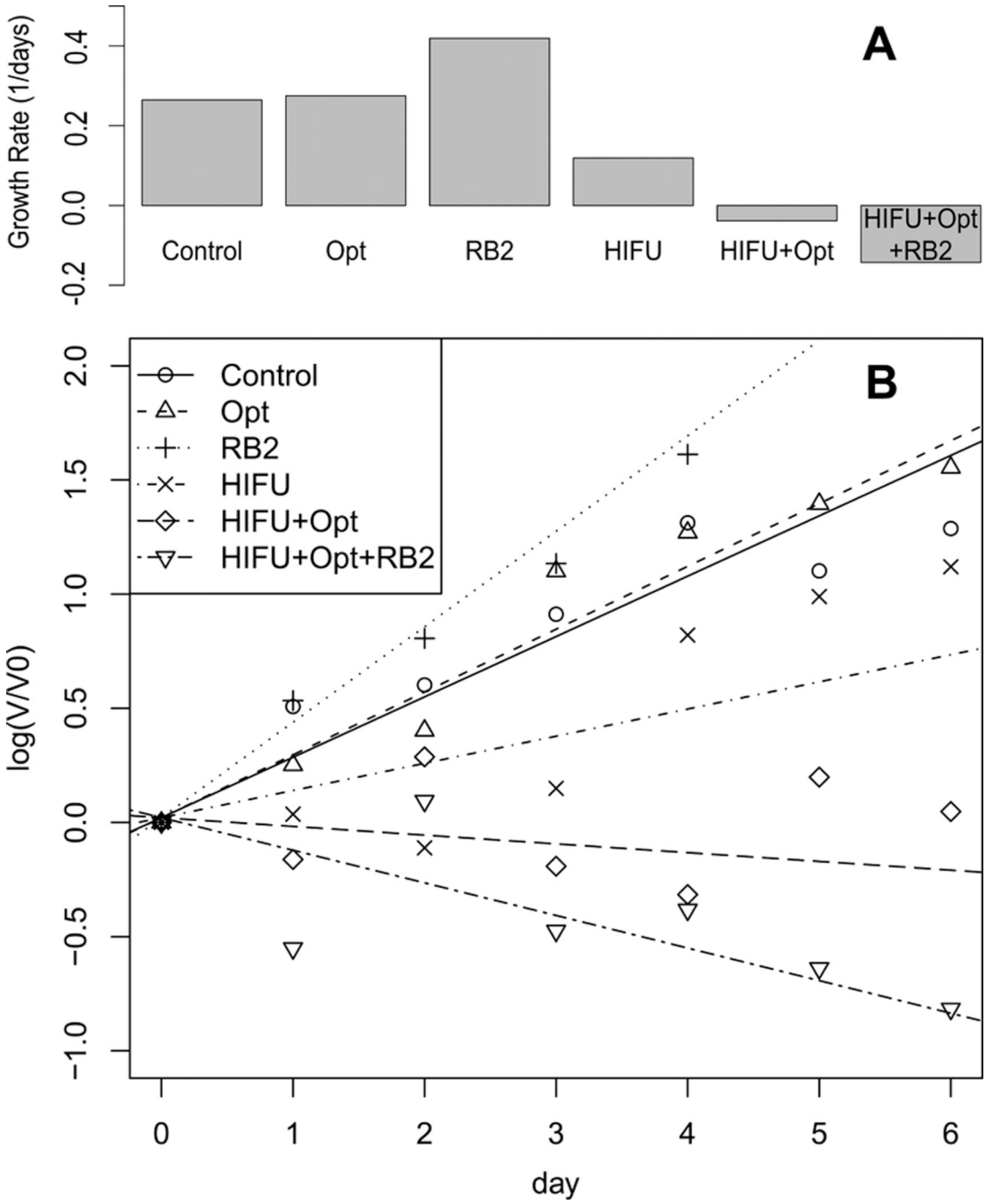

Figure 7.

Exponential tumor growth rates. A) Growth rates for various treatment combinations, calculated from Table 1 and Eq. 2, with O=50 μl, R=0.5 mg, and H=7 MPa. The growth rate for the full combination treatment with RB2 is 154% less than the Control group growth rate. B) Log-normalized tumor volume vs. time up to 6 days following treatment. Shown here are mean values for each day (points) and linear fit (lines) (see Methods).

Table 1

Tumor growth model coefficients with various treatments. The significance of a given term in explaining tumor growth under various conditions is given in the usual way by the p-value (see Methods); the alternative hypothesis is that the coefficient is zero. Thus, coefficients such as a_{13} and a_{123} are significant only if the effect of the combination treatment is not explained as the additive effect of individual components.

Group : Coefficient	Value ± Std. Error (unit)	p-value	
: b	0.02±0.05	0.67	
Control : a_0	0.26±0.03 /day	1.4e-15	***
Optison : a_1	4.3e-04±2.3e-03 /(μl day)	0.85	
RB2 : a_2	0.31±0.12 /(mg day)	0.021	*
HIFU : a_3	−2.1e-02±7.9e-03 /(MPa day)	0.0089	**
Opt+HIFU : a_{13}	−9.6e-04±6.3e-04 /(μl MPa day)	0.13	
Opt+RB2+HIFU : a_{123}	−3.0e-03±1.3e-03 /(μl mg MPa day)	0.024	*