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14. ABSTRACT High intensity focused ultrasound (HIFU) has been combined with a 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 30s) of pulsed HIFU with power sufficient for cavitation destroys >95% of breast cancer cells in suspension with 15uM of the compound. Neither the pulsed HIFU nor the RB2 compound was found to have any impact on the viability of the cells when used alone. Introducing an antioxidant (N-acetylcysteine, NAC) reduced the effectiveness of the treatment. In vivo tests using these same cells growing as a xenograft in mice were also done. Ultrasound contrast agents were used to initiate cavitation. We were able to demonstrate rapid tumor regression for tumors with cavitation. It was not clear if the addition of the RB2 yielded any substantial improvement over the contrast agent alone. In some animals, ablating a tumor with cavitation resulted the subsequent regression of both treated and control tumors.					
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Introduction

High power ultrasound creates and interacts with microbubbles, causing extremes in pressure and temperature that can reportedly “activate” molecules known as “sonosensitizers”. Called “sonodynamic therapy”, this technique is often seen as the ultrasonic analog of the clinically tested photodynamic therapy, and historically researchers have used the same compounds for both therapies¹. However, many of the problems associated with photodynamic therapy, including side effects resulting from exposure to ambient light, could be solved by treating with a compound that is activated by sound (a “sonosensitizer”) but not by light. In our study, we have introduced and tested a potent new sono-sensitive compound based on Rose Bengal which is not photo-sensitive. This compound (RB2) was tested *in vitro* and *in vivo* in combination with cavitation driven by high intensity focused ultrasound (HIFU). Applying HIFU in pulsed mode (to avoid overheating) has shown promise in improving the penetration of large therapeutics into tumors. It is also capable of producing consistent cavitation activity even deep in the tissue. This project was designed to test whether using pulsed HIFU for delivery and activation of a sonosensitizer might result in an effective targeted chemotherapy that could be useful for treating breast tumors without the side effects associated with traditional untargeted chemotherapy or photodynamic therapy. The *in vitro* work consisted of looking for a synergistic cytotoxicity between RB2 and pulsed HIFU treatment of a breast cancer cell line. The *in vivo* studies were designed to test for systemic toxicity of the compound and synergistic anti-tumor effects when applied to a breast cancer xenograft model.

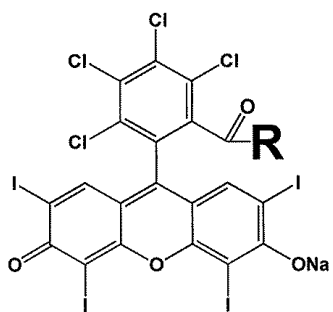
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Jianjun Qi, PhD; Postdoctoral Fellow, chemist
Rongmin Xia, PhD; Postdoctoral Fellow, elec. engineer

Body

Chemistry

As described in our Annual Report² of 8/15/2009, a Rose Bengal derivative was produced and used as a sonosensitizer in our studies. This compound was designed to be activated by ultrasound but not by light. The structure of the compound is shown in Fig. 1. Other compounds that we studied included

hematoporphyrin IX (HP), mesoporphyrin IX (MP), protoporphyrin IX (PP), and isohematoporphyrin IX (IP), obtained from Frontier Scientific, Inc.



R: RB: -OH
RB1: -O-(CH₂)₁₂-CH₃
RB2: -NH-(CH₂)₁₂-CH₃

Rose Bengal

Figure 1: Chemical structure of Rose Bengal analogs.

In Vitro Studies

In vitro testing continued as described in the first Annual Report², except that the polystyrene tubes were replaced with ones made of borosilicate glass. A comparison between porphyrin compounds and RB2 was repeated. A particular focus was the relative efficacy of MP and RB2. The compounds were tried in varying doses, applying varying acoustic powers, and varying treatment times. Both compounds showed similar synergy with the ultrasound. The other porphyrin compounds were not nearly as effective as MP.

In an effort to better understand the nature of the sonodynamic synergy, various other comparison studies were conducted. A control study looked at the effect of adding just the DMSO to the medium in combination with the ultrasound. DMSO was used to assist in dissolving the hydrophobic RB2 and porphyrin compounds in the aqueous solution. Surprisingly, it turned out that 30 μ l DMSO alone had cytotoxic effect in combination with the ultrasound at 7 MPa, however, at 6.5 MPa, it required RB2 or MP to be effective ($p < 0.05$) (Fig. 2).

One constant observation across the various compounds has been that test tubes showed cytolysis if and only if they had good bubble production, and that bubble initiation was not entirely governed by the ultrasound parameters. The sonodynamic therapy literature^{3,4,5} suggests that the bubble production is what creates reactive oxygen species, resulting in cell death. Cytolysis may indeed be caused by lipid peroxidation of the cell membrane¹. However, it is also known that the physical effects of cavitation can directly destroy cell membranes⁶. It would appear that doping the cell culture medium with micromolar amounts of various compounds can result in bubble initiation at lower powers. Cell lysis might then be caused by the reactive oxygen species, the physical action of the bubbles, or possibly some combination of the two. One way to approach this question has been to test whether the introduction of free radical scavengers can dampen the effect⁵. We therefore set up an experiment using NAC (N-acetylcysteine) in excess as a reactive oxygen scavenger⁷ in combination with RB2. At the acoustic pressure of 7 MPa, we were able to show a clear difference between the RB2 group and the group of RB2 plus NAC (Fig. 3, $p = 0.015$, $n = 6$). Adding the scavenger reduced the probability of cell lysis by about 50%. This suggests that reactive oxygen species might be involved. However, is still not entirely possible to rule out the possibility that the extra compound simply resulted in a reduction in bubble production.

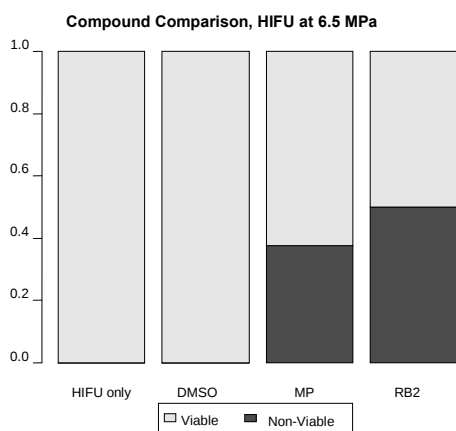


Figure 2. Viable vs. non-viable tubes following HIFU treatment at 6.5 MPa with various compounds. At 7 MPa, DMSO, MP and RB2 all produce significant cell death.

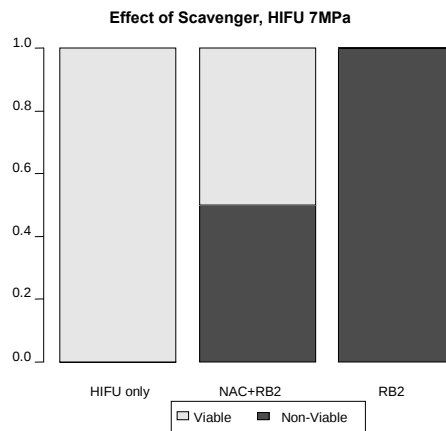


Figure 3. Tubes of viable and non-viable cells following HIFU treatment at 7.0 MPa with and without the antioxidant NAC.

In Vivo Studies

In the Annual Report², we were able to show a trend towards regression of small tumors treated with RB2 in conjunction with HIFU and Optison vs. HIFU alone. This study was repeated with an additional 15 animals. Unfortunately, there were again problems with growing tumors in some of these animals. The tumors grew in two cohorts, each of which behaved differently under our experimental conditions. The first, faster-growing cohort continued the trend mentioned above. The untreated control tumors grew normally, while the treated tumors regressed. Combined with the previous data set, we were able to show a significant reduction in growth ($p=0.03$, $n=6$, non-parametric paired test) by day 4 (Fig. 4) following treatment. By contrast, the HIFU only group did not show a significant reduction in growth ($p=0.125$, $n=4$, non-parametric test) (Fig. 5). There was insufficient data to do the same test with the HIFU plus Optison (without RB2) group on any given day, however, the trend appears very similar to the animals with the RB2 injection. Fitting an exponential growth model to the three groups results in treated tumors having insignificant growth for all groups receiving Optison and HIFU, with or without RB2 IV injection. Without Optison, the HIFU treated tumor growth is only slightly retarded compared untreated controls in the same animal. This would suggest that, at the acoustic power used, the anti-tumor effect of the Optison mediated cavitation is quite good, and the addition of the RB2 could not significantly improve upon it. At a lower acoustic power the difference might more pronounced.

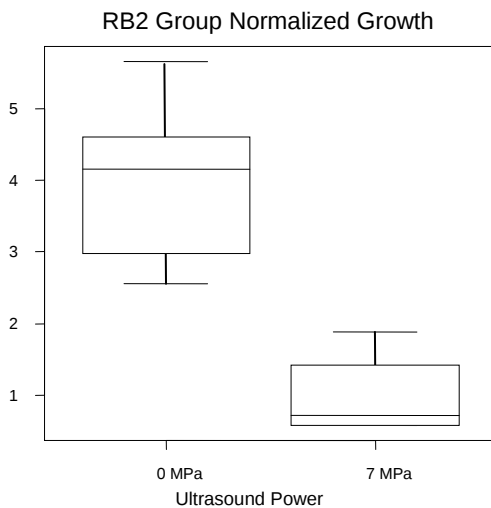


Figure 4. Tumor growth for HIFU treated (right) and untreated, bilateral controls (left), in group with RB2 IV and Optison IT injection.

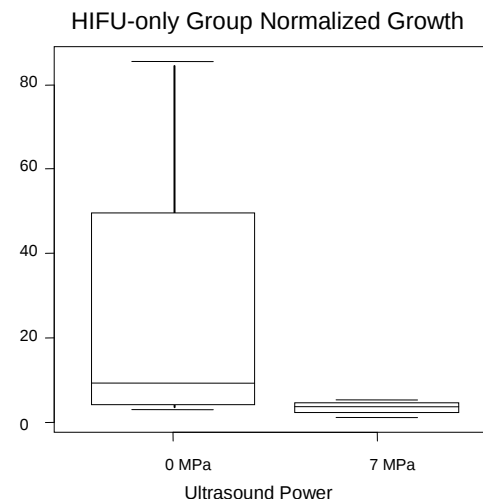


Figure 5. Tumor growth for HIFU treated (right) and untreated, bilateral controls (left) in group without RB2 or Optison injection.

The second cohort of animals in the last study group had tumors that required an additional 10 days to develop (to somewhat smaller average size). These animals were then treated precisely the same way as the first cohort; however, the results were significantly different (See Figs. 6 and 7). In fact, following treatment with either Optison or RB2, both the treated and control tumors started to regress. It would appear that treatment of one tumor resulted in an immunological boost that also cleared the control tumor. Why some animals reacted this way while others did not is uncertain since all animals were athymic nu/nu mice from Charles River

of the same age, but it is probably linked to the slower initial rate of growth. It is possible the slow growth was indicative of an already heightened immune system, which the in vivo lysis of the tumor cells then boosted. Unfortunately, the splitting of our remaining animals into two cohorts reduced the total number of animals in each group, as well as our chances of finding a significant statistically result for either set.

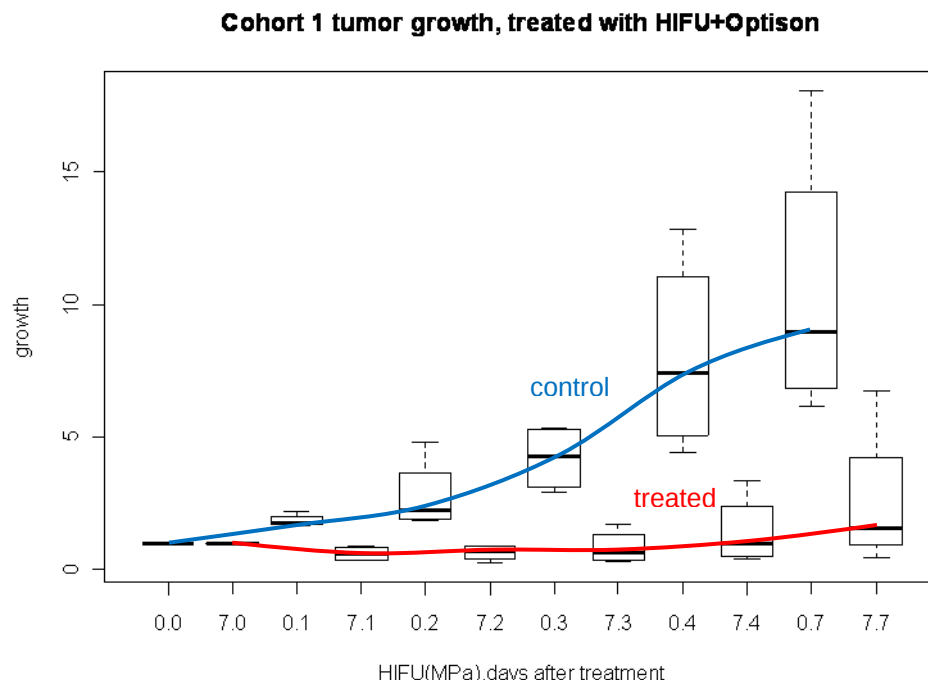


Figure 6. Tumor growth of first cohort (four animals), receiving HIFU treatment with Optison injection and possibly RB2 IV injection (2 animals). For all four animals, the treated tumors regressed while the control tumors grew normally.

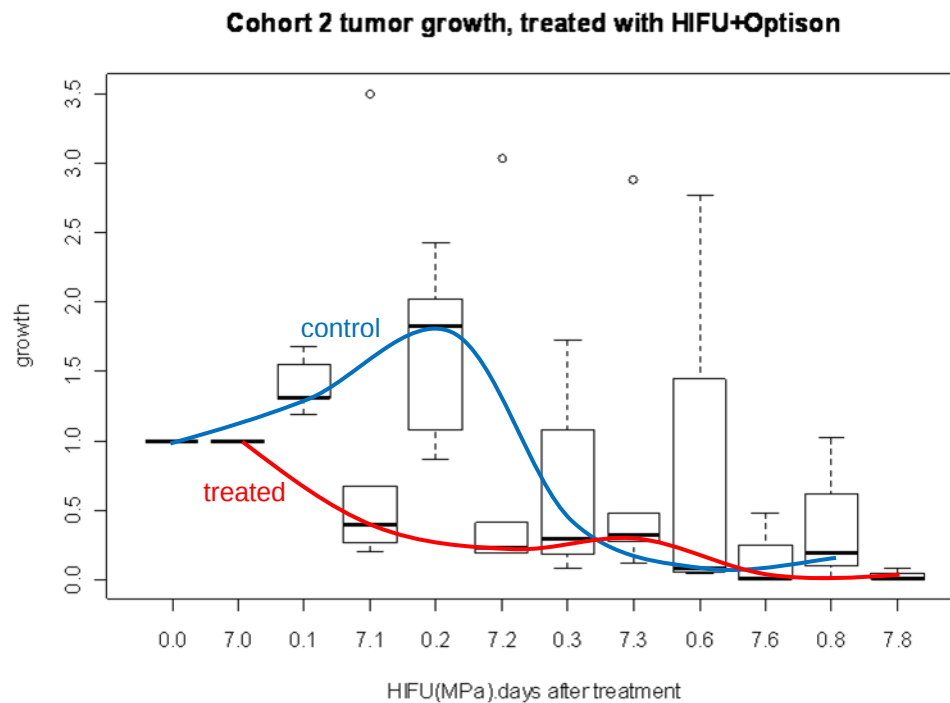


Figure 7. Tumor growth of second cohort (four animals), receiving HIFU treatment with Optison injection and possibly RB2 IV injection (2 animals). For all four animals, both treated and control tumors regressed over time. The tumor regression was most likely caused by an immune response induced by the cavitation lysis of the treated tumor.

Key Research Accomplishments

- Created a novel sono-sensitive compound based on Rose Bengal. RB2 is based on a photosensitive drug, but was altered to:
 - have greater affinity to the cell membrane, and
 - have no light associated toxicity.
- Tested the new compound in vitro against breast cancer cell lines, using pulsed high intensity focused ultrasound for activation. Demonstrated strong synergistic effect, with the combination of compound and ultrasound cavitation killing over 95% of cells, while neither the drug nor the ultrasound alone showed any significant effects.
- Compared RB2 to various sonosensitive porphyrins mentioned in the literature.
- Found HIFU activation is, in general, more lethal than the activation reported in sonodynamic literature using unfocused ultrasound.
- Demonstrated that cavitation is required for this effect.
- Demonstrated that the method of killing is direct membrane disruption (lysis).
- Showed that the addition of NAC, a known antioxidant, reduces but does not eliminate the effect.
- Tested the combination of cavitation, RB2 and HIFU (7MPa only) in vivo, and showed that cavitation, whether with or without the sonosensitizer, resulted in a significant drop in tumor growth
- Discovered a potential immunological response due to cavitation ablation

Reportable Outcomes

1. Provisional patent on RB2 compound for use as a sonosensitizer.
2. Dr. Rongmin Xia is now Assistant Professor at the University of Arkansas.

Conclusion

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. The cytotoxicity is based on cell lysis and necrosis rather than apoptosis. 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. This result is correlated with increased bubble production. It is unclear whether the physical action of the bubbles themselves is responsible for the cytotoxicity, or whether reactive species generated in the bubbles are also partially responsible. Traditional photo-sensitive sonodynamic compounds (porphyrins) were compared with the RB2. Only one of these (mesoporphyrin) demonstrated a very similar effect at nearly the same concentration. This 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 cavitation was initiated in that tube. Cavitation initiation near the threshold appears to be somewhat stochastic in nature. Once cavitation is initiated, cell viability drops by about 11%/second of treatment time (at 50% duty cycle, 1 Hz repetition rate). The cavitation threshold appears to be dependent on the presence of the RB2 or other compounds, making. The cavitation in turn lyses the cells either directly or in concert with reactive species produced from the sonosensitizer, possibly as described in Ref. 8. It is not clear how or why the cavitation threshold is reduced by the addition of very small amounts of these compounds.

The translation of this synergistic effect to an in vivo model is complicated by the difficulty of initiating cavitation in vivo. We used a microbubble injection (Optison) to assist in this. Ultimately one would like to get to a point where this is not needed. As it happened, the resulting cavitation did yield a significant tumor ablation by itself without the assistance of the RB2 compound, as manifested clinically by regression of implanted tumors in mice. Unfortunately, the tumor model was plagued with issues, which has reduced the significance of the results. We were not able to distinguish any additional effects of the RB2 compound administration beyond the damage caused by the cavitation induced by the Optison, perhaps because the cavitation ablation was already quite effective. This was only tested for ultrasound powers similar those that worked in vitro; it is possible that there would be a difference at lower powers. The form of “cavitation ablation” studied here may find useful application compared to the more typical thermal ablation precisely because it is not thermal and therefore does not cause protein denaturation. The tumor proteins are then available to be scavenged by the immune system, possibly resulting in a strengthened immune reaction against the tumor and against metastases. Indeed, this is the likely explanation for at least one anomalous set of results, where animals treated with cavitation ablation saw subsequent regression of their untreated control tumors.

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