# Enhancing cell kill *in vitro* from hyperthermia through pre-sensitizing with ultrasound-stimulated microbubbles

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**Abstract:** Ultrasound-stimulated microbubbles (MBs) were demonstrated to enhance cell kill from hyperthermia. Definity MBs were injected into wells containing 4T1 cells in culture media and scanned with 1-MHz ultrasound, an exposure duration of 30 s and a negative pressure of 0.5 or 1.3 MPa. Some cell samples were placed in a water bath heated to 42 °C for 5 min. Cell death was quantified. When combining MBs, ultrasound at 1.3 MPa and hyperthermia, more than 58.8% ± 7.21% of cells were nonviable. When exposed to hyperthermia alone or exposure to MBs and ultrasound but no hyperthermia, cell death was less than 10.1% ± 6.96% and 30.1% ± 10.8%, respectively.


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## 1. Introduction

In recent years, ultrasound in combination with microbubbles (MBs) has been explored for its ability to produce therapeutic effects. McDannold *et al.* (2006) showed that MBs could enhance the ability of focused ultrasound to create lesions in the brain at lower power levels. Ultrasound in combination with MBs has been used to enhance thrombus destruction (Culp *et al.*, 2011) (sonothrombolysis) and to transfect cells through sonoporation (Bao *et al.*, 1997).

Other studies have demonstrated that exposure of cells to ultrasound and MBs followed by exposure to ionizing radiation resulted in increased cancer cell death with much lower radiation dose (Karshafian *et al.*, 2009). Acute myeloid leukemia (AML-5) cells in suspension were exposed to ultrasound pulses, ultrasound contrast agents (Definity) and ionizing radiation. The ultrasound contrast agents were MBs with diameters ranging from 0.5 to 10 $\mu$m. Clonogenic viability assays were conducted to quantify cell death from the combined therapy. Cell death increased by ~35% when combining ultrasound with MBs and radiation compared to ultrasound alone or radiation treatment alone. Therefore, it was concluded that ultrasound-stimulated microbubbles (USMBs), i.e., collapsing MBs through ultrasound exposure, could enhance the therapeutic effects of ionizing radiation, thereby possibly reducing the required ionizing radiation dose for therapy.

In a similar experiment, prostate cancer cells and breast cancer cells *in vitro* were treated with a chemotherapy agent (Taxotere) with exposure to ultrasound and MBs to demonstrate a synergistic effect (Karfashian *et al.*, 2010). Clonogenic assays were used to determine if a synergistic effect was observed. A statistically significant increase in therapy efficacy was observed when using chemotherapy combined with ultrasound and MBs.

Follow on studies *in vivo* using mouse xenografts demonstrated the ability of ultrasound-mediated MB vascular disruption to synergistically enhance the response of tumors to radiation therapy (Czarnota *et al.*, 2012; Tran *et al.*, 2012). Pre-treating tumors with ultrasound and MBs followed by radiation therapy resulted in a synergistic effect. A smaller dose of radiation could be given to achieve a large tumoricidal response when using the pre-sensitization from ultrasound and MBs. Therefore, the *in vitro* radiation results have been demonstrated to translate *in vivo*.

In a study conducted by ter Haar *et al.* (1980), V79 cells and HeLa cells were irradiated with continuous ultrasound (3 W cm$^{-2}$). Some cells were irradiated at a temperature of 37 °C for 6 hours and some cells were irradiated at temperatures in the

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hyperthermia (HT) range (42 °C–45 °C). At 37 °C, no cell killing was observed. However, at the HT ranges, greater cell kill was observed than could be accounted for by HT alone.

The results from these studies suggest that first exposing cells to ultrasound and MBs can pre-condition cells and tumors to be more sensitive to subsequent therapy. In this study, cells in culture were exposed to ultrasound and Definity MBs followed immediately by low-grade HT treatment. The effects of the combined therapy were quantified and compared to HT treatment alone and treatment with USMBs alone.

## 2. Methods

### *2.1 Cell culture*

A mouse mammary tumor cell line (4T1 [CRL-2539]) was purchased from American Type Culture Collection (ATCC, Manassas, VA). The 4T1 cells were stored at −70 °C, thawed at 37 °C in a water bath, grown in RPMI 1640 medium with 10% fetal bovine serum and antibiotic/antifungal supplements (ATCC, Manassas, VA), and incubated at 37 °C at 100% humidity and 5% $CO_2$. Cells were grown in 75 $cm^2$ tissue culture flasks (T-75, Corning Incorporated, Corning, NY). When cells were 80% confluent, they were replated in 96 well plates (BD Falcon, San Jose, CA) for subsequent therapy. Each well in the plate held 0.37 mL of liquid and had a diameter of 6.4 mm. Approximately 14 560 cells were added in each well such that after incubating the plate overnight a monolayer of greater than 90% confluence was present in each well.

### *2.2 Ultrasound and MB exposure*

In the experiments, the 96 well cell plates were filled with culture medium, covered by MicroAmp optical adhesive film (Applied Biosystems, Foster City, CA) to act as an acoustic window, and placed inverted in a tank of degassed water. For cells being exposed to ultrasound and MBs, Definity MBs were first injected into the cell plates before covering with the optical adhesive film. By inverting the cell plate, this ensured that the rising MBs would be at the surface to which the cells were adhered. Definity contains octafluoropropane and is stabilized by a phospholipid shell. The concentration of Definity in the vial is $120 \times 10^8$ $mL^{-1}$ gas bodies. The mean diameter ranges from 1.1 to 3.3 $\mu$m, with 98% of the MBs smaller than 10 $\mu$m. For cells being exposed to ultrasound and MBs, Definity MBs of concentration 1%/vol were used. Therefore, approximately $4.44 \times 10^7$ MBs ($0.37 \times 120 \times 10^8 \times 1/100$) were present in each well. For uniform mixing of MBs in the culture medium, a mixture of culture medium and MBs were first prepared separately and then pipetted into each well.

An ultrasonic transducer was then used to expose the surface of the cell plate. The transducer had a center frequency of 1 MHz, focal length of 5.7 cm and f-number of 3 (Model: E1051, Valpey Fisher Instrument Inc, Hopkinton, MA). The −6-dB beam width at the focus was 4.5 mm as measured using a National Physical Laboratory calibrated polyvinylidene difluoride bilaminar shielded membrane hydrophone (diameter of the active element, 0.5 mm; Marconi 699/1/00001/100; GEC Marconi Ltd., Great Baddow, England). A schematic of the experimental setup is shown in Fig. 1. The transducer was positioned such that the focal point was at the backside of a plate of the 96 well plate, i.e., at the cell surface. The transducer was held stationary and exposed a single well plate at a time. Once one well plate was exposed, the transducer was aligned with another well plate by moving the 96 well plate using a computer-controlled micropositioning system (Daedal, Inc., Harrisburg, PA). The transducer was driven using a pulser/receiver (RAM500, Ritec, Warwick, RI). The Ritec pulser-receiver generated a five-cycle sinusoidal tone bursts with a pulse repetition rate of 100 Hz. An exposure duration of 30 s per well was used for the experiments. A peak rarefactional pressure amplitude (PRPA) of 0.5 or 1.3 MPa was used for all the exposures and designated as low and high pressure settings. These pressure level estimates accounted for the normal incidence reflection from the back wall of the culture plate.

### *2.3 HT treatment*

In the experiments, immediately after exposure to ultrasound, the cell culture plates were submerged in a tank of degassed water heated to 42 °C for 5 min. The temperature at a well plate wall was monitored by placing a thermocouple at the wall of the plate. Next the plate was removed from the water bath and the optical adhesive film was removed. The culture medium was then removed from each well and 200 $\mu$l of

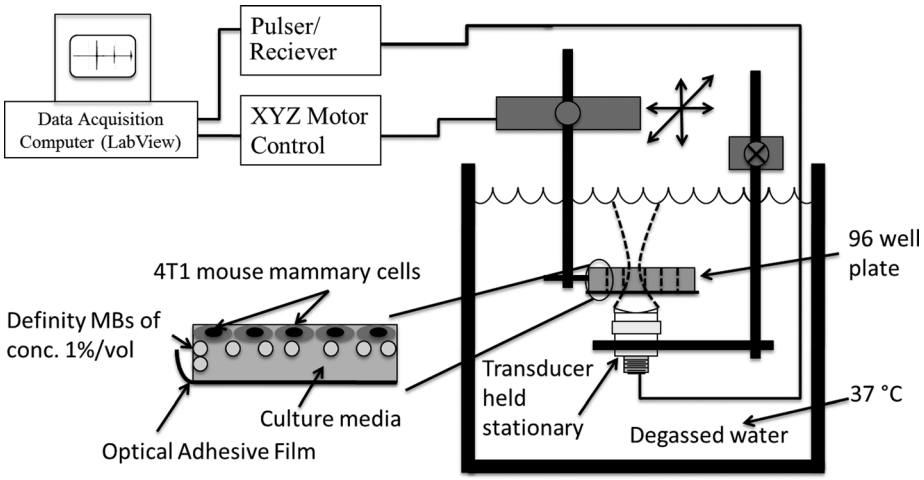


Fig. 1. Experimental setup.

fresh culture media was pipetted into each well. The plates were then incubated for 24 h at 37 °C at 100% humidity and 5% $CO_2$.

*2.4 MTT cell proliferation assays*

Cell kill in the cell culture studies was quantified through the MTT Cell Proliferation Assays. Yellow tetrazolium salt MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)) is reduced in metabolically active cells, which forms purple colored crystals that can be quantified by spectroscopic analysis (van de Loosdrecht *et al.*, 1994). The MTT assay measures cell proliferation rates. After incubating the cells for 24 h after HT treatment, 20 $\mu$L of MTT reagent was added into each well and incubated for additional 4 h. Each well was then inspected under the microscope to clearly visualize the presence of purple precipitate. Then 100 $\mu$L of detergent reagent was added to each well and incubated for another 12 h. The absorbance was measured using a 540 nm microplate spectrophotometer (ELx800 Absorbance Microplate Reader and Gen5, BioTek, Winooski, VT), to estimate the concentration of the dissolved purple crystals. The percentage cell viability was calculated based on the optical density of the solutions compared to the control cell plates.

*2.5 Statistical analysis*

Seven experimental conditions were tested: (1) ultrasound PRPA of 0.5 MPa in presence of MB followed by HT (0.5+MB+HT), (2) ultrasound PRPA of 1.3 MPa in presence of MB followed by HT (1.3+MB+HT), (3) presence of MB followed by HT (MB+HT), (4) HT only (HT), (5) ultrasound PRPA of 0.5 MPa in presence of MB (0.5+MB), (6) ultrasound PRPA of 1.3 MPa in presence of MB (1.3+MB), and (7) control. A one-way analysis of variance was used to test for statistically significant differences in cell viability between different therapy configurations. Statistically significant differences were quantified through $p$ values $<0.05$.

## 3. Results

The percentage cell viability using the different experimental configurations is listed in Table 1. The lowest cell viability of 41.2% ± 7.21% was observed when using exposure pressure of 1.3 MPa combined with MBs and HT. When the cells had no exposure to ultrasound but exposure to HT alone or exposure to MBs and high pressure ultrasound but no HT, cell viability was observed to be 89.9% ± 6.96% and 69.9% ± 10.8%,

Table 1. Cell viability as a percent compared to control of 4T1 cells for various experimental configurations.

| Therapy | Mean (%) | Standard Deviation (%) |
|---|---|---|
| 0.5+MB+HT | 69.4 | 12.4 |
| 1.3+MB+HT | 41.2 | 7.21 |
| MB + HT | 85.8 | 8.22 |
| HT | 89.9 | 6.96 |
| 0.5 + MB | 91.6 | 8.24 |
| 1.3 + MB | 69.9 | 10.8 |

respectively. The positive control samples, which did not undergo any therapy, were assumed to have 100% viability for comparison with the treated samples. Each experimental configuration was repeated at least three times to estimate the variance in the percentage of cell viability for the respective configuration. The 0.5 MPa exposures had similar trends but with reduced cell kill observed. The combined therapy using the 0.5 MPa pressure level resulted in 69.4% ± 12.4% viability of cells. Using only USMBs at 0.5 MPa without HT resulted in 91.6% ± 8.24% viability. The statistical significance between any two pair of experimental configuration is tabulated in Table 2. The treatment when using exposure pressure of 1.3 MPa combined with MBs and HT resulted in the maximum cell death, which had statistically significant differences ($p$ value $<0.05$) compared with all other therapy configurations.

## 4. Discussion and conclusion

The results from the cell culture studies suggest that the combined therapy synergistically increased the cell kill. If the effects were merely additive, then the expected kill (using the 1.3 MPa results) from the combined therapy would be 30% kill for USMBs times another 10% kill for the HT treatment or a total additive kill rate of 40% (a resulting viability of 60%). The observed viability of 41% means that the kill rate was 59% for the combined therapy. For the 0.5 MPa exposures, an 8% kill rate of the USMBs combined with the 10% kill rate of the HT would suggest a total additive kill rate of 18% (a resulting viability of 82%). However, the viability of the 0.5 MPa exposures was 70%. This means the kill rate was 30% versus the additive effect estimate of 18% for the 0.5 MPa exposure. Therefore, the kill rate when using ultrasound and MBs followed by HT was more than additive.

When exposing the cell plates to ultrasound, the entire surface of an individual cell plate was not exposed to ultrasound and MBs. The diameter of an individual cell plate was 6.4 mm and the −6-dB beamwidth was estimated at 4.5 mm. Therefore, the beam of the ultrasound only covered the center of an individual cell plate well and a portion of the cells were not exposed to ultrasound. For cells not exposed to ultrasound, it is possible that no effect would be observed. The killing effects of exposing cells to USMBs may potentially be greater than what was assessed in these experiments because some fraction of the cells assayed would only be exposed to HT. Therefore, the synergistic effect in the cell culture studies is likely higher than was observed considering the ultrasound beam size was smaller than the well diameter.

Synergistic effects of USMBs on HT were investigated using *in vitro* experiments. It is hypothesized that the USMBs will sensitize the cells such that the therapeutic effect is enhanced. At 1.3 MPa, the 90% threshold for collapse of Definity is reached, whereas at 0.5 MPa the collapse threshold is closer to 50% (King and O'Brien, 2014). As the pressure was increased, the effect was increased. Likewise, as the pressure increases the MBs are collapsed more rapidly. Therefore, our data would suggest that collapsing MBs were involved with the observed effects, i.e., as more MBs collapsed a larger effect was observed.

The HT dose (42 °C for 5 min) provided in the cell culture studies represents a very low dose compared to what is typically provided clinically. Employing the formulation of Sapareto and Dewey the thermal dose is given by (Sapareto and Dewey, 1984; Perez and Sapareto, 1984)

$$\mathrm{CEM}_{43} = \sum_{t=0}^{t=\mathrm{final}} R^{(43-T_t)}\Delta t, \quad \begin{cases} R = 0.25 & \text{for} \quad T < 43\,^{\circ}\mathrm{C} \\ R = 0.50 & \text{for} \quad T \geq 43\,^{\circ}\mathrm{C}, \end{cases} \tag{1}$$

where $T_t$ is the average temperature recorded by the thermocouple during time $\Delta t$. The cells were exposed to a temperature of 42 °C for 5 min corresponding to thermal dose

Table 2. $p$ values of pairwise comparison for statistical significance.

| Therapy | 0.5+MB+HT | 1.3+MB+HT | MB+HT | HT | 0.5+MB | 1.3+MB |
|---|---|---|---|---|---|---|
| 0.5+MB+HT | 1.0000 | 0.0077 | 0.0706 | 0.0280 | 0.0246 | 0.9554 |
| 1.3+MB+HT | 0.0077 | 1.0000 | 0.0002 | 0.0001 | 0.0001 | 0.0045 |
| MB + HT | 0.0706 | 0.0002 | 1.0000 | 0.4700 | 0.3531 | 0.0579 |
| HT | 0.0280 | 0.0001 | 0.4700 | 1.0000 | 0.7625 | 0.0206 |
| 0.5 + MB | 0.0246 | 0.0001 | 0.3531 | 0.7625 | 1.0000 | 0.0186 |
| 1.3 + MB | 0.9554 | 0.0045 | 0.0579 | 0.0206 | 0.0186 | 1.0000 |

of $CEM_{43} = 1.25$ min. Typically most tissues are undamaged if treated for 1 h up to a temperature of 44 °C (van der Zee, 2002).

As a result, with this low dose alone, very little cell death was obtained (10%). However, when combining the low dose HT with USMBs, a significant killing enhancement was observed. Therefore, it is expected that these effects would be even more extensive if a higher HT dose were utilized. However, the use of lower HT doses to achieve dramatic cell kill suggests that the USMB pre-sensitization can reduce the need for long HT treatments.

Caution should be used when inferring how tumors *in vivo* would respond to the combination of USMBs and HT. The enhanced killing of cells *in vitro* does not necessarily mean that these techniques will translate *in vivo* and further testing must be conducted to determine the *in vivo* effects. If this technique can be successfully translated to *in vivo*, one benefit of using USMBs for pre-treatment is that the therapy can be spatially targeted to a tumor region. The ultrasound fields used to activate the MBs and perturb the vasculature in tumors can be targeted by focusing to a small volume. Therefore, the effects of the combined therapy can be confined to just the tumor volume and surrounding healthy tissues can be spared. HT more effectively treats regions in tumors with low blood perfusion or hypoxic regions because in these regions the heat provided by HT is not carried away by blood flow (Moyer and Delman, 2008). By treating a tumor with USMBs, it is possible that vascularized regions of the tumor can be made more sensitive to HT treatment. This suggests that combining USMBs with HT will mitigate some of the weaknesses associated with traditional HT treatment of vascularized portions of tumors.

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## References and links

Bao, S., Thrall, B. D., and Miller, D. L. (**1997**). "Transfection of a reporter plasmid into cultured cells by sonoporation in vitro," Ultrasound Med. Biol. **23**, 953–959.

Culp, W. C., Flores, R., Brown, A. T., Lowery, J. D., Roberson, P. K., Hennings, L. J., Woods, S. D., Hatton, J. H., Culp, B. C., Skinner, R. D., and Borelli, M. J. (**2011**). "Successful microbubble sonothrombolysis without tissue-type plasminogen activator in a rabbit model of acute ischemic stroke," Stroke **42**, 2280–2285.

Czarnota, C. J., Karshafian, R., Burns, P. N., Wong, S., Mahrouki, A. A., Lee, J. W., Caissie, A., Tran, W., Kim, C., Furukawa, M., Wong, E., and Giles, A. (**2012**). "Tumor radiation response enhancement by acoustical stimulation of the vasculature," Proc. Natl. Acad. Sci. U.S.A. **109**, E2033–E2041.

Karshafian, R., Almasri, F., Giles, A., and Czarnota, G. J. (**2010**). "Enhancing chemotherapy by ultrasound and MBs: Effect of acoustic pressure and treatment order in in vitro suspension of breast and prostate cancer cells," in *Proceedings of the 2010 IEEE International Ultrasonics Symposium*, San Diego, CA, pp. 1582–1585.

Karshafian, R., Giles, A., Burns, P. N., and Czarnota, G. J. (**2009**). "Ultrasound stimulated microbubbles as novel enhancers of radiotherapy in Leukemia cells in vitro," in *Proceedings of the 2009 IEEE International Ultrasonics Symposium*, Rome, Italy, pp. 1792–1795.

King, D. A., and O'Brien, W. D., Jr. (**2014**). "Quantitative analysis of ultrasound contrast agent postexcitation collapse," IEEE Trans. Ultrason. Ferroelect. Freq. Contr. **61**, 1237–1241.

McDannold, N. J., Vykhodsteva, N. I., and Hynynen, K. (**2006**). "Microbubble contrast agent with focused ultrasound to create brain lesions at low power: MR imaging and histologic study in rabbits," Radiology **241**, 95–106.

Moyer, H. R., and Delman, K. A. (**2008**). "The role of hyperthermia in optimizing tumor response to regional therapy," Int. J. Hyperthermia **24**, 251–261.

Perez, C. A., and Sapareto, S. A. (**1984**). "Thermal dose expression in clinical hyperthermia and correlation with tumor response/control," Cancer Res. **44**, 4818–4825.

Sapareto, S. A., and Dewey, W. C. (**1984**). "Thermal dose determination in cancer therapy," Int. J. Radiation Oncol. Biol. Phys. **10**, 787–800.

ter Haar, G., Stratford, I. J., and Hill, C. R. (**1980**). "Ultrasonic irradiation of mammalian cells in vitro at hyperthermic temperatures," Br. J. Radiol. **53**, 784–789.

Tran, W. T., Iradji, S., Sofroni, E., Giles, A., Eddy, D., and Czarnota, G. J. (**2012**). "Microbubble and ultrasound radioenhancement of bladder cancer," Br. J. Cancer **107**, 469–476.

van de Loosdrecht, A. A., Beelen, R. H. J., Ossenkoppele, G. J., Broekhoven, M. G., and Langenhuijsen, M. M. A. C. (**1994**). "A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukimc cells from cell lines and patient with acute myeloid leukemia," J. Immunol. Methods **174**, 311–320.

van der Zee, T. (**2002**). "Heating the patient: A promising approach?," Ann. Oncol. **13**, 1173–1184.