

# Ultrasound-Stimulated Microbubbles Enhance Radiation-induced Cell Killing

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## Research

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## Abstract

**Background:** There has been recent interest in the use of ultrasound-stimulated microbubbles (USMB) as a localised radiosensitiser, with *in vivo* studies to date demonstrating impressive results. *In vitro* results have focussed primarily on Human Umbilical Vein Endothelial Cells (HUVEC), and studies using other cell lines have shown varying results. This study aims to further investigate any increases in radiation-induced cell killing *in vitro* using HUVECs and two carcinoma cell lines; and to study the effect different radiation energies have on cells treated with ultrasound-stimulated microbubbles and radiation.

**Methods:** HUVEC, Metastatic Follicular Thyroid Carcinoma cells (FTC-238) and Non-Small Cell Lung Carcinoma cells (NCI-H727) were treated using a combination of 1.6% v/v microbubbles (Definity®); ~90 seconds of 2MHz ultrasound with a mechanical index of 0.8; and 0-6Gy of kV or MV x-rays. Cell viability was measured using a cell viability assay 72 hours post-treatment, and normalised survival calculated relative to untreated controls. Statistical significance was determined using a Three-Way Analysis of Variance (ANOVA) after confirming equal variance existed across all groups.

**Results:** All cells treated with combined USMB and radiation demonstrated decreased normalised survival, with statistically significant effects observed for the NCI-H727 cells. Statistically significant differences in the effect of kV and MV radiation were also observed for FTC-238 cells.

**Conclusions:** USMB and Radiation Therapy significantly reduced the viability of NCI-H727 cells when compared to cells exposed to radiation treatment only. Decreased cell viability was also observed in HUVEC and FTC-238 cells, but this was not significant. Further studies using increased Microbubble concentrations may be required to achieve statistically significant results as reported elsewhere. Similarly, further research is needed to understand the differing effect of kV and MV x-rays on the survival of FTC-238 cells.

## Background

Microbubbles (MBs) have long been used as contrast agents to improve image quality in ultrasound (US) cardiac imaging [1]. Their size is similar to the average erythrocyte, meaning they remain within the endovascular borders during their travel from the site of injection to the site of interest. This feature has resulted in MBs also being investigated for a variety of focussed therapeutic purposes, as they can be 'burst' using higher US intensities once at the site of interest, creating localised biophysical disruptions in the vicinity [2, 3], and if loaded with a treatment agent, delivering a localised therapeutic payload [4].

## In vivostudies to date

Consequently, there has been recent interest in using Ultrasound-Stimulated Microbubbles (USMB) as localised radiosensitisers of radiation treatment (RT), with *in vivo* studies demonstrating some promising results. Czarnota et al. [5] reported a statistically significant increase in mean animal survival from 19 days with RT alone to 28 days using USMB and RT together (USMB + RT). PC3 human prostate cancer xenografts in mice were treated twice weekly with USMBs in conjunction with 24Gy in 12 fractions of 160kVp x-rays delivered four times a week for three weeks. Daecher et al. [6] reported a mean animal survival of 11 days with RT alone compared to 35 days using USMB + RT in treating human hepatocellular carcinoma xenografts in nude rats. In this study the xenografts were treated with USMBs, followed by a single dose of 5Gy kV x-rays delivered using 4x 1.25Gy beams. Two animals treated with USMB + RT showed complete tumour control, with tumour reduction or stability observed 50 days post-treatment. Eisenbrey et al. [7] observed a statistically significant growth delay for MDA-MB-231 human adenocarcinoma breast xenografts in mice using USMB + RT compared to the control of US and oxygen MBs alone ( $p = 0.03$ ), or US and RT in the absence of MBs ( $p = 0.01$ ). Animals were treated with in-house manufactured oxygen or nitrogen USMBs and a single fraction of 5Gy using 310 kV x-rays.

Other *in vivo* studies have also demonstrated that USMB + RT increased tumour cell death 24 hours after treatment. Al-Mahrouki et al. [8], using clonogenic assays from excised PC3 xenografts in mice, showed decreases in cell survival from 45.5% for 2Gy alone compared to 26.8% for USMB + 2Gy; and 38.2% for 8Gy alone compared to 14.4% for USMB + 8Gy, respectively. Lai et al. [9], using *in situ* end-labelling (ISEL) staining, reported increased rates of tumour death for MDA-MB-231 xenografts in Swiss nude mice of 3.4- and 2.3-fold respectively, for USMB + 2Gy or USMB + 8Gy treatments compared to that of 2Gy or 8Gy RT alone.

It has been proposed that USMB + RT acts as a biophysical vascular disruptor *in vivo*, acting primarily on endothelial cells to disturb tumoural blood supply [10]. As a result of downstream vascular disruption, overall tumour control can be achieved at much lower radiation doses as microvascular collapse and rapid vascular shut down leads to secondary tumour cell death once these cells become starved of nutrients and oxygen. Consequently, three *in vitro* studies were centred around human umbilical vein endothelial cells (HUVECs) using 3.3% v/v MBs, 30 seconds of US with a mechanical index of 0.8, and between 0 and 8Gy of 160 kVp x-rays. Clonogenic assays (normalised to control) demonstrated an increase in cell death from 67% for 8Gy alone ( $p < 0.014$ ) to 95% for USMB + 8Gy ( $p < 0.002$ ) [11], and a decrease in cell survival from 8% using 2Gy alone, to 1% using USMB + 2Gy [12]. The third study using HUVECs [13], found that treatment with USMB alone or USMB + RT caused cell membrane distortion, which was not evident in cells treated using RT alone, suggesting multiple mechanisms of cell damage may be achieved through the combined USMB + RT treatment.

## In vitro studies to date

Other *in vitro* studies investigating direct tumour cell radiosensitisation using USMB, have also been conducted. Karshafian et al. [14] exposed acute myeloid leukaemia (AML-5) cells to varying concentrations of MBs, differing US pressures and 2-8Gy of 160kVp x-rays; and observed that cell viability decreased with increased MB concentration, until maximal death was achieved at ~ 1.6% v/v. Higher USMB levels did not increase cell killing but rather conferred a slightly protective effect. When AML-5 cells were exposed to 570kPa US Peak Negative Pressure (PNP), and 3.3% v/v USMB, cell viability was  $71 \pm 7\%$  which was higher than that seen for cells only exposed to 4Gy radiation ( $55 \pm 5\%$ ). The order of treatment (i.e. USMB followed by RT compared to RT followed by USMB) was shown to have minimal impact on cell viability. Clonogenic assays were 11% for 2Gy alone vs 2.5% for USMB + 2Gy normalised to control (no treatment). Clonogenic survival rates also fell in another study [15], when PC3 and KHT-C murine fibrosarcoma cells were treated with USMB + 3Gy (PC3 17% and KHT-C 34%, respectively) compared to RT-alone (PC3 50% and KHT-C 75%, respectively). Treatment order and time between treatments also impacted clonogenic survival, with greatest decreases in clonogenic viability seen when PC3 cells were exposed to USMB followed by immediate 3Gy treatment (17%), compared to that of KHT-C cells treated with USMB followed by a 3-hour gap before being exposed to 3Gy (30%).

Deng et al. [16] treated NCE-2 nasopharyngeal carcinoma cells with 3% v/v perfluoropropane-based USMBs, administered 24 hours prior to exposure to 2Gy or 8Gy RT. Cell viability was measured using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay 24, 48 and 72 hours post-treatment. Significant ( $p < 0.01$ ) decreases in viability for NCE-2 cells treated with USMB + 2Gy compared to 2Gy was observed at 48 hours; and at 72 hours USMB + RT significantly increased cell death for both radiation doses. However, in a study of human pharyngeal squamous carcinoma (FaDU) cells, no differences between cells treated with USMB + RT and RT alone were observed in clonogenic assays [17]. In this study the cells were exposed to  $1\mu\text{g/ml}$  Cisplatin and USMB (700 $\mu\text{l}$  of SonoVue™) 24 hours before being treated with varying doses of MV  $\gamma$ -rays (2, 4, 6, 8 or 10Gy). The addition of USMB did not enhance any nuclear damage to that elicited by RT alone in these cells.

Given the variation between results reported for *in vivo* studies compared to *in vitro*, the purpose of this study was to investigate the role of USMBs in directly enhancing radiation-induced tumour cell killing *in vitro*. Two tumour cell lines not previously investigated before were used to determine whether direct USMB radiosensitisation could be achieved for both a primary and metastatic cell line. HUVECs were also investigated to allow for comparisons to existing published data. Definity® MBs were selected as they are the only commercially available MB approved for clinical use in Australia, and clinical hardware was used to provide US simulation. Both kV and MV x-rays were used to investigate if different radiation energies enhance the cytotoxic effect of USMBs, as previously it was shown that MV energies did not [17].

## Methods

### Cell lines and Culture Conditions

Experiments were performed using HUVEC (Human Umbilical Vein Endothelial Cells), FTC-238 (lung metastasis of Follicular Thyroid Carcinoma) and NCI-H727 (Non-Small Cell Lung Carcinoma). HUVECs (catalogue number C2519A, pooled donor) were purchased from Lonza (Walkersville, USA). FTC-238 and NCI-H727 cell lines were supplied by the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom) as catalogue numbers 94060902 and 94060303 respectively, and were purchased from CellBank Australia (Westmead, Australia). All cell lines have been previously described [18–20].

Cells were maintained according to the manufacturer's instructions and incubated at 37°C, 5% CO<sub>2</sub> and 95% humidity. HUVEC cells were cultured using EGM-2 Endothelial Cell Growth Medium-2 BulletKit™ from Lonza (catalogue number CC-13162). FTC-238 cells were cultured using DMEM:F12 (catalogue number D8437, Sigma-Aldrich, Sydney, Australia), supplemented with 5% FBS (Corning, catalogue 35-076-CV supplied by Fisher Biotec, Wembley, Australia) and 1% Penicillin-Streptomycin (Pen-Strep) (catalogue number 15070063, Sigma-Aldrich). NCI-H727 cells were cultured in RPMI 1640 (catalogue number 11875093, Thermo Fisher, Scorsby Australia), supplemented with 10% FBS and 1% Pen-Strep.

## Plating and MTS Assay optimisation

Cell Proliferation Assays were initially optimised in the absence of treatments using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (catalogue number G3582, Promega, Australia). The MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is similar to the MTT compound used in previous studies [16], and is bio-reduced by viable cells into a coloured formazan product that can be analysed via colorimetry. Cells are incubated with the reagent for between 1-4 hours, and then absorbance readings taken at 490nm in a 96-well plate reader. The amount of formazan product measured by absorbance is directly proportional to the number of viable cells in the culture [21].

Cell seeding densities and MTS development times were optimised to achieve 80% confluence at 72 hours post-treatment for each cell line, as summarised in Table 1. This level of confluence was chosen in order to avoid any potential effects of contact-dependent cell signalling or contact inhibition, and 72 hours is consistent with the manufacturer's instructions for cell proliferation studies and other experiments using this assay [21–23] or the MTT assay [16].

Table 1  
Cell seeding densities and MTS development times

Cell Line	Flask Size	Seeding Density	MTS development time
HUVEC	T25cm	8,000 cells / flask	25min
FTC-238	T25cm	12,000 cells / flask	25min
NCI-H727	T12.5cm	50,000 cells / flask	15min

HUVEC and FTC-238 cells from stock cultures were plated into T25cm<sup>2</sup> Tissue culture flasks (Sigma-Aldrich, catalogue number CLS430639). Due to supply issues, NCI-H727 cells were plated into T12.5cm<sup>2</sup> flasks (Bio-Strategy, catalogue number BDAA353107, Tullamarine, Australia). Cells at the designated seeding densities were allowed to adhere overnight in their respective flasks for ~14-16 hours before treatment. Eight flasks were prepared for each experiment – three controls (no treatment), one USMB-alone (USMB), and two flasks for each radiation dose level – one with USMB and one without (ie. 3Gy, USMB+3Gy; 6Gy, and USMB+6Gy). Each experiment was performed in triplicate, with separate experiments run for kV and MV x-ray energies using the HUVEC and FTC-238 cell lines. Concurrent experiments were undertaken for NCI-H727 cells where 12 flasks were prepared for each experiment – four for kV and four for MV in addition to four 0Gy flasks (ie. three controls and one USMB). This experiment was also performed in triplicate.

## Pre-Treatment Preparation

Two hours prior to treatment, cells grown in T25cm<sup>2</sup> flasks (volumes used for the T12.5cm<sup>2</sup> flasks were half that described here) were washed using 5ml Phosphate-Buffered Saline (PBS) (Thermo Fisher, catalogue number 70011069), and detached from their adherent state using 2ml 0.25% Trypsin-EDTA (Thermo Fisher, catalogue number 25200056). Both flask sizes were then filled with 30ml of cell-line specific complete media. Definity® Microbubbles (Lantheus Medical Imaging, Inc, supplied by Global Medical Solutions, Keilor Park, Australia) were activated using the Vialmix® activation device (Lantheus Medical Imaging, Inc) following the manufacturer's instructions [24], and 480µl added to the relevant treatment flasks to give a final microbubble concentration of 1.6% v/v, which was close to that described in other *in vitro* studies [11–16].

The culture flasks including the untreated controls were then transported offsite to the treatment facility at either the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA) (Yallambie, Australia) or the GenesisCare Epping Radiation Oncology Centre (EPROC) (Epping, Australia). Travel time to and from these facilities was around 20min each way, with the cells out of the 37°C incubator for ~90min.

## Ultrasound Treatment

Once onsite at the treatment facility, Ultrasound sonication was applied using the LOGIQ i Portable Ultrasound with the 4C-RS transducer (GE Healthcare, Paramatta, Australia). Here, 2MHz frequency ultrasound was applied directly to the flask using a coupling gel, with a focal point of 3.25cm Focal Point and depth of 4cm to achieve a Mechanical Index of 0.9. These settings were the closest match possible to the mechanical index of 0.8 reported in previous studies [11–13]. The transducer was moved across the flask for ~90 seconds until the opaque, milky-white microbubbles had burst and the media had returned to its normal transparency. Previous preliminary studies using colorimetry and microscopy had shown that this visual change correlated with the bursting of the microbubbles (Data not shown). The flasks were then immediately exposed to either 0Gy, 3Gy or 6Gy of kV or MV x-rays. The three control flasks were left untreated, whilst the USMB only flask received ultrasound sonication in the absence of ionising radiation.

## Radiation Treatment

6MV x-rays were delivered using either a Varian iX (EPROC) or an Elekta Synergy Linear Accelerator (ARPANSA), with the same linac used for each repeat of an individual experiment. Flasks were laid flat on the treatment couch on top of 10cm of solid water, with a source-to-surface distance (SSD) of 100cm set to the top of the solid water from a gantry angle of 0°. Next 2cm of solid water was then placed on top of the flasks to provide a build-up region, and both the RT±USMB flasks irradiated simultaneously using a 20x20cm field size to cover both flasks. A schematic diagram of this setup is shown in Figure 1.

100kVp x-rays (average energy 42.5kV) were delivered using a Model X80 X-Ray Beam irradiator by Hopewell Designs (ARPANSA). A 100cm SSD was set to the baseplate of the unit before the RT±USMB flasks were taped vertically to the baseplate (see Figure 2). A 10cm circle field size was used to expose both flasks concurrently. As the dose rate from this unit was 6mGy/s, the 3Gy and 6Gy doses took 491.8 and 983.6 seconds to deliver respectively, which was the determining factor in the selection of x-ray doses used in this study. The 3Gy dose was chosen as an approximate clinical fractional dose consistent with that used in an earlier study [15], while the 6Gy dose selected as the highest dose deliverable in a reasonable timeframe within the limitations of the 6mGy/s dose rate. Radiation field uniformity and coverage was validated in the x- and y-planes for all radiation setups using Gafchromic film (data not shown).

## Post-Treatment

Following treatments, the flasks were transported back to the laboratory where they were returned into the incubator for a few minutes whilst the laminar flow hood was prepared. Once flasks were examined under the microscope to confirm cells were still detached, flask contents were transferred to a 50ml tube and centrifuged (200g for 5min) to remove any residual treatment compounds. Cells were then resuspended in a T25cm<sup>2</sup> flask containing 10ml complete media and returned to the incubator. After 24 hours, the tissue culture media was replenished, and 48 hours after which cell viability was measured using MTS assay.

## Cell viability measurements

At ~72 hours after treatment, the media was removed from each flask, and replaced with 1ml supplement free (ie. Incomplete) tissue culture media and 200µl MTS at a ratio of 5:1 per the manufacturer's instructions [21]. Four technical replicates of 100µl incomplete media plus 20µl MTS were plated in a 96 well plate to act as media blanks. Flasks and plates were then returned to the incubator for the development times stated earlier. Four technical replicates of 120µl from each flask were then transferred to the 96 well plate and the plate read at 490nm using a CLARIOstar Plus Plate reader (BMG Labtech, Mornington, Australia). The average of the four technical replicates was calculated for each condition, and the average of the four blank wells subtracted to give the final Raw

Absorbance reading for each condition. Values for the three control flasks were averaged within each experiment, and the standard deviation (S.D.) calculated. Normalised survival was then calculated by dividing the Raw Absorbance values by the averaged control for each experiment.

## Statistical Analysis

Overall normalised survival was averaged across the three repeats of the same experiment, and the S.D. calculated using Excel. The key assumptions of the ANOVA test were initially validated in SPSS V26 (International Business Machines Corporation (IBM), USA), using the Shapiro-Wilk (SW) test to confirm normality of distributions; Levine's test to confirm homogeneity of Error Variances (LHEV); and outliers identified visually on the box-and-whisker plot as flagged by the software [25,26]. Statistical significance between treatment groups was then determined via a Three-Way ANOVA using a 2x3x2 design to report on the two levels of USMB (ie. presence or absence), three radiation dose levels of 0, 3 and 6Gy, and two radiation energies of kV and MV x-rays. Post-Hoc analysis included pair-wise t-tests, with p values <0.05 reported as statistically significant [27]. Where LHEV revealed unequal variances, data was re-analysed using a One-Way Welch's ANOVA with Games-Howell Post-Hoc testing after re-expressing the 2x3x2 design as a one-way subprogram [26,28].

A coefficient of variation was calculated using the standard error of the Raw Absorbance values from each experiment and dividing this by the overall average. This value is used to give an indication of any variation arising from differences in plating between experiments [27].

## Results

The effect of USMB on the viability of cells exposed to either kV or MV radiation was examined using three different cell lines to observe if ultrasound stimulated microbubbles directly enhance radiation-induced cell killing *in vitro*. NCI-H727 cells are from a primary non-small cell lung carcinoma (NSCLC), while FTC-238 cells were from a metastatic follicular thyroid carcinoma deposit in lung tissue. Human umbilical vein endothelial cells (HUVECs) were chosen to allow for comparisons to previously published results [11, 13].

Plating between untreated control flasks was fairly uniform, with coefficients of variation for each experiment ranging from 6–11% as summarised in Table 2, suggesting reasonable consistency in cell numbers was achieved for each repeat of the same experiment.

Table 2  
Comparison of the plating efficiency for flasks used in the radiation experiments.

X-rays Treatments	Exp 1	Exp 2	Exp 3	Average	Coefficient of Variation
NCI-H727 - kV & MV	0.42 ± 0.04	0.51 ± 0.03	0.51 ± 0.04	0.48 ± 0.03	7%
FTC-238 - kV	0.43 ± 0.06	0.37 ± 0.03	0.46 ± 0.03	0.42 ± 0.03	8%
FTC-238 - MV	0.48 ± 0.03	0.61 ± 0.01	0.60 ± 0.08	0.57 ± 0.04	8%
HUVEC - kV	0.70 ± 0.06	0.57 ± 0.05	0.68 ± 0.03	0.65 ± 0.04	6%
HUVEC - MV	0.72 ± 0.02	0.54 ± 0.02	0.84 ± 0.05	0.70 ± 0.08	11%

*Three control flasks were used in each experiment, and the cell number determined using the MTS assay. The results expressed as the mean ± SD for 3 replicates for each experiment. The overall average for the experimental repeats is expressed as the mean ± SEM for 9 replicates. The coefficient of variation for each experiment was calculated as SEM / Overall average.*

## NCI-H727 cells

Statistically significant decreases in irradiated cell survival were demonstrated across all RT dose levels and energies through the addition of USMB (Figure 3). Results were very highly significant ( $p < 0.001$ ) for USMB-alone in the absence of RT, with the highest Partial Eta Squared ( $\eta_p^2$ ) value of 0.764 suggesting the presence of USMB had a large effect on survival. The  $\eta_p^2$  for the Radiation dose was 0.728 and for radiation energy it was 0.028, with the later suggesting radiation energy did not influence normalised

survival for the NCI-H727 cells. This was further supported when no statistical difference between kV or MV radiation groups at any dose level was observed ( $p=0.972$ ,  $\eta_p^2<0.001$  for 3Gy,  $p=0.225$ ,  $\eta_p^2=0.058$  for 6Gy). Three-Way ANOVA revealed equal variance across the entire dataset (LHEV=0.097).

## FTC-238 cells

Decreases in survival when the irradiated cells were pre-treated with USMB was more pronounced in FTC-238 cells exposed to kV compared to MV x-rays (Figure 4). These decreases were not significant at any dose level. Between the 2 cohorts, kV radiation had an overall greater impact on cell survival than did MV radiation, however there was no statistically significant difference observed between equivalent groups (eg. The mean difference between 3Gy kV-alone vs 3Gy MV-alone was -0.13,  $p=0.913$ ; and between 6Gy kV-alone and 6Gy MV-alone was also -0.13,  $p=0.523$ ). Unirradiated cells treated with USMB-alone demonstrated no significant impact on survival compared to control in either the kV or MV cohorts. The exclusion of an outlier observed within the 0GyMV-control group resulted in LHEV=2.417,  $p=0.023$ , hence a One-Way ANOVA with Games-Howell Post-Hoc testing was used for the analysis of this cell line after the Welch's test of Equality of Means was confirmed as statistically significant ( $p<0.001$ ). SW testing revealed the USMB+6Gy-MV subgroup was non-normally distributed ( $p<0.0001$ ), however this was only the case for one subgroup, and there is literature to support the robustness of the ANOVA to violations of normality [29,30].

## HUVEC cells

When the unirradiated HUVEC cells were treated with USMB there was no significant effect on cell viability (Figure 5). There was a slight, non-significant decrease in survival when the irradiated cells were pre-treated with USMB. This effect was similar irrespective of whether the cells were exposed to kV or MV ionising radiation. At equivalent radiation dose levels, cell survival was slightly higher in those cells exposed to MV x-rays compared to kV x-rays, however this was not significant. LHEV revealed unequal variances, so these cells were analysed using a One-Way ANOVA with Games-Howell Post-Hoc testing. The resulting Welch's test of Equality of Means was highly statistically significant ( $p<0.001$ ), overcoming the limitations of the initial LHEV result. A single violation of the normality assumption was again observed, this time for the 6Gy kV-alone subgroup so additional caution was applied in the interpretation of these results.

## Discussion

USMB had a significant effect on the survival of irradiated NCI-H727 cells. This effect could be cell-type dependent, which has been suggested in an earlier study [10]. These cell-type-specific responses to USMB + RT have been linked to ASMase gene expression and ceramide signalling associated with apoptosis. This was demonstrated by Nofiele et al. [12] using ASMase deficient<sup>(-/-)</sup> astrocytes and ASMase<sup>(+/+)</sup> astrocytes treated with Sphingosine-1-Phosphate (S1P) to counteract the ceramide-mediated apoptosis pathway. ASMase<sup>(+/+)</sup> astrocytes were more sensitive to USMB + RT compared to ASMase<sup>(-/-)</sup> or S1P-ASMase<sup>(+/+)</sup> astrocytes where ASMase and Ceramide production had been genetically or chemically inhibited. As NCI-H727 cells elicited a strong response to USMB + RT, changes in ASMase gene expression and ceramide levels produced in response to this treatment should be investigated to determine whether this could be used as an indicator of cell-sensitivity to such treatments. In a study of pre-operative blood serum samples extracted from 61 patients diagnosed with NSCLC, significantly increased levels of ASMase activity was detected compared to healthy controls, which may explain why the NSCLC cell line investigated here demonstrated such a strong response compared to the other two cell lines [31]. However HUVEC cells have also been reported to have ~ 20-fold higher levels of ASMase compared to other cells [32], and so should have also demonstrated decreased viability when treated using USMB + RT in this study if ASMase gene expression is the primary mechanism involved. Further investigations on what other factors may be responsible for the dramatic effect USMB have on the viability of irradiated NCI-727 cells are underway.

These factors under investigation include the use of T12.5cm<sup>2</sup> flasks, as the acoustic field applied to the NCI-H727 cells may have been different than that applied to the HUVEC and FTC-238 cells treated in T25cm<sup>2</sup> flasks. Kinoshita et al. [33] demonstrated that cell viability was substantially decreased in the presence of standing waves, and the interaction of the US field with the T12.5cm<sup>2</sup> flask may have created such a phenomenon which was absent in the T25cm<sup>2</sup> flasks. It is also interesting to note that Kinoshita et al. reported differences in cell viability when cells were exposed to USMB in a suspended state compared to an adherent state.

Lammertink et al. [17] also exposed the cells in an adherent state, further introducing yet another point of difference to all other research using USMB + RT which may also explain why their results were so different.

Although slight dose enhancement was noted for HUVECs treated with USMB + RT compared to that of RT, these were not significant and less than that seen in previous studies [11–13]. This difference could be related to the experimental setup, where different cell numbers and culture vessels; MB concentrations; US applications; and x-ray fields were used. The 1.6% v/v MB concentration used in this current study was less than the 3.3% v/v used in previous studies on HUVEC [11–13]. MB concentrations was shown to be optimal at 1.6% v/v in enhancing the effect of RT on Acute Myeloid Leukaemia cells [14]; however Lammertink et al. [17] observed no significant difference in the survival of FaDU cells when MB at 7% v/v were added to cells exposed to RT. Unlike HUVECs, the effect of MB on the cytotoxic effect of RT has not been examined for either FTC-238 and NCI-H727 cells, and as such 1.6% v/v was chosen as the MB concentration to use in these experiments. As the effects elicited with 1.6% v/v MB on the cytotoxic effect of RT on HUVEC cells reported here was less than those seen in earlier studies [11–13], repeat experiments using 3.3% v/v is now warranted.

The FTC-238 cells are derived from a metastatic tumour deposit of a follicular thyroid carcinoma. The addition of MB at 1.6% v/v did not have a significant effect on the viability of these secondary tumour cells when exposed to either kV or MV radiation. Of interest was that at the same dose (3 or 6Gy), kV radiation was significantly more cytotoxic than MV radiation. This difference between the cytotoxic effect elicited by kV and MV radiation was not seen in HUVEC or NCI-H727 cells. It is unlikely that such responses to differences in radiation energy are cell-type dependent and is more likely due to the differences in raw absorbance readings recorded for the control flasks, which varied by ~26% between the two x-ray treatment types for the FTC-238 cell line (Table 2). This could have arisen due to differences in travel times to the various treatment facilities resulting in these cells being out of the incubator for different lengths of time. Subsequent research is required to investigate the impact of this further. In the study conducted by Lammertink et al. [17], FaDU cells were exposed to MV x-rays and the survival curves for cells treated with USMB + RT compared to that of RT were similar. This differs to other studies where cells exposed to kV x-rays were treated with USMB and there was a reduction in cell survival rates [14–16]. In our study, flasks of FTC-238 cells were exposed to kV and MV x-rays at different times and it is unknown if a similar result would be obtained if the flasks were irradiated by both forms of x-rays concurrently, as done for the NCI-H727 cells. We observed that in the NCI-727 cells, there was minimal difference in survivals at similar dose levels for kV and MV x-rays, compared to the differences seen when the different cohorts were treated separately for FTC-238 cells.

However, the most likely explanation for the NCI-H727 results here is that these cells are inherently more sensitive to the effects of USMB, which is supported by the very highly significant difference ( $p < 0.001$ ) in normalised survival between the control and USMB only cohorts. Where the HUVEC and FTC-238 cells may benefit from increases in MB concentration, further experiments at lower concentrations for the NCI-H727 cells may be required to establishing toxicity profiles for US, MB and USMB-alone in the absence of RT.

## Conclusions

While USMB did enhance radiation-induced cell killing *in vitro* in the cell lines examined it was only statistically significant for NCI-H727 non-small cell lung carcinoma cells. This suggests that enhancement is most likely cell-type dependent, which is consistent with previous findings in the literature. However, there may also be other factors influencing these results, such as variations in applied acoustic fields and differences in the cytotoxicity profiles for USM-alone. The differences in cell killing elicited by kV and MV x-rays in FTC-238 cells are likely to be due to other factors, as similar observations were not seen in NCI-H727 or HUVEC cells. Given the overall trends observed here, this study demonstrates that this type of research is feasible using clinical hardware, and that further opportunities exist to understand the specific mechanisms involved in the direct radiosensitisation of tumour cells using USMB.

## Abbreviations

AML-5 – Acute Myeloid Leukaemia cells

ANOVA – Analysis of Variance

ARPANSA – Australian Radiation Protection and Nuclear Safety Agency

ASMase – Acid Sphingomyelinase

EPROC – Epping Radiation Oncology Centre, GenesisCare

FaDU – Human pharyngeal squamous carcinoma

FTC-238 – Lung metastasis of Follicular Thyroid Carcinoma cells

Gy – Gray

HUVEC – Human Umbilical Vein Endothelial cells

ISEL – *In situ* end-labelling

KHT-C – Murine fibrosarcoma cells

kPa – kiloPascal

kV – kilovoltage

kVp – kilovoltage peak

LHEV – Levine's test of homogeneity of Variances

MB – microbubbles

MDA-MB-231 – Human adenocarcinoma breast cells

MI – Mechanical Index

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium compound

MTT - 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H- tetrazolium bromide compound

MV – megavoltage

NCE-2 – Nasopharyngeal carcinoma cells

NCI-H727 – Non-Small Cell Lung Carcinoma cells

NSCLC – Non-Small Cell Lung Carcinoma

PC3 – Human prostate cancer cells

PNP – Peak Negative Pressure

RT – Radiation Treatment

SD – Standard Deviation

SE – Standard Error (calculated as  $SD/\sqrt{n}$ )

SSD – Source to Skin distance

US – ultrasound

USMB – ultrasound-stimulated microbubbles

USMB+RT – ultrasound-stimulated microbubbles and radiation

## Declarations

### Ethics approval and consent to participate

Not Applicable

### Consent for publication

Not Applicable

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

### Competing Interests

The authors declare that they have no competing interests

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### Authors' contributions

GM conducted all experiments and data analysis, with support and assistance from MN. BF provided biological expertise and assisted with the experimental design and data analysis. MG designed the project, provided medical radiation science expertise and assisted with the experimental design and data analysis. TP provided authoring and proof-reading support. GM wrote this article, with contribution from all authors. All authors read and approved the final manuscript.

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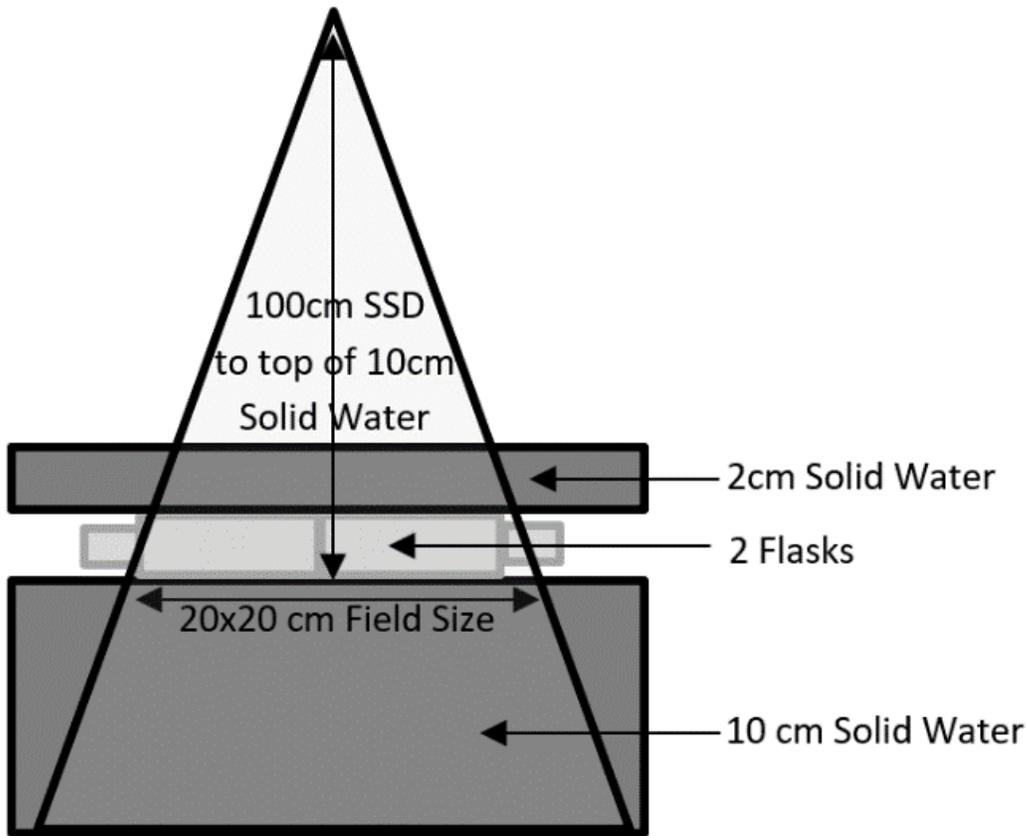
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## Figures



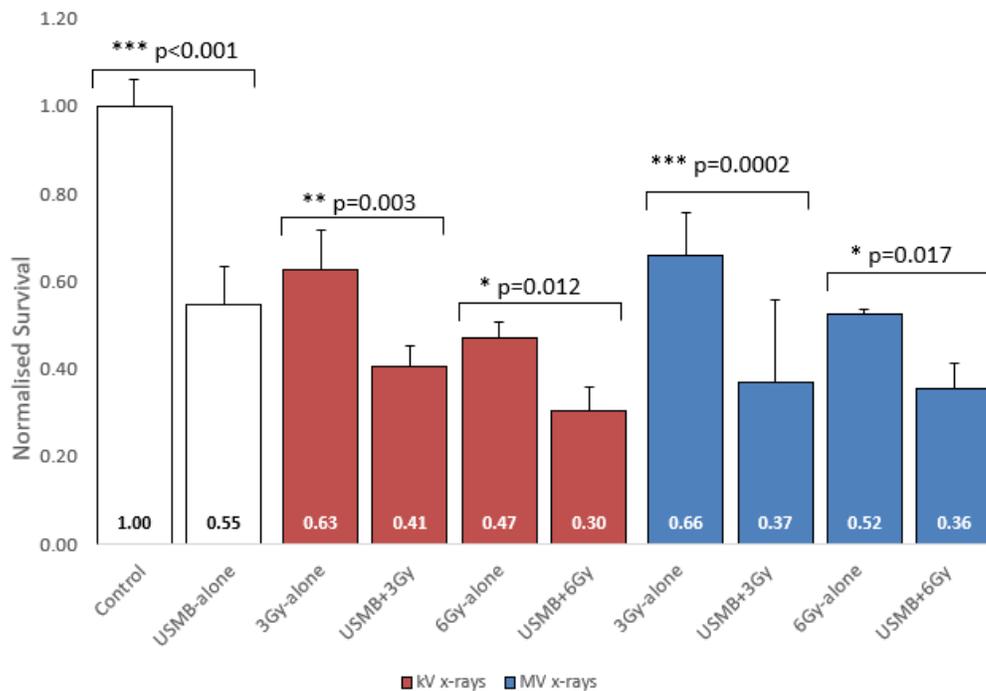
**Figure 1**

Schematic diagram demonstrating experimental setup for MV x-ray exposures. RT±USMB flasks were placed on top of 10cm Solid Water, with 100cm SSD set to top of solid water. Then 2cm solid water was placed on top of flasks to provide a build-up region. The flasks were exposed to a 6MV x-ray beam, at a gantry angle of 0° and field size 20 x 20cm.



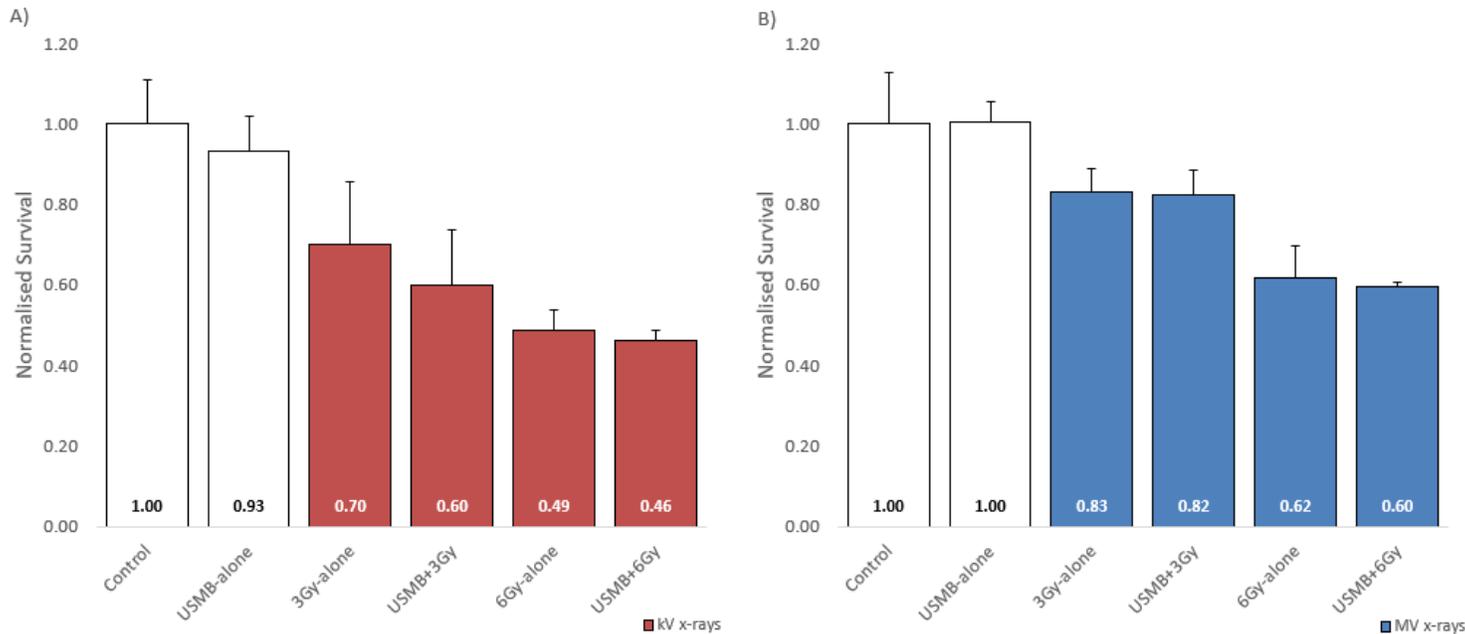
**Figure 2**

Image of experimental setup for kV x-ray exposures. Treatment flasks taped vertically to the baseplate (A) of the Model X80 X-Ray Beam irradiator (B). The Source-to-Surface distance was set at 100cm to the front of the baseplate.



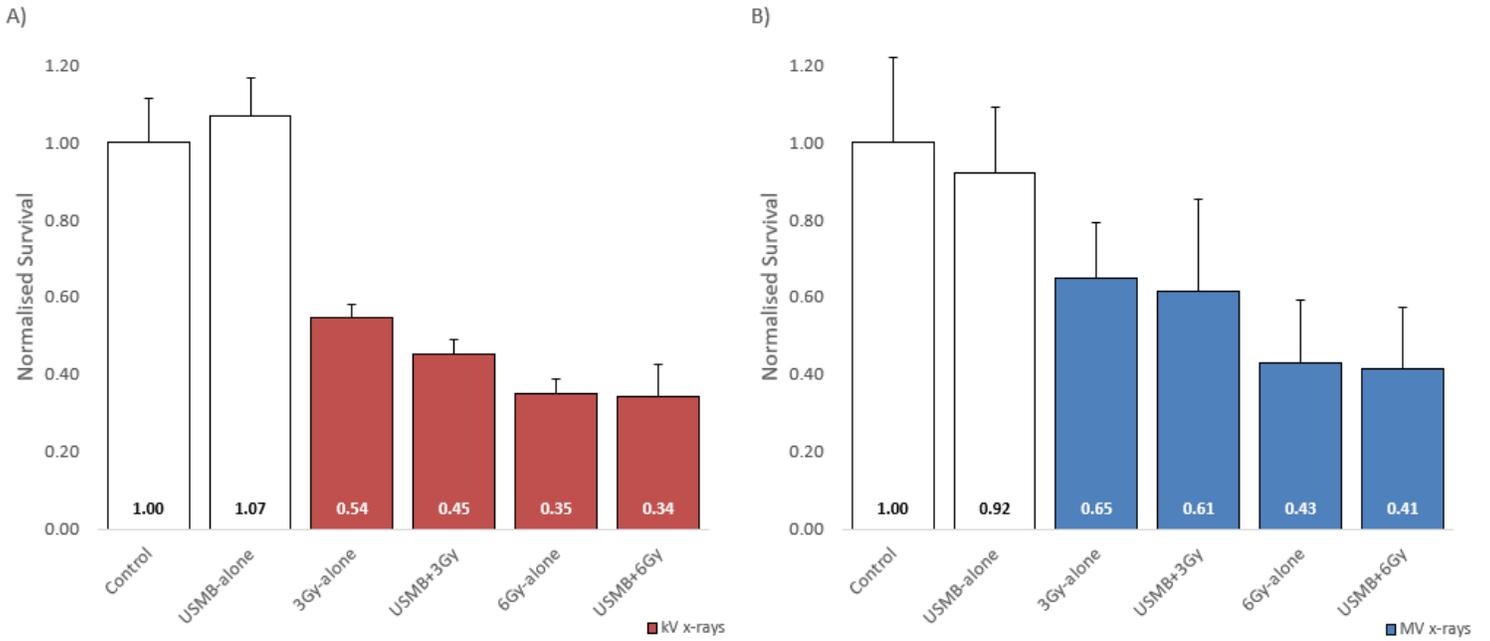
**Figure 3**

Effect of USMB+RT on the viability of NCI-H727 cells. Cells were treated with 1.6% MB, ~90 seconds of US, and either 0, 3 or 6Gy of A) kV and B) MV x-rays. Results expressed are the mean+SD for 3 replicates. The value for the untreated controls was assigned as unity (1), and the values for the treated cells were expressed as a fraction of this number. Both kV and MV experiments were carried out concurrently for this cell line. Significance values were calculated using a Three-Way Analysis of Variance (ANOVA).



**Figure 4**

Effect of USMB+RT on the viability of FTC-238 cells. Cells were treated with 1.6% MB, ~90 seconds of US, and either 0, 3 or 6Gy of A) kV and B) MV x-rays. Results expressed are the mean+SD for 3 replicates. The value for the untreated controls was assigned as unity (1), and the values for the treated cells were expressed as a fraction of this number. Significance values were calculated using a Three-Way Analysis of Variance (ANOVA)



**Figure 5**

Effect of USMB+RT on the viability of HUVEC cells. Cells were treated with 1.6% MB, ~90 seconds of US, and either 0, 3 or 6Gy of A) kV and B) MV x-rays. Results expressed are the mean+SD for 3 replicates. The value for the untreated controls was assigned as unity (1), and the values for the treated cells were expressed as a fraction of this number.