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Breast Cancer Cell Line Phenotype Affects Sonoporation Efficiency Under Optimal Ultrasound Microbubble Conditions

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Statistical Analysis C
Data Interpretation D
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Background: Ultrasound/microbubble (USMB)-mediated sonoporation is a new strategy with minimal procedural invasiveness for targeted and site-specific drug delivery to tumors. The purpose of this study was to explore the effect of different breast cancer cell lines on sonoporation efficiency, and then to identify an optimal combination of USMB parameters to maximize the sonoporation efficiency for each tumor cell line.

Material/Methods: Three drug-sensitive breast cell lines – MCF-7, MDA-MB-231, and MDA-MB-468 – and 1 multidrug resistance (MDR) cell line – MCF-7/ADR – were chosen. An orthogonal array experimental design approach based on 3 levels of 3 parameters (A: microbubble concentration, 10%, 20%, and 30%, B: sound intensity, 0.5, 1.0, and 1.5 W/cm^2, C: irradiation time, 30, 60, and 90 s) was employed to optimize the sonoporation efficiency.

Results: The optimal USMB parameter combinations for different cell lines were diverse. Under optimal parameter combinations, the maximum sonoporation efficiency differences between different breast tumor cell lines were statistically significant (MDA-MB-231: 46.70±5.79%, MDA-MB-468: 53.44±5.69%, MCF-7: 59.88±5.53%, MCF-7/ADR: 65.39±4.01%, $P<0.05$), so were between drug-sensitive cell line and MDR cell line (MCF-7: 59.88±5.53%, MCF-7/ADR: 65.39±4.01%, $p=0.026$).

Conclusions: Different breast tumor cell lines have their own optimal sonoporation. Drug-resistant MCF-7/ADR cells had higher sonoporation efficiency than drug-sensitive MCF-7 cells. The molecular subtype of tumors should be considered when sonoporation is applied, and optimal parameter combination may have the potential to improve drug-delivery efficiency by increasing the sonoporation efficiency.

MeSH Keywords: Genes, MDR • Triple Negative Breast Neoplasms • Ultrasonography

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Background

Breast cancer is one of the most common malignancies in women, and in 2012 over 1.7 million new cases were diagnosed by ultrasound alone [1]. Chemotherapy has been widely used for breast cancer treatment. However, chemotherapy has several limitations, including lack of targeted destruction, serious systemic toxicity, and acquired multidrug resistance (MDR) [2,3]. Ultrasound in combination with microbubbles (USMB) has grown rapidly as a new strategy with minimal procedural invasiveness for targeted and site-specific drug delivery to tumors [4–6]. The SonoVue, a stabilized sulfur hexafluoride (SF_6) microbubble, is commonly used to investigate sonoporation in various studies. Ultrasound as a physical vector may be more attractive than previous chemical approaches from a clinical viewpoint [7,8]. Herein, USMB was considered to be a 'gentle' technique compared with various other drug delivery systems [9]. A number of studies have focused on how USMB could enhance the delivery efficiency of chemotherapeutics [10,11]. The general principle of this physical method is considered to be sonoporation [12].

Microbubbles have been shown to create transient or lethal pores in cell membranes under the influence of ultrasound, known as USMB-mediated sonoporation. Sonoporation may result from oscillations of the microbubbles [13]. These microbubbles, oscillating in the presence of ultrasound, create localized shear stress or expand and collapse to render intense local heating and pressure, which cause cavitation and subsequent disruption of cell membranes and creates some temporary pores in the cell membrane. Through these pores, drugs, gene products, and some other particles can enter the cells [14–16]. This phenomenon has been demonstrated by high-speed camera images [9]. A correlative study also suggested that the increase of membrane permeability was enhanced by sonoporation [17]. Moreover, in a USMB-mediated delivery system, drug delivery efficiency has been reported to result in a 20–80% improvement in tumor response to drug treatment compared with administration of drugs alone in clinical murine models [18,19].

Some studies have investigated microbubble and ultrasound parameters, including microbubble concentration, sound intensity, irradiation time, fundamental transmission frequency and duty cycle [20–24]. However, most of these studies did not investigate the different combinations of various levels of parameters, and few considered the influence of different phenotypic cell lines of a certain tumor on sonoporation efficiency, especially for breast cancer cell lines. In addition, few studies compared the sonoporation between drug-sensitive and drug-resistant tumor cells.

Therefore, in the present study, 3 drug-sensitive and 1 drug-resistant breast cancer cell lines were selected to investigate whether cell phenotype and drug sensitivity affect sonoporation efficiency in breast cancer *in vitro*. In addition, the optimal parameter combination for each tumor cell line were determined, which could enhance sonoporation efficiency while minimizing cell death, using an orthogonal array experimental design (OAD) based on 3 levels of 3 parameters (L9 (3^3)).

Material and Methods

Cell culture

Four human breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, and MCF-7/ADR) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. MCF-7 cells were routinely cultured in DMEM (Gibco, Grand Island, NY, USA) and MDA-MB-231, MDA-MB-468, and MCF-7/ADR cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) medium, all supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 100 U/mL penicillin-streptomycin solutions at 37°C in humidified air with 5% CO_2. To maintain the drug-resistant phenotype, MCF-7/ADR cells were cultured in the presence of 1 μg/mL of the broad-spectrum chemotherapy drug Adriamycin (ADR, Hisun Pharmaceutical, Zhejiang, China). One week before each experiment, MCF-7/ADR cells were cultured in a drug-free medium to avoid the influence of ADR in the treatment. All cells used in the experiments were in exponential phase.

Ultrasonic exposure

A low-intensity therapeutic ultrasound system (US10, Cosmogamma Corporation, Italy) was used in this study. Ultrasound frequency (1 MHz), duty cycle (70%), and pulse repetition rate (100 Hz) were chosen and maintained consistently during all ultrasound studies. SonoVue (Bracco Research SA, Geneva, Switzerland) was prepared by diluting the powder in a sterile 0.9% NaCl solution. Microbubble concentration, irradiation time, and acoustic intensity were adjusted to observe differences between the bioeffects demonstrated.

Treatment protocol

According to the method of Zhai et al. [25], a water tank (20×20×15 cm) was filled with degassed water maintained at 37°C. A flow cytometry tube was used for cell suspension. Cells in exponential phase were collected and randomly divided into 9 experimental groups and 1 control group and each group has 3 samples. Each flow cytometry tube was filled with 1 mL of 5×10^5 cell suspension. For the experimental groups, each tube was lowered into the water tank and situated 1 cm from the transducer source. The ultrasound transducer was placed above the bottom of the water tank pointing upward, with its

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Table 1. Levels and factors of orthogonal design.

Level	Factors		
	Microbubble concentration (A), %	Sound intensity (B), W/cm²	Irradiation time (C), s
1	10	0.5	30
2	20	1.0	60
3	30	1.5	90

Table 2. Designing scheme of orthogonal experiment.

Test no.	Factors		
	Microbubble concentration (A),%	Sound intensity (B), W/cm²	Irradiation time (C), s
1	30	1.50	30.00
2	10	1.00	90.00
3	30	0.50	90.00
4	10	1.50	60.00
5	20	1.50	90.00
6	30	1.00	60.00
7	20	1.00	30.00
8	20	0.50	60.00
9	10	0.50	30.00

beam aligned axially with the tube as the orthogonal array experiment design one by one. The space between the transducer and tube was filled with degassed water to facilitate ultrasound transmission. The acquired data were subjected to orthogonal analysis to obtain the optimal USMB parameter combination for each group of cells. Cells prepared as above were irradiated under the optimal combination of parameters. Each group of experiment was repeated 3 times.

Orthogonal analysis

Three parameters in 3 levels (microbubble concentration: 10%, 20%, and 30%); irradiation time: 30 s, 60 s, and 90 s; acoustic intensity: 0.5 W/cm², 1.0 W/cm², and 1.5 W/cm²; Table 1) were selected for L9 (3^3) orthogonal analysis as shown in Table 2. Experiment results were subjected to intuitive analysis (Tables 3, 4). K_j and k_j were the sum and mean value of sonoporation efficiency or cell viability results for a certain parameter at level j, respectively. k_j reflected the effect of level j of a certain parameter on test index. Higher k_j values for sonoporation efficiency and cell viability rate represented optimal conditions. For each parameter, R (range) was calculated as k_{max}–k_{min}. R value reflected the order of parameters which affected the test index. Higher R values represented a parameter with more impact on the test index. Trend graphs were plotted with k_j on the Y axis and parameter levels on the X axis, as shown in Figure 1.

Flow cytometry

Sonoporated cells were counted by flow cytometry and the transference percentage (sonoporation efficiency) was evaluated. Cells were irradiated with ultrasound in the presence of 1 mg/mL fluorescein isothiocyanate-dextran (FD500, Sigma-Aldrich, St. Louis, MO, USA). After ultrasound irradiation, cells were washed with PBS, then immediately analyzed by flow cytometry (Beckman Coulter, Miami, USA). The percentage of FD500 fluorescent-positive cells were calculated as sonoporation efficiency using FlowJo 7.6.1 software.

Cell viability assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit (Beyotime, Wuhan, China) [26]. Cell suspension treated with ultrasound was grown in 96-well plates. Cells were incubated with 20 µL MTT reagent in each well for 4 h at 37°C, which was subsequently replaced by 100 µL DMSO. Following 10-min agitation on a plate shaker, the optical density of the samples was measured at a wavelength of 490 nm (A_{490}) using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The results were calculated using the following equation: Cell viability (%)=(A_{490} of experimental group/A_{490} of control group)×100%.

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Table 3. Intuitive analysis of sonoporation efficiency.

Value	MDA-MB-231			MDA-MB-468			MCF-7			MCF-7/ADR		
	A	B	C	A	B	C	A	B	C	A	B	C
K1	130.81	114.04	133.59	118.63	86.21	111.43	108.30	101.87	120.26	140.60	105.88	142.28
K2	138.31	110.05	118.45	112.13	129.83	117.04	126.98	115.71	124.09	157.28	189.73	143.48
K3	80.91	125.94	97.99	140.29	155.01	142.58	132.39	150.09	123.32	174.59	176.86	186.71
k1	43.60	38.01	44.53	39.54	28.74	37.14	36.10	33.96	40.09	46.87	35.29	47.43
k2	46.10	36.68	39.48	37.38	43.28	39.01	42.33	38.57	41.36	52.43	63.24	47.83
k3	26.97	41.98	32.66	46.76	51.67	47.53	44.13	50.03	41.11	58.20	58.95	62.24
R	19.13	5.30	11.87	9.39	22.93	10.38	8.03	16.07	0.26	11.33	23.66	14.81

A – microbubble concentration; B – sound intensity; C – irradiation time; K – sum of sonoporation efficiency for the factors at each level; k – the mean values of sonoporation efficiency for the factors at each level; R – $k_{max}k_{min}$.

Table 4. Intuitive analysis of cell survival rate.

Value	MDA-MB-231			MDA-MB-468			MCF-7			MCF-7/ADR		
	A	B	C	A	B	C	A	B	C	A	B	C
K1	229.90	234.88	236.93	244.46	248.19	240.79	209.62	255.34	228.30	257.91	264.90	246.48
K2	219.46	226.58	221.02	239.97	215.16	226.70	216.99	217.09	205.39	254.41	247.13	266.14
K3	225.40	213.30	216.81	192.94	214.02	209.88	216.93	171.11	209.85	232.62	232.91	232.32
k1	76.63	78.29	78.98	81.49	82.73	80.26	69.87	85.11	76.10	85.97	88.30	82.16
k2	73.15	75.53	73.67	79.99	71.72	75.57	72.33	72.36	68.46	84.80	82.38	88.71
k3	75.13	71.10	72.27	64.31	71.34	69.96	72.31	57.04	69.95	77.54	77.64	77.44
R	3.48	7.19	6.71	17.17	11.39	5.61	0.02	28.08	6.15	8.43	10.66	11.27

A – microbubble concentration; B – sound intensity; C – irradiation time; K – sum of sonoporation efficiency for the factors at each level; k – the mean values of sonoporation efficiency for the factors at each level; R – $k_{max}k_{min}$.

Statistical analysis

All data are expressed as the mean ± standard deviation. SPSS 19.0 software (IBM, Armonk, NY, USA) was used to analyze the data, and the pairwise comparison between any 2 groups was performed with one-way ANOVA with *LSD-t* method. $P<0.05$ was considered statistically significant.

Results

Optimization of USMB parameters

Using the L9 (3^3) orthogonal experiment design based on 3 levels of 3 parameters chosen from the literature [17,25,27,28] (Tables 1, 2), were investigated how different USMB parameter combinations affected the sonoporation efficiency and cell viability of 3 drug-sensitive (MDA-MB-231, MDA-MB-468, and MCF-7) and 1 MDR breast cancer cell lines (MCF-7/ADR), and the results are shown in Table 5. Intuitive analysis of sonoporation efficiency and cell viability are shown in Tables 3 and 4. Higher-range values of the 3 parameters demonstrated greater influence on sonoporation efficiency and cell viability. Based on the experimental data (Table 3–5), the optimal parameter combinations are summarized in Table 6.

Sonoporation efficiency and cell viability analysis in 4 breast cancer cell lines

The maximum sonoporation efficiencies were further obtained under the optimal parameter combination for each cell line, which were: MDA-MB-231: 46.70±5.79%, MDA-MB-468: 53.44±5.69%, MCF-7: 59.88±5.53%, and MCF-7/ADR: 65.39±4.01%. The survival rate of the cells in the experimental group was the relative survival rate calculated with the survival rate of the cells in the control group as 100%. Cell survival rates under the optimal parameter combination were MDA-MB-231: 84.24±5.83%, MDA-MB-468: 81.03±3.43%,

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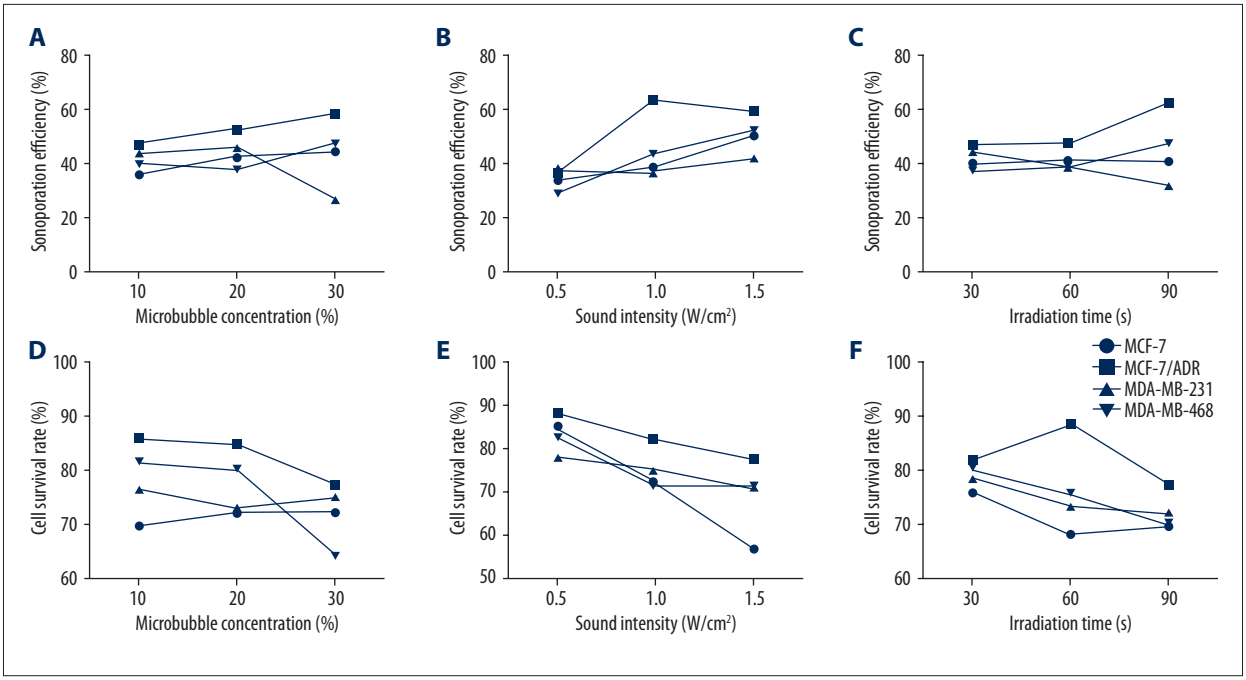

Figure 1. Effect of microbubble concentration, sound intensity, and irradiation time in 3 levels on the sonoporation efficiency (**A–C**) or cell viability (**D–F**) in 4 breast cancer cell lines. Experiments were repeated 3 times.

Table 5. Experimental results based on L9 (3^3) orthogonal design.

Test no.	Sonoporation efficiency (%)				Cell survival rate (%)			
	MDA-MB-231	MDA-MB-468	MCF-7	MCF-7/ADR	MDA-MB-231	MDA-MB-468	MCF-7	MCF-7/ADR
1	33.43	48.48	58.30	60.08	82.72	63.84	64.59	69.25
2	32.90	43.25	39.83	67.85	81.94	71.09	71.33	77.70
3	22.76	45.28	39.79	51.38	72.36	70.23	82.22	79.71
4	50.18	52.48	48.09	49.30	68.07	81.62	50.22	88.75
5	42.33	54.05	43.70	67.48	62.51	68.56	56.30	74.91
6	24.72	46.53	34.30	63.13	70.32	58.87	70.12	83.66
7	52.43	40.05	41.58	58.75	74.32	85.20	75.64	85.77
8	43.55	18.03	41.70	31.05	82.63	86.21	85.05	93.73
9	47.73	22.90	20.38	23.45	79.89	91.75	88.07	91.46

Table 6. The optimal experimental parameter combination and results for four breast tumor cell lines.

Cell line	Factors			Results	
	Microbubble concentration, %	Sound intensity, W/cm²	Irradiation time, s	Sonoporation efficiency, %	Cell survival rate, %
MDA-MB-231	10	1.5	90	46.70±5.79	84.24±5.83
MDA-MB-468	20	0.5	30	53.44±5.69	81.03±3.43
MCF-7	30	0.5	30	59.88±5.53	80.48±3.22
MCF-7/ADR	30	1.0	60	65.39±4.01	82.91±2.83

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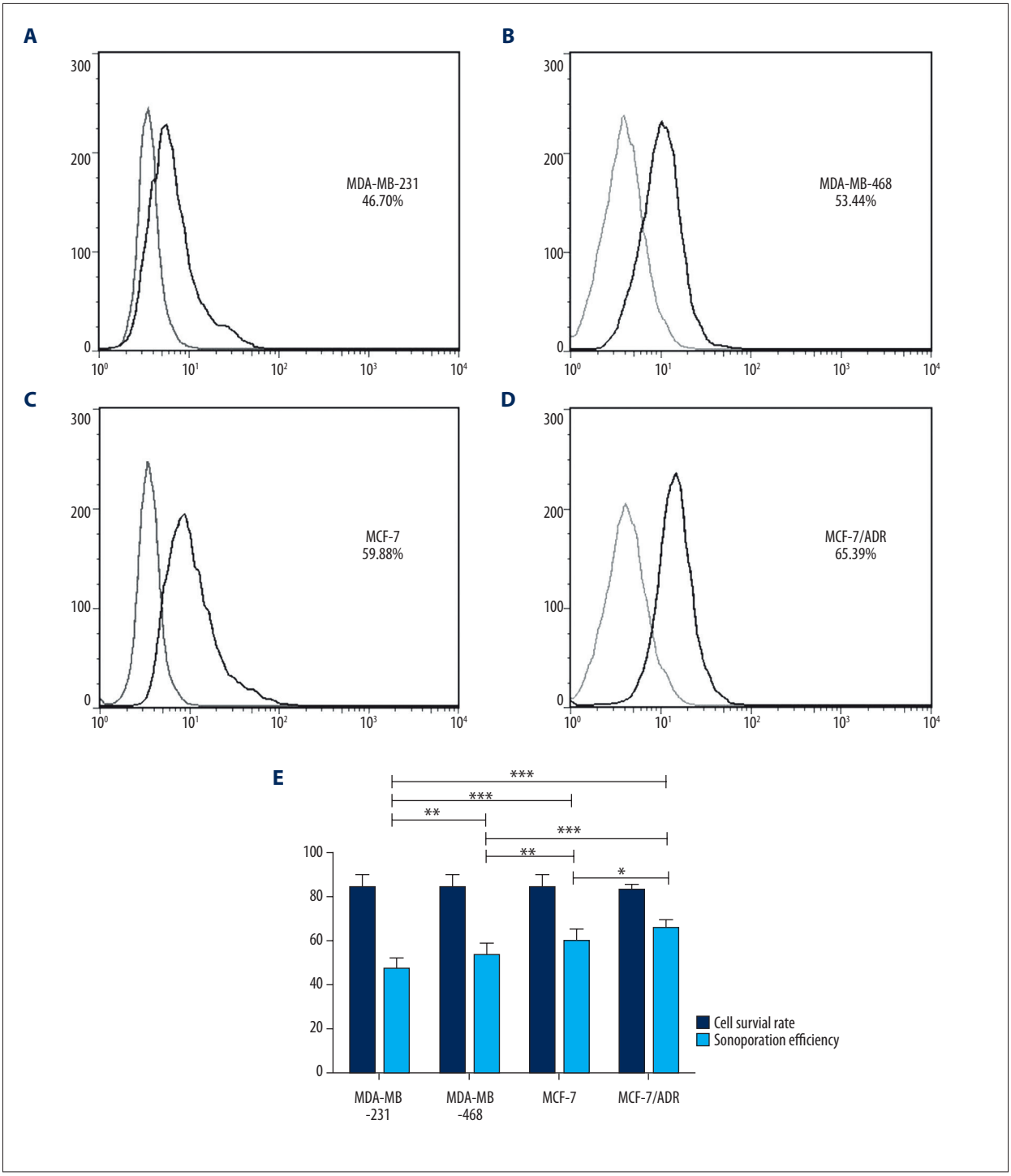

Figure 2. Maximum sonoporation efficiency and cell survival rate of 4 tumor cell lines under their own optimum parameter combination. (**A–D**) Flow cytometry analysis of the maximum sonoporation efficiency (**A**, MDA-MB-231 cell line; **B**, MDA-MB-468 cell line; **C**: MCF-7 cell line; **D**, MCF-7/ADR cell line). Gray lines indicate the control groups, and black lines indicate the experimental groups. (**E**) The maximum sonoporation efficiency and cell survival rate of 4 breast cancer cell lines under their own optimal parameter combination. Experiments were repeated 3 times. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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and MCF-7: 80.48±3.22%, MCF-7/ADR: 82.91±2.83% (Table 6, Figure 2A–2D). One-way ANOVA followed by multiple comparison with *LSD-t* test showed that the differences in sonoporation efficiency between any 2 of the 4 cell lines were statistically significant ($P<0.05$), while differences in cell survival rate had no significance ($P>0.05$) (Figure 2E).

Influence of each parameter on sonoporation efficiency and cell viability

Crosswise analysis

The effects of the 3 parameters (A: microbubble concentration, B: sound intensity, and C: irradiation time) on sonoporation efficiency and cell viability are shown in Tables 3 and 4 by comparing the R values of the optimization results. Table 3 illustrates the rank order of the 3 factors' influence on sonoporation efficiency for each cell line (MDA-MB-231: A>C>B, MDA-MB-468 and MCF-7/ADR: B>C>A, and MCF-7: B>A>C). Table 4 illustrates the rank order of these factors' influence on cell viability (MDA-MB-231 and MCF-7: B>C>A, MDA-MB-468: A>B>C, and MCF-7/ADR: C>B>A).

Vertical analysis

The response curves of the 3 parameters are exhibited in Figure 1. As the microbubble concentration increased (Figure 1A), the sonoporation efficiency of the MCF-7, MCF-7/ADR and MDA-MB-468 cells ultimately increased, while that of the MDA-MB-231 cells dropped at high microbubble concentration, following a slight increase. With the increase in sound intensity (Figure 1B), the sonoporation efficiency of all 4 cell lines increased. The sonoporation efficiency of MCF-7/ADR and MDA-MB-468 cells both increased with irradiation time (Figure 1C), while the efficiency of MDA-MB-231 cells decreased, and that of the MCF-7 cells did not change significantly. High microbubble concentration, sound intensity, and irradiation time negatively affected cell survival rates of all cell lines except for the MCF-7 cells, although an irradiation time of 60 s increased the viability of MCF-7/A cells (Figure 1D–1F).

Discussion

Improving cellular membrane permeability to enhance chemotherapeutic drug uptake in cancer yields the potential to improve drug delivery and treatment efficacy. Development of more effective strategies for systemic delivery of these agents could permit lower drug dose throughout a therapeutic regimen, thereby decreasing patient toxicity. USMB-mediated sonoporation has gained considerable attention for potentiating the delivery of exogenous vectors at the specific biological site in a safe and efficient way [11]. In this study, we assessed and compared the sonoporation efficiency in drug-sensitive and MDR breast cancer cells *in vitro*, and then optimized the parameter combination for maximal sonoporation efficiency and minimal cell death.

The results of this study are the first to indicate that the phenotype of different human breast cancer cell lines affects sonoporation efficiency (Table 6, Figure 2). Previously, Pichardo et al. reported that various cell lines from the same tumor tissue (cervical-carcinoma-derived cells) affected sonoporation [29]. One possible explanation is that cell physiology affects the internalization pathways of non-viral vectors, leading to cell line-dependent drug delivery efficiency, so different internalization mechanisms may lead to disparate sonoporation efficiencies in diverse cell lines [30].

Secondly, based on our results, higher potential values of sonoporation are suggested for drug-resistant tumor cells, as the sonoporation efficiency of the MCF-7/ADR cell line was higher than that of the MCF-7 cell line. This reflects the higher sensitivity to enhanced sonoporation mediated by USMB exposure for MCF-7/ADR cells than MCF-7 cells, which is consistent with a previous study [31]. The underlying mechanism might involve the enhancement of cell membrane permeability and downregulation of MDR-related genes and proteins [32]. In addition, a reduction in the amount of permeability glycoprotein (P-gp) due to USMB might prevent chemotherapeutic drugs from leaving the tumor cells [33]. Furthermore, the levels of sound intensity and irradiation time of the optimal parameter combination for the MCF-7/ADR cell line (A3B2C2) were higher than those for the MCF-7 cell line (A3B1C1). One possible explanation i that MDR cells have higher membrane rigidity than sensitive cells due to overexpression of P-gp [34,35].

Our study also optimized the physical parameters of USMB for different breast cancer cell lines. Compared with the non-optimized combination in Table 5, the sonoporation efficiency of each cell line under the optimal USMB combination was enhanced, while preserving over 80% cell viability (Tables 5, 6). The difference in viability between cell lines was not significant ($P>0.05$) (Table 6 and Figure 2E). This observation is in agreement with a previous report that cell viability was not dependent on cell lines [29]. Among the 3 parameters analyzed, sound intensity was the main factor influencing the sonoporation efficiency of MDA-MB-468, MCF-7, and MCF-7/ADR cell lines, while microbubble concentration was the main factor affecting the MDA-MB-231 cell line. The ranges of the 3 parameters and the 3 levels were chosen based on previous studies. Shapiro et al. reported that the concentration of microbubbles must be kept in a very tight range to achieve enhanced sonoporation [36]. Ultrasound intensity ranged from 0.064 to 3 W/cm^{2,} and ultrasound exposure time from 2 s to 10 min were recommended to deliver drugs in tumor tissue without

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injuries [37,38]. Therefore, in our study, the microbubble concentrations were chosen as 10%, 20%, and 30%, the sound intensity was increased from 0.5 W/cm^2 to 1.5 W/cm^2, and the range of irradiation time was increased from 30 s to 90 s.

Finally, for different molecular subtypes of breast cancer, the maximum sonoporation efficiency was different. MDA-MB-231 cell line was the least susceptible to sonoporation, indicating that the sonoporation conditions required to induce the permeabilization of MDA-MB-231 cell membrane should be modified to create the same level of drug uptake as in other cell lines. Taken together, these results might have implications for a better design of individualized treatment for breast cancer based on sonoporation.

This study establishes the foundation for further exploring the feasibility to reverse MDR by USMB combined with anticancer drug and for elucidating the underlying mechanism involved. One limitation of this study is that only 1 drug-resistant cell line was explored. Comparisons of different MDR cell lines could be performed in in-depth research in the future. In addition, using acoustically transparent plates would further increase the accuracy of the sonoporation experiments. Currently, our study is at *in vitro* stage using microbubbles for sonoporation efficiency of different breast cancer cell lines. In the future we will further study sonoporation in different subtypes of breast cancer *in vivo* using the more permeable nanobubbles, and explore the underlying mechanism.

Conclusions

This study demonstrates that cell phenotype affects sonoporation efficiency under optimal conditions, but does not affect cell viability. The optimal sonoporation was different for each breast cancer cell line, despite having the same tissue origin. Optimization of the USMB parameter combinations can effectively increase sonoporation efficiency. The optimal parameter combination and maximum sonoporation efficiency achieved in the present study may represent an important developmental step toward USMB-mediated non-invasive drug/gene delivery for the personalized treatment of different pathological types of breast cancer in the future.

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Conflict of interest

None.

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