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Doxorubicin Delivery into Tumor Cells with Ultrasound and Microbubbles

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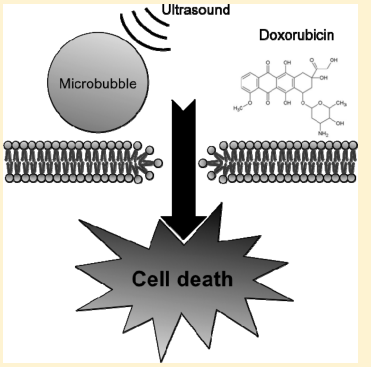
ABSTRACT: Doxorubicin is a potent chemotherapeutic whose severe side effects limit its application. Drug-targeted delivery with noninvasive techniques is required to increase the drug concentration locally and to reduce systemic side effects. Microbubble-assisted ultrasound has become a promising strategy for noninvasive local drug delivery. The aim of this study is to evaluate the applicability and the effectiveness of administration of doxorubicin combined with microbubble-assisted ultrasound in human U-87MG glioblastoma and MDA-MB-231 breast cancer cells. In the present study, the doxorubicin delivery aided by microbubble-assisted ultrasound enhanced the death of breast cancer and glioblastoma cells, including the induction of apoptosis. Various microbubbles were evaluated including Vevo Micromarker, BR14, SonoVue and experimental polymer shelled microbubbles. The results showed that Vevo Micromarker microbubble-assisted ultrasound could induce an enhancement of doxorubicin in glioblastoma and breast cancer cell death. Polylactide-Shelled PEG and Vevo Micromarker microbubbles were the best microbubbles for efficient doxorubicin delivery in the U-87 MG and MDA-MB-231 cells, respectively. Moreover, the induction of apoptosis by doxorubicin and Vevo Micromarker microbubble-assisted ultrasound was examined and results showed a positive increment for acoustic pressures above 600 kPa. The conclusions drawn from *in vitro* study show the potential of this strategy for an *in vivo* application.



KEYWORDS: doxorubicin, ultrasound, microbubble, tumor therapy, apoptosis

INTRODUCTION

Doxorubicin, also known as adriamycin, is one of the most powerful antineoplastic drugs prescribed on its own or in a combination with other agents. Doxorubicin is a compound of the anthracycline class that has the broadest spectrum of activity. Therefore, anthracyline is widely used for the treatment of solid tumors and hematological malignancies, including breast, prostate, uterus, ovary, stomach and liver tumors, childhood solid tumors, osterosarcomas and soft tissue sarcomas, and Kaposi's sarcoma, as well as acute myeloblastic and lymphoblastic leukemia.[1] The antitumor activity of doxorubicin has been attributed to its intercalation into the nuclear and mitochondrial DNA,[2,3] the production of reactive oxygen species[4] and the inhibition of topoisomerase II.[5]

The early use of doxorubicin in clinical application revealed major undesired effects, such as the development of resistance in tumor cells (e.g., P-glycoprotein and toposiomerase II resistance)[6,7] and side effects, such as the toxicity in healthy tissues (e.g., heart, brain, liver and kidney toxicities).[1] To overcome these problems, the development of an efficient and targeted delivery of doxorubicin is required to reduce the therapeutic dose of doxorubicin. Recent research has demonstrated that the application of high frequency ultrasound (1−10 MHz) combined with ultrasound contrast agents enhances the intracellular delivery of drugs on cells and tissues. Microbubble oscillations are assumed to play a major role in the increased uptake of the drugs by the cells.[8−11] When microbubbles are exposed to ultrasound, they shrink and expand alternately during the respective phases of the high and the low pressures of the ultrasound wave. These oscillations induce intense liquid flow around the microbubbles, so-called microstreaming.[12,13] At higher ultrasound pressures, the microbubbles grow rapidly during the rarefaction phase, until they collapse and/or fragment. The collapse of microbubbles might be accompanied by the generation of shock waves in the medium close to the microbubbles.[14] In the case of an asymmetrical collapse, jet formation may occur when a collapsing microbubble is located near a surface such as a cell membrane.[15] As described in the available literature, microstreaming, shock waves and microjets can transiently perforate the plasma membranes of nearby cells and, therefore, enhance the intracellular uptake of drugs.[16−18]

The synergetic effect of ultrasound on doxorubicin delivery is reported in several studies.[19−21] However, these investigations focused mainly on the intracellular delivery of free doxorubicin using ultrasound alone. Whereas it was reported that the combination of ultrasound and microbubbles increased the effectiveness of gene delivery compared to ultrasound alone, this combination has not been yet explored to date to enhance the doxorubicin delivery.[22−24] The aim of our study is to investigate whether the coadministration of an approved drug and clinically approved microbubbles in combination with ultrasound waves is

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Table 1. Description of Microbubbles[a]

name	shell's composition	gas	mean diameter (μm)	manufacturer
Vevo Micromarker	phospholipids, PEG, fatty acids	N_2, C_4F_{10}	2.3−2.9	Bracco Research, Geneva, Switzerland
BR14	phospholipid	C_4F_{10}	2.6	Bracco Research, Geneva, Switzerland
SonoVue	DSPC/DPPG/PA	SF_6	≤2	Bracco Research, Geneva, Switzerland
Polylactide shelled microbubbles	polylactide	N_2	2	Philips Research, Eindhoven, The Netherlands
Polylactide-Shelled PEG microbubbles	polylactide, PEG	N_2	2	Philips Research, Eindhoven, The Netherlands

[a] Abbreviations: DPPG, dipalmitoyl-phosphatidylglycerol; PA, palmitic acid; DSPC, distearoyl-phosphatidylcholine; PEG, polyethylene glycol; C_4F_{10}, perfluorocarbon; SF_6, sulfur hexafluoride; N_2, nitrogen.

an efficient strategy for drug delivery. Therefore, we investigated the therapeutic potential of the combination of doxorubicin and ultrasound with various types of microbubbles in glioblastoma and breast adenocarcinoma cells. In particular, we sought to compare the effectiveness of different microbubbles for doxorubicin delivery (including Vevo Micromarker, BR14, SonoVue, Polylactide Shelled and Polylactide-Shelled PEG microbubbles) (Table 1). The cell viability and apoptosis were monitored by Trypan blue and annexin V assays. This methodology addresses the following research: (i) Does the microbubble-assisted ultrasound improve the cell death in comparison to doxorubicin alone? (ii) Does the combination of doxorubicin and microbubble-assisted ultrasound induce cell apoptosis? (iii) Which is the most efficient microbubble for doxorubicin delivery? (iv) Is there cell specificity in the doxorubicin delivery by microbubble-assisted ultrasound? The outline of this paper is as follows: the experimental methodology is presented in the next section (Experimental Section). Then, results from the tests on doxorubicin-induced tumor cell death and apoptosis using microbubble-assisted ultrasound are presented and a therapeutic ratio that quantifies the methodology efficiency is defined. Finally, these results are discussed before the main conclusions on this study are drawn.

EXPERIMENTAL SECTION

Chemicals. Doxorubicin in HCl salt (Sigma-Aldrich, St. Louis, MO) was dissolved in a phosphate buffered saline solution (PBS) (Gibco-Invitrogen, Carlsbad, CA) at a concentration of 100 μM, and then sterilized by filtration (mesh size 0.22 μm, Fisher Bioblock, Illkirch, France). Afterward, it was stored frozen to −20 °C and then thawed just prior to being used. For each *in vitro* experiment, the doxorubicin concentration was 5 μM.[25] The commercial concentration of propidium iodide solution (Sigma-Aldrich, St. Louis, MO) was 10 mg/mL and for these experimental set, it was reduced to 0.5 μg/mL.

Cell Culture. Human glioblastoma astrocytoma cells (U-87 MG) were derived from a malignant glioma (ECACC European Collection of Cell Cultures, Salisbury, U.K.). These cells were capable of developing a malignant tumor (i.e., glioblastoma) in nude mice. Human breast adenocarcinoma cells (MDA-MB-231) were derived from a metastatic site in the form of pleural effusion (ECACC European Collection of Cell Cultures, Salisbury, U.K.). Cells were grown as a monolayer in Dulbecco's modified Eagle medium (DMEM) (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-Invitrogen, Carlsbad, CA) and 2 mM L-glutamine (Gibco-Invitrogen, Carlsbad, CA). The cells were routinely subcultured every 4 days and incubated at 37 °C in humidified atmosphere with a 5% CO_2 incubator.

Ultrasound Setup. Ultrasound waves were generated from an unfocused single element transducer (Vermon SA, Tours, France) with a center frequency of 1 MHz. The transducer had a diameter of 14 mm and was naturally focused at 3 cm. The transducer was driven with an electrical signal generated by an arbitrary waveform generator (Agilent, City, CA, USA) and amplified with a power amplifier (ADECE, Artannes sur Indre, France). The peak negative pressure of the acoustic wave was measured in a separate setup using a calibrated PDVF needle hydrophone (0.2 mm diameter, Precision Acoustics, Dorchester, United Kingdom) at the natural focal distance of the transducer.

Doxorubicin Delivery by Microbubble-Assisted Ultrasound. Cells were trypsinized, washed once and resuspended in Opti-MEM (Gibco-Invitrogen, Carlsbad, CA) supplemented with 1% FCS. During the procedure, the cell suspension was maintained at 37 °C in a water bath (Grant Instruments Ltd., Cambridge, U.K.). Then, the cell suspension (5×10^5 cells in 1.5 mL) was placed in a polystyrene cuvette (Fisher Scientific SAS, Illkirch, France) (45 mm height, 10 mm internal width and 12 mm external width). The doxorubicin solution was added at the required concentration of 5 μM.[25] All microbubble suspensions were prepared *in situ* by mixing with NaCl 0.9%. To achieve the same microbubble-cell ratio (i.e., 5 microbubbles per cell) a defined volume of microbubbles was added to the plastic cuvette just before ultrasound application: 12.5 μL for BR14, 12.5 μL for SonoVue, 2.2 μL for Vevo Micromarker, 1.8 μL for Polylactide Shelled and 89 μL for Polylactide-Shelled PEG (Table 1). The center of the plastic cuvette was positioned at the focal distance of the transducer in a deionized water tank maintained at room temperature. The cell suspension was kept uniform through a gentle magnetic stirring during ultrasound application. Subsequently, the cell suspension was exposed to 1 MHz sinusoidal ultrasound waves with a pulse repetition period of 100 μs, 40 cycles per pulse and for 30 s. Acoustic pressures from 400 to 800 kPa were applied. Five experimental groups were selected: (1) nontreated (Ctrl), (2) doxorubicin-treated (Doxo), (3) US-microbubble treated (US-microbubbles), (4) combination of doxorubicin and US (Doxo-US), (5) combination of doxorubicin and US-microbubbles (Doxo-US-microbubbles).

After ultrasound application, 500 μL of cells were cultured in a 24-well cell culture plate (Corning Life Science B.V., Amsterdam, The Netherlands) and incubated at 37 °C in a humidified atmosphere with a 5% CO_2 incubator. Four hours later, 1 mL of 10% FCS-Opti-MEM medium was added to each well and incubated at 37 °C in humidified atmosphere with a 5% CO_2 incubator for 48 h.

Doxorubicin Uptake. After doxorubicin delivery, the cell medium was removed and the cells were washed with PBS and collected through centrifugation (i.e., 3 min, 800*g*). The cells were resuspended in 500 μL of PBS before measurements were

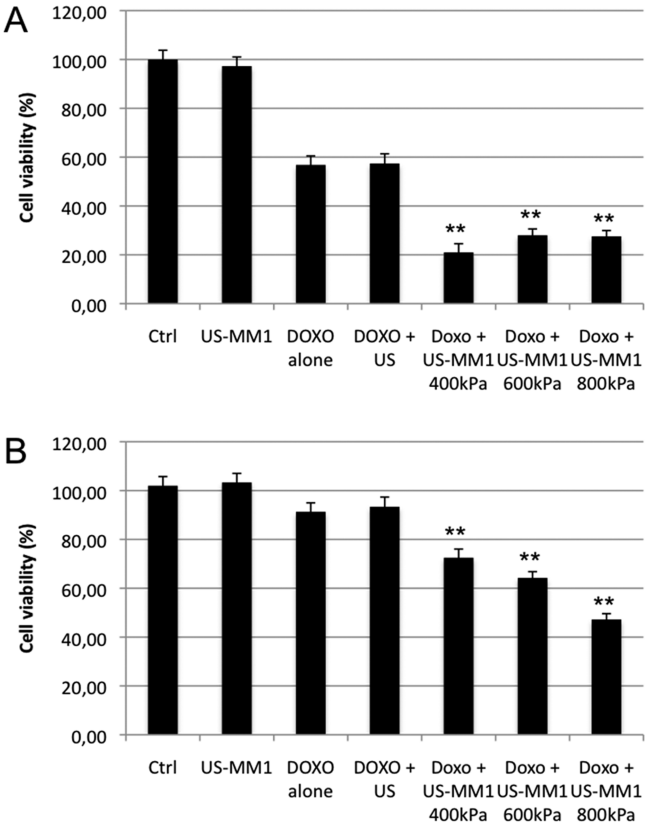

Figure 1. Enhancement of doxorubicin-induced cell death by ultrasound combined with Vevo Micromarker microbubbles. U-87 MG (A) and MDA-MB-231 (B) cells were incubated with 5 μM doxorubicin alone (Doxo alone), or with ultrasound at 600 kPa (Doxo+US), or with microbubble-assisted ultrasound (Doxo+US-MM1) at 400 kPa (Doxo+US-MM1-400 kPa), 600 kPa (Doxo+US-MM1-600 kPa) and 800 kPa (Doxo+US-MM1-800 kPa) for 30 s. 48 h after treatment, cell viability was measured by a Trypan blue assay. Data expressed as mean ± SEM was calculated from five independent experiments. Statistical analysis was performed using the nonparametric Mann−Whitney test. Significance was defined as $p < 0.05$ (NS, nonsignificance, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

made. Fluorescence histograms were recorded with flow cytometer (Beckman, Coulter, Fullerton, CA, USA) and analyzed using Kaluza software supplied by the manufacturer. Minimums of 10,000 events were analyzed to generate each histogram. The gate was arbitrarily set for the detection of red fluorescence.

Cell Viability Assay. A Trypan blue assay evaluated cell viability.[26] After 48 h of treatment, the cell medium containing dead cells was removed but kept for further counting. The cells were harvested by trypsinization and with the preserved medium. The cell suspension was centrifuged (i.e., 3 min, 800*g*) and was then resuspended in phosphate saline buffer. The cells were stained in an equal volume of Trypan blue stain (0.4% w/v) (Molecular Probes-Invitrogen, Carlsbad, CA) and incubated for 3 min. After that, live and dead cells were accurately counted by a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA). Cell survival rate was calculated as the ratio of the number of surviving cells in the treated group to the number of surviving cells in the control group.

Apoptosis Assay. The doxorubicin-induced apoptosis was determined by annexin V assay.[27] After 48 h of treatment, the cells were stained with an annexin V−FITC apoptosis kit (MACS, Miltenyi Biotec SAS, Paris, France) according to the manufacturer's instructions. The propidium iodide was added at 0.5 μg/mL concentration to the cell suspension immediately before flow cytometry analysis (Beckman, Coulter, Fullerton, CA, USA). Early apoptotic cells were only stained by annexin V−FITC, and late apoptotic cells were stained by annexin V−FITC and propidium iodide.

Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM) of five independent experiments. Statistical analysis was performed using the nonparametric Mann−Whitney test. Significance was defined as $p < 0.05$ (NS, nonsignificance, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

RESULTS

Enhancement of Doxorubicin-Induced Tumor Cell Death by Vevo Micromarker Microbubble-Assisted Ultrasound. Cell viability was assessed by the Trypan blue dye exclusion test at 48 h after doxorubicin treatment in combination with Vevo Micromarker microbubble and ultrasound. When the cells were insonated at 600 kPa with only the presence of Vevo Micromarker microbubbles, the cell viability was 97 ± 4% for U-87 MG cells and 100 ± 5% for MDA-MB-231 cells (Figure 1). Similar cell viability was obtained at 400 and 800 kPa with or without Vevo Micromarker microbubbles for the two types of cell lines (data not shown). As shown in Figure 1, the cell viability after doxorubicin treatment alone and only in combination with ultrasound was 57 ± 4% for U-87 MG cells. On the contrary, for MDA-MB-231 cells, it was 91 ± 4% for doxorubicin treatment alone and 93 ± 4% when only combined with ultrasound (Figure 1B). In the case of U-87 MG cells, the combination of doxorubicin treatment with microbubble-assisted ultrasound at 400 kPa induced a 2.5-fold decline of cell viability compared to only doxorubicin treatment or only in combination with ultrasound ($^{**}p < 0.01$ compared to doxorubicin treatment alone or in combination with ultrasound alone). The increase of the acoustic pressure from 600 to 800 kPa caused similar cell viability as for an acoustic pressure of 400 kPa (Figure 1A), whereas for MDA-MB-231 cells, the increase of the acoustic pressure from 400 to 800 kPa induced a significant, gradual decrease of the cell viability, 47 ± 4% at 800 kPa ($^{**}p < 0.01$ compared to doxorubicin treatment alone or in combination with ultrasound alone) (Figure 1B). These results showed that the doxorubicin treatment with Vevo Micromarker microbubble-assisted ultrasound induced a synergistic increment of cell death.

Enhancement of Doxorubicin-Induced Apoptosis by Vevo Micromarker Microbubble-Assisted Ultrasound. The induction of apoptosis was assessed by flow cytometry after annexin V−FITC and propidium iodide staining at 48 h after doxorubicin treatment in combination with Vevo Micromarker microbubble-assisted ultrasound. Two types of apoptotic cells were analyzed: the early apoptotic cells were only stained by annexin V−FITC, and the late apoptotic cells were stained by annexin V−FITC and propidium iodide.

As shown in Figure 2A, the percentage of U-87 MG cells with apoptotic features induced by an insonation at 600 kPa with only Vevo Micromarker microbubbles was 7 ± 1% for early apoptotic cells and 8 ± 1% for late apoptotic cells (Figure 2A). A similar cell apoptosis was achieved at 400 and 800 kPa with or without Vevo Micromarker microbubbles (data not shown). The doxorubicin treatment alone increased 1.5 times higher the presence of early apoptotic cells compared to late apoptotic cells (20 ± 1% vs

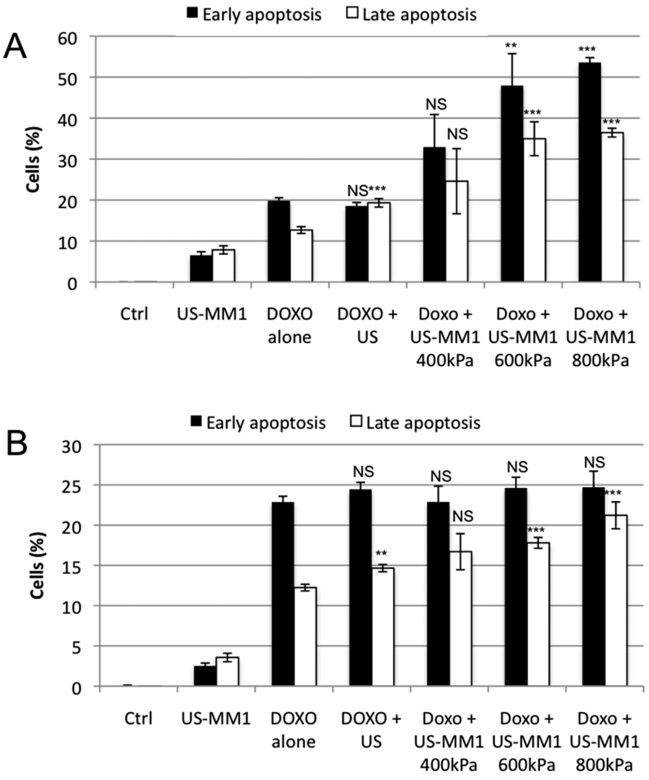

Figure 2. Enhancement of doxorubicin-induced apoptosis by ultrasound combined with Vevo Micromarker microbubbles. U-87 MG (A) and MDA-MB-231 (B) cells were incubated with 5 μM doxorubicin alone (Doxo alone), or with ultrasound at 600 kPa (Doxo+US), or with microbubble-assisted ultrasound (Doxo+US-MM1) at 400 kPa (Doxo+US-MM1-400 kPa), 600 kPa (Doxo+US-MM1-600 kPa) and 800 kPa (Doxo+US-MM1-800 kPa) for 30 s. 48 h after treatment, cell viability was measured by an annexin V−FITC and propidium iodide assay. Data expressed as mean ± SEM calculated from five independent experiments. Statistical analysis was performed using the nonparametric Mann−Whitney test. Significance was defined as $p < 0.05$ (NS, non-significance, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

13 ± 1%) (Figure 2A). When the U-87 MG cells were treated with doxorubicin in combination with ultrasound at 600 kPa, similar levels of early and late apoptotic cells were obtained (19 ± 1%) (Figure 2A). At 400 kPa acoustic pressure, doxorubicin in combination with microbubble-assisted ultrasound did not induce a significant increase of apoptotic cells. The enhancement of acoustic pressure from 600 to 800 kPa induced a 2.5-fold increase of early and late apoptotic cells when compared to doxorubicin treatment alone ($^{**}p < 0.01$) (Figure 2A). In the microbubble-assisted ultrasound condition, the percentages of early and late apoptotic cells were 2 and 2.5 times higher than those of doxorubicin treatment in combination with ultrasound alone ($^{**}p < 0.01$) (Figure 2A).

The insonation of MDA-MB-231 breast cancer cells at 600 kPa with Vevo Micromarker microbubbles alone induced a low level of apoptosis (i.e., 3 ± 1% of early apoptotic cells; 4 ± 1% of late apoptotic cells) (Figure 2B). As for human glioblastoma cells, similar cell apoptosis was achieved at 400 and 800 kPa with or without Vevo Micromarker microbubbles (data not shown). The treatment of MDA-MB-231 cells by the doxorubicin alone induced 8- and 3-fold increase of early and late apoptotic cells, respectively, compared to the ultrasound treatment alone

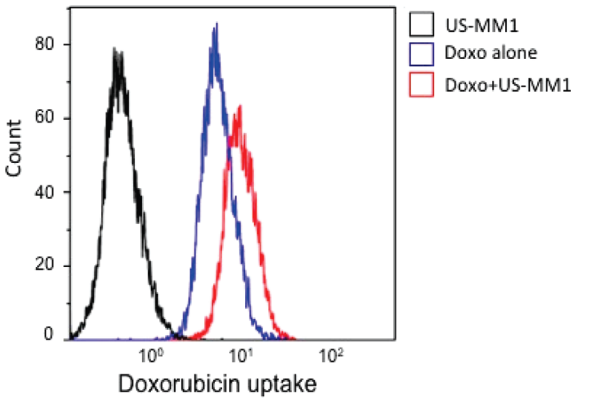

Figure 3. Enhancement of doxorubicin uptake into MDA-MB-231 cells after microbubble-assisted ultrasound. MDA-MB-231 cells were incubated with 5 μM doxorubicin alone (Doxo alone), or with ultrasound (at 600 kPa) in combination with Vevo Micromarker microbubbles (Doxo+US-MM1). After treatment, doxorubicin uptake is measured by flow cytometer.

(Figure 2B). The association of doxorubicin with ultrasound and/or Vevo Micromarker microbubbles led to a level of early apoptotic cells comparable to that obtained with doxorubicin treatment alone (Figure 2B). However, when the MDA-MB-231 cells were treated with doxorubicin in combination with ultrasound at 600 kPa, the level of late apoptotic cells was significantly increased to reach 25 ± 1% ($^{**}p < 0.01$) (Figure 2B). As shown in Figure 2B, the microbubble-assisted ultrasound induced a significant rise of late apoptotic level at 600 kPa and at 800 kPa ($^{***}p < 0.01$) (Figure 2).

These results showed that the doxorubicin treatment with microbubble-assisted ultrasound induced cell death by apoptosis.

Enhancement of Doxorubicin Uptake by Vevo Micromarker Microbubble-Assisted Ultrasound. Flow cytometry has been used for quantitative determination of doxorubicin uptake in the cells. Indeed, since doxorubicin itself is fluorescent, it was used directly to measure cellular uptake without additional markers, fluorescence intensity being directly proportional to the internalized amount of doxorubicin.

The insonation of MDA-MB-231 breast cancer cells at 600 kPa with Vevo Micromarker microbubbles alone did not show an increase of the fluorescence intensity (Figure 3B). This result was similar to that obtained with untreated cells (data not shown). As shown in Figure 3B, the doxorubicin was successfully transported into cells. The cellular uptake of doxorubicin was significantly ($^{*}p < 0.05$) higher in the cells insonated with ultrasound with Vevo Micromarker microbubbles than in those treated with free doxororubicin.

Therapeutic Ratio. In order to assess advantage in the use of microbubble-assisted ultrasound, we defined a therapeutic ratio as the ratio of the cell viability obtained with doxorubicin in association with microbubbles and ultrasound to the cell viability when applying only doxorubicin (Figure 4). The therapeutic ratios obtained with different types of microbubbles were compared at 48 h post-treatment.

As shown in Figure 4A, independent of the microbubble type, the therapeutic ratio was higher than 1, which confirmed that the combination of the doxorubicin with microbubble-assisted ultrasound was more efficient than the doxorubicin treatment on its own to induce U-87 MG glioblastoma cell death. However, the efficiency of microbubble-assisted ultrasound for drug delivery

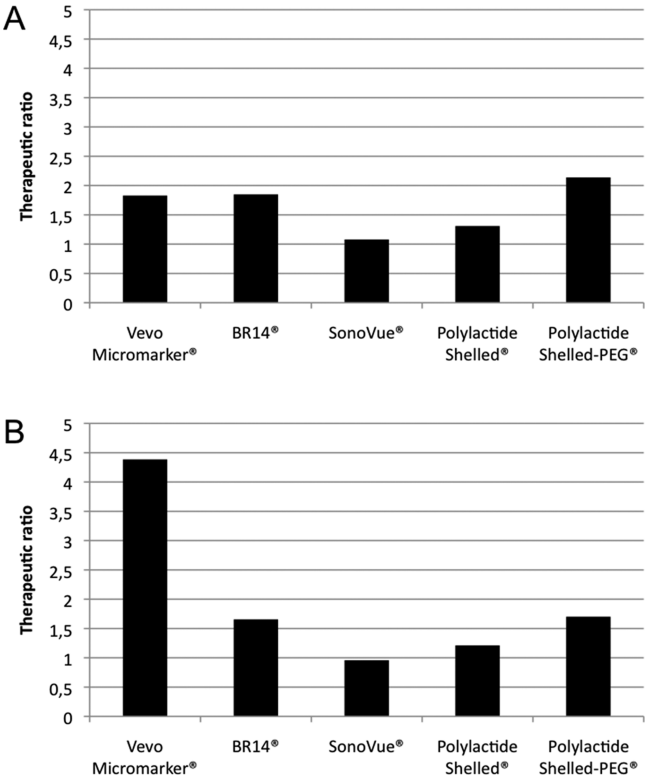

Figure 4. Comparative study of doxorubicin-induced cell death by only doxorubicin versus doxorubicin with microbubble-assisted ultrasound. U-87 MG (A) and MDA-MB-231 (B) cells were incubated with 5 μM doxorubicin alone, or with ultrasound (at 600 kPa) in combination with different kinds of microbubbles. 48 h after treatment, cell viability was measured by a Trypan blue assay.

depends on the type of microbubbles used. Polylactide-Shelled PEG, Vevo Micromarker and BR14 microbubbles were 1.5 to 2 times more efficient than SonoVue and Polylactide-Shelled microbubbles for doxorubicin-induced cell death (Figure 4A).

Likewise, the combination of doxorubicin with microbubble-assisted ultrasound was more efficient than the doxorubicin treatment alone when inducing MDA-MB-231 breast cancer cell death (Figure 4B). However, surprising differences were observed between the two cell lines under study. In fact, SonoVue microbubbles and ultrasound did not enhance the effect of doxorubicin on MDA-MB 231 cells (Figure 4B). However, Vevo Micromarker microbubbles in combination with ultrasound showed a therapeutic ratio of 4.3 (Figure 4B).

These results demonstrated that, for both cell lines, the doxorubicin delivery by microbubble-assisted ultrasound was more efficient than doxorubicin treatment alone or only in combination with ultrasound alone. However, the efficiency of this approach was dependent on the type of microbubbles.

DISCUSSION

In the present study, an enhanced effect of doxorubicin on cancer cell death has been shown when it is coadministered with microbubbles and insonified with appropriate ultrasound parameters. This effect has been evaluated on glioblastoma and breast cancer cells and is directly related to the induction of apoptosis.

First, we validated the enhanced cancer cell death with ultrasound and microbubbles. The doxorubicin treatment with Vevo Micromarker microbubble-assisted ultrasound induced a synergistic enhancement in both glioblastoma and breast cancer cell death. In agreement with data available in the literature, these results demonstrated indirectly that the enhancement of cell death could be ascribed to an increased doxorubicin uptake through ultrasound-induced hydrophilic pores.[8] As shown In Figure 3, the enhancement of MDA-MB-231 cell death is correlated to an increase of doxorubicin uptake into the cells. Moreover, and based on the increased uptake or release of marker compounds and by measuring changes in ionic conductivity, previous studies showed that the microbubble-assisted ultrasound induced transient nanopores into the plasma membrane.[16−18] Thus, the intracellular uptake of small molecules (i.e., $\leq$4 kDa) such as propidium iodide or doxorubicin is likely governed by passive diffusion through membrane pores with a size range from 30 to 100 nm. The small molecules accessed directly into the cytosol of targeted cells. The duration of intracellular uptake is naturally dependent on the membrane recovery time, i.e. a few seconds for small molecules.[16,18] Subsequently, we demonstrated that the efficiency of microbubble-assisted ultrasound was dependent on the type of microbubbles. Our results showed that Polylactide-Shelled PEG and Vevo Micromarker microbubbles were the most optimal microbubbles for an efficient doxorubicin delivery in the U-87 MG and in MDA-MB-231 cells. Our results are in agreement with previously published studies, which showed that the efficiency of drug delivery was dependent on the nature and type of microbubbles.[11,28] Indeed, the efficiency of membrane permeabilization and drug uptake induced by microbubble-assisted ultrasound depends strongly on microbubble parameters: size, shell composition, gas and concentration. These parameters control the acoustic properties of the microbubbles and influence the microbubble response to the ultrasound excitation. Indeed, Kudo and collaborators showed that microbubbles with different shell compositions and sizes induced different physical forces (i.e., microstreaming, shock waves and microjets) that could transiently and differently perforate the plasma membrane of nearby cells and, therefore, enhance the intracellular uptake of drugs.[29] We showed in a recent article proceedings that insonation at experimental conditions similar to those used in this study induce a total microbubble destruction after 30 s.[28] Moreover, we have demonstrated that the microbubble destruction is correlated to the rate of cell membrane permeabilization. Hence, we believe that microbubble destruction plays a major role in membrane permeabilization and drug delivery. Finally, when the induction of apoptosis by doxorubicin and Vevo Micromarker microbubble-assisted ultrasound was examined, a positive enhancement was observed for acoustic pressures above 600 kPa, though no enhancement was observed at 400 kPa. Given that microbubble-assisted ultrasound on its own did not stimulate the apoptosis, these results confirmed that the apoptosis is induced by doxorubicin uptake during the application of microbubble-assisted ultrasound.

The cell lines used in this study respond differently to the treatment with doxorubicin and ultrasound and microbubbles. The difference might be due to a different sensitivity to doxorubicin between human glioblastoma (U-87 MG) cells and breast carcinoma (MDA-MB-231), which might be explained by the different membrane properties (e.g., lipid, protein and carbohydrate composition) that could influence the membrane fluidity and hence the membrane permeabilization induced by microbubble-assisted ultrasound.

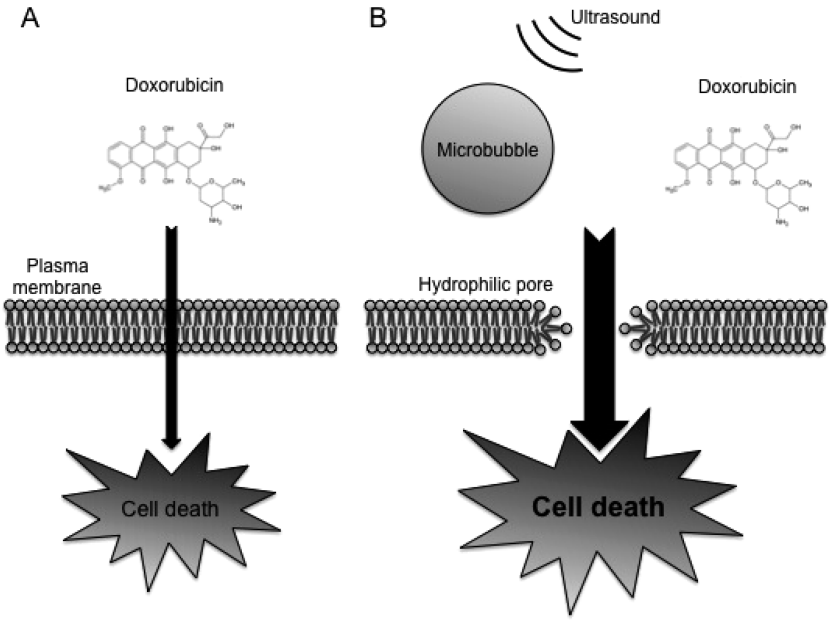

Figure 5. Schematic representation of the combination of doxorubicin treatment with microbubble-assisted ultrasound. (A) Doxorubicin molecules cross the plasma membrane by simple diffusion. (B) During the application of ultrasound, the cavitating and imploding microbubbles improved the plasma membrane permeability (i.e., formation of pores), which enhanced the amount of uptaken doxorubicin by tumor cells. (C) Doxorubicin uptake by U-87MG cells using microbubbles (BR14) and ultrasound: contrast phase (left) and fluorescence (right) microscopy.

Based on the results drawn from the present study, we hypothesize that free doxorubicin in combination with microbubble-assisted ultrasound may significantly improve the *in vivo* efficacy of free doxorubicin for tumor treatment as shown in the schematic drawing in Figure 5. This novel strategy is compatible with current chemotherapy protocols (i.e., intravenous injection of a single dose of doxorubicin).[1] A targeted and controlled ultrasound-triggered release of free doxorubicin in the tumor may enhance the cell death and tumor regression. Moreover, the cavitation and the destruction of microbubbles by ultrasound in the tumor microvascular network may promote permeation on the tumor endothelium, and consequently enhance the extravasation of free doxorubicin.[30−32] The permeabilization of endothelial cells may increase the uptake of free doxorubicin in these cells. Thus, this new therapeutic strategy might potentiate the destruction of tumor vasculature and reduce the nutrient supply (i.e., oxygen, nutrients, etc.) of cancer cells.[33] However, further research is necessary to validate the therapeutic association of doxorubicin with microbubble-assisted ultrasound *in vivo,* and ultimately to reduce the doxorubicin dose required to achieve a high therapeutic ratio.

As mentioned earlier, the use of microbubble-assisted ultrasound to increase drug release from micro- and nanoparticles and to enhance drug uptake has been previously described. However, only a few studies have reported the combination of ultrasound and doxorubicin-loaded microbubbles[34,35] or doxorubicin-encapsulated liposomes loaded microbubbles[8] to improve antitumor effect and diminish side effects of doxorubicin. While these approaches seem to be promising, the amount of encapsulated doxorubicin encountered was major drawback. Tinkov et al. reported that the combination of doxorubicin-loaded microbubbles and ultrasound led to a significant decrease of pancreas carcinoma.[34] However, the administered dose of 0.7 mg/kg doxorubicin was 1.6 times smaller than the therapeutic dose used in human chemotherapy protocols.[34] Consequently, the use of doxorubicin-loaded microbubbles required either enhancement of the doxorubicin loading efficiency or administration of more microbubbles to reach human therapeutic dose provided that this administration is well tolerated or application of consecutive treatments. Lentacker et al. were the first to report on the design of doxorubicin-encapsulated liposomes attached to microbubbles.[8] They showed that ultrasound induces the release of the doxorubicin. The authors estimated the amount of encapsulated doxorubicin attached to one microbubble to be 3.35×10^{-8} μg. They compared this doxorubicin dose with liposomal doxorubicin formulation, Doxil, approved by FDA for the treatment of Kaposi's sarcoma and ovarian cancer. To reach this therapeutic dose (i.e., one dose of Doxil corresponds to 40 mg of doxorubicin for a patient of about 80 kg), a total number of 1.23×10^{12} doxorubicin-encapsulated liposome loaded microbubbles have to be injected. However, the recommended diagnostic dose of Definity microbubbles is 10^{10} for a patient of 80 kg. This dose is about 100 times lower than that of doxorubicin-encapsulated liposome loaded microbubbles, which have to be administered to reach 40 mg of doxorubicin. However, as underlined by the authors, it has been demonstrated that doses of Definity microbubbles that are 1,000 times higher than the recommended dose are well tolerated in primates.[36−38] Consequently, the injection of a high dose of microbubbles could be considered for drug delivery. Moreover, a recent investigation showed that repeated treatments of epirubicin (i.e., a byproduct of doxorubicin) and microbubble-assisted ultrasound induced an efficient inhibition of marrow leukemic HL-60 tumor growth followed by tumor eradication.[39]

■ CONCLUSIONS

In summary, our data suggest that the combination of doxorubicin treatment with microbubble-assisted ultrasound enhanced the doxorubicin cytotoxicity compared with only doxorubicin treatment. This improvement could be ascribed to

the increase of doxorubicin uptake into cancer cells. Further research is necessary to understand the mechanisms involved in doxorubicin uptake. Our results demonstrated that the coadministration of microbubbles and approved anticancer drugs in combination with ultrasound might be a new strategy to improve the efficiency and the safety of conventional chemotherapy treatment.

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