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# 1 Delivery of Anti-Cancer Drugs using Microbubble- 2 Assisted Ultrasound in a 3D Spheroid Model

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17 RUNNING TITLE

18 Acoustically mediated drug delivery in spheroids

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22 version of the manuscript.

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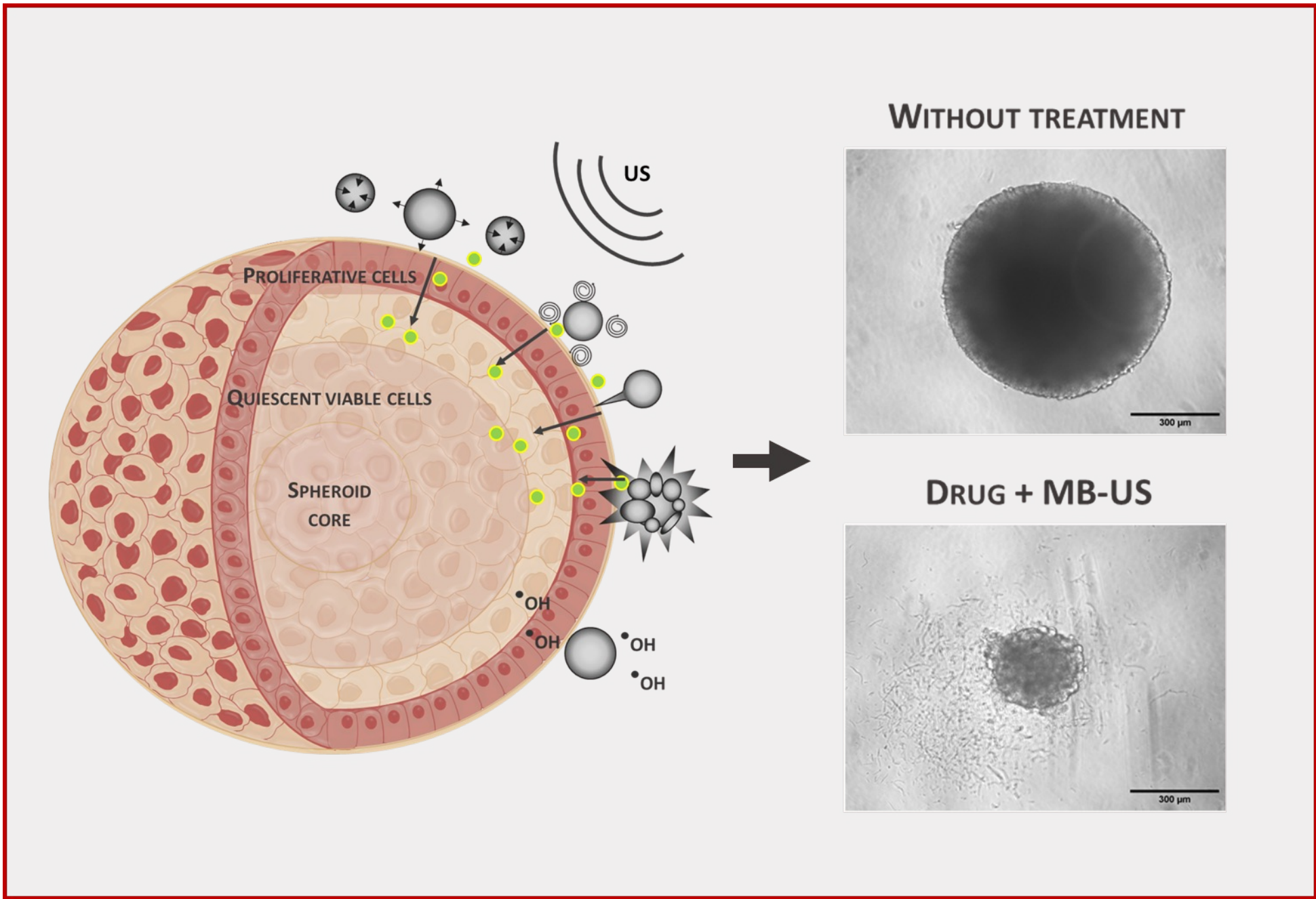
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38 ABSTRACT (200 WORDS)

39 Tumor spheroids are promising 3D *in vitro* tumor models for the evaluation of drug delivery  
40 methods. The design of noninvasive and targeted drug methods is required to improve the  
41 intratumoral bioavailability of chemotherapeutic drugs and to reduce their adverse off-target  
42 effects. Among such methods, microbubble-assisted ultrasound (MB-assisted US) is an innovative  
43 modality for noninvasive targeted drug delivery. The aim of the present study is to evaluate the  
44 efficacy of this US modality for the delivery of bleomycin, doxorubicin and irinotecan in colorectal  
45 cancer (CRC) spheroids. MB-assisted US permeabilized the CRC spheroids to propidium iodide,  
46 which was used as a drug model, without affecting their growth and viability. Histological analysis  
47 and electron microscopy revealed that MB-assisted US affected only the peripheral layer of CRC  
48 spheroids. The acoustically mediated bleomycin delivery induced a significant decrease in CRC  
49 spheroid growth in comparison to spheroids treated with bleomycin alone. However, this US  
50 modality did not improve the therapeutic efficacy of doxorubicin and irinotecan on CRC spheroids.  
51 In conclusion, this study demonstrates that tumor spheroids are a relevant approach to evaluate the  
52 efficacy of MB-assisted US for the delivery of chemotherapeutics.

53 KEYWORDS: Microbubble – Ultrasound – Sonoporation – Drug delivery – Spheroid – Colorectal  
54 Cancer.

55



Graphical abstract

## 56 INTRODUCTION

57 Ethical considerations in animal experimentation have led researchers to consider and  
58 design new 3D *in vitro* tumor models for drug screening, drug design, drug targeting and drug  
59 toxicity, especially when the promising results obtained in 2D *in vitro* tumor models (*e.g.*, cell  
60 monolayers, coculture systems, *etc.*) have not been reproduced in animal tumor models. Among  
61 these 3D models, tumor spheroids are the most exploited for the design and validation of anticancer  
62 strategies. As reported in [1], tumor spheroids are 3D aggregates of tumor cells that may or may  
63 not be associated with other cell types, such as fibroblasts or immune cells. These cells interact  
64 not only with themselves but also with an extracellular matrix (*i.e.*, endogenous or exogenous),  
65 thus creating a dense molecular and cellular network, which limits drug access to tumor cells [2,3].  
66 The main physiological consequence of such gradients is the establishment of a peripheral layer  
67 of proliferative cells, an intermediate layer of quiescent cells and a necrotic core, as described for  
68 tumors *in vivo* [4,5]. Thus, these spheroids partially mimic *in vivo* tumor physiology,  
69 heterogeneity, microenvironment, and drug resistance [6]. These intrinsic properties of the tumor  
70 and its microenvironment restrict the extravasation, penetration, targeting and retention of drugs  
71 into the tumor tissue, thus resulting in low therapeutic efficacy and severe off-target effects. To  
72 overcome these limitations, the design and evaluation of efficient and targeted drug delivery  
73 modalities are required to increase the local dose of anticancer drugs at the desired site while  
74 reducing side effects to healthy tissues.

75 Among these modalities, microbubble-assisted ultrasound (MB-assisted US) is a promising  
76 physical method for the noninvasive and targeted delivery of various types of anticancer drugs,  
77 including chemotherapeutic drugs, nucleic acids (*e.g.*, plasmid DNA, mRNA, siRNA),  
78 immunotherapeutics, kinase inhibitors, tumor sensitizers and oncolytic viruses [7–10]. These

79 therapeutics and MBs (*i.e.*, bare or targeted ones) can be administered intratumorally and  
80 intravenously depending on the pharmacological properties of both agents and/or the desired  
81 therapeutic effect [11]. The therapeutics are either coadministered or administered successively  
82 with MBs *in vivo* and can also be charged on or into the MBs and be administered together. Then,  
83 US is applied to the tumor tissue when a quantity of MBs and drugs are sufficiently accumulated  
84 [12]. In response to US waves, MBs oscillate close to biological barriers, such as the plasma  
85 membrane of tumor cells (after their intratumoral administration) or the blood-tumor barrier (after  
86 their intravenous administration), therefore promoting acoustic phenomena (*e.g.*, pulling/pushing  
87 processes, microstreaming, shock waves, microjet) [13]. These phenomena transiently  
88 permeabilize these barriers to the therapeutics through the stimulation of intracellular (*e.g.*,  
89 formation of membrane pores and endocytosis), paracellular (*e.g.*, disruption of tight junctions)  
90 and transcellular (*e.g.*, transcytosis) pathways. Thus, this US modality enhances the therapeutic  
91 efficacy of anticancer drugs by improving their intratumoral bioavailability while minimizing their  
92 off-target effects [14].

93         Since the advent of spheroids, fewer than ten published *in vitro* studies have used spheroids  
94 to either design and evaluate US protocols and new formulations of drugs and MBs or to  
95 investigate the influence of the tumor microenvironment on therapeutic efficacy [1,15,16]. In this  
96 context, the aims of the present study are (i) to evaluate the influence of acoustic pressure and MB  
97 concentration on the delivery of a drug model into colorectal cancer (CRC) spheroids and (ii) to  
98 investigate whether MB-assisted US is able to deliver different types of chemotherapeutic drugs  
99 in these CRC spheroids.

## 100 EXPERIMENTAL SECTION

### 101 *Cell culture*

102           The IVISbrite<sup>®</sup> HCT-116 Red F-luc tumor cell line (Perkin-Elmer<sup>®</sup>, Codolet, France) is a  
103 bioluminescence-producing cell line derived from HCT-116 human colorectal carcinoma. These  
104 cells were stably transduced with the redshifted firefly luciferase gene from *Luciola Italica*. They  
105 were cultured in McCoy's 5A (Thermo Fisher Scientific, Waltham, MA) supplemented with 10%  
106 fetal calf serum (FCS; Eurobio, Courtaboeuf, France) and 1% penicillin/streptomycin (Thermo  
107 Fisher Scientific), and they were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.  
108 This cell line has been chosen because it is the most common cell line used to generate CRC  
109 spheroids uniformly and reproducibly. In addition, the physiological properties of these spheroids  
110 are well documented [17,18]

#### 111 *Spheroid formation*

112           Adapted from Griseti et al., [19] the cells were seeded at a density of 500 cells/well in a  
113 96-well clear round bottom ultralow attachment (ULA) microplate (Dutscher, Bernolsheim,  
114 France) and centrifuged at 500 × g for 3 min. Then, the microplate was incubated at 37 °C in a  
115 humidified atmosphere in a 5% CO<sub>2</sub> incubator. The plastic surface of these wells is treated to  
116 prevent the attachment of cells to this surface and to promote their aggregation. This ULA surface  
117 enables uniform and reproducible 3D multicellular spheroid formation after 3 days. In a  
118 preliminary study, a groupwise analysis of spheroid area using a Kruskal-Wallis test showed no  
119 significant difference in spheroid uniformity in microplates under our experimental conditions (p  
120 > 0.05; n > 400 spheroids from 6 independent experiments). In addition, the intragroup coefficients  
121 of variation of spheroid area for each experiment are small (4.6 to 4.8) indicating a greater  
122 reproducibility of spheroids in our experimental conditions. The spheroids were spherical and  
123 cohesive and have with an average diameter of 300 ± 10 μm.

#### 124 *Anticancer drugs and microbubbles*



125 Bleomycin sulfate (B1141000; Sigma–Aldrich, St Louis, MO) and doxorubicin  
126 (D2975000; Sigma Aldrich, St Louis, MO) were dissolved in Dulbecco’s phosphate-buffered  
127 saline solution (DPBS; Thermo Fisher Scientific) at a 1 mM stock solution. Irinotecan (Accord  
128 Health care AG, Bottmingen, Switzerland) was a generous gift from Dr. Virginie André (Regional  
129 Center of Cancerology, Henry Kaplan, CHRU de Tours, France), and its concentration was 20  
130 mg/mL. Vevo MicroMarker® contrast agents were purchased from VisualSonics-Fujifilm Inc.  
131 (Toronto, Canada) [20,21]. These agents are MBs consisting of a gaseous core of nitrogen and a  
132 perfluorobutane mixture encapsulated in by a PEGylated phospholipid shell [22]. As previously  
133 reported in our *in-vitro* and *in-vivo* studies, these MBs are the most effective in delivering  
134 therapeutic molecules including anticancer drugs and plasmid DNA[21–23]. They were prepared  
135 according to the manufacturer’s instructions at a final concentration of  $2 \cdot 10^9$  MB/mL.

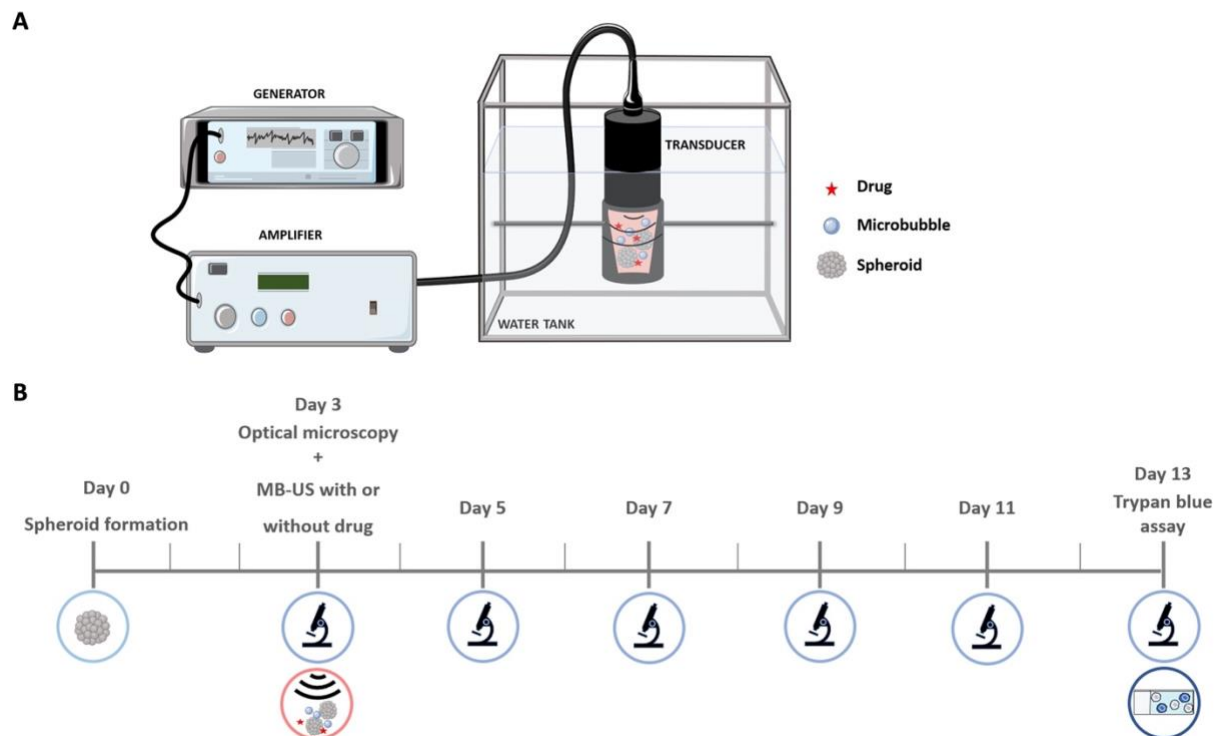
#### 136 *Ultrasound device*

137 A 1-MHz single-element transducer (IBMF014; NDT Systems, Nashua, NH, USA) was  
138 used for membrane permeabilization and drug delivery. The transducer had a diameter of 12.7  
139 mm, a natural focal distance at 27 mm and a focal spot of 6 mm. An arbitrary waveform generator  
140 (Agilent, Santa Clara, CA) was used to generate an electrical sinusoidal signal with a central  
141 frequency of 1 MHz, pulse repetition frequency (PRF) of 10 kHz and a duty cycle of 40%. The  
142 signal was then amplified using a power amplifier (AAP-500-0.2-6-D; ADECE, Veigné, France)  
143 before its transmission to the transducer. A separate system using a calibrated capsule hydrophone  
144 (HGL-0085; ONDA Corporation, Sunnyvale, CA, USA) was used to determine the lateral and  
145 axial acoustic profiles and the sensitivity curve of the transducer. The peak negative pressures  
146 (PNP) measured in the cuvette on the axis of transducer were 126, 252, 378 and 504 kPa while  
147 these PNP were 100, 200, 300 and 400 kPa in water, respectively.

148 *Spheroid permeabilization*

149 Propidium iodide (PI, P4864; Sigma–Aldrich) was used as a drug model (669.39 Da;  
150 hydrodynamic radius of 0.6 nm) to investigate the influence of peak negative pressure (PNP; 100,  
151 200, 300 and 400 kPa) and MB concentration ( $2 \cdot 10^7$ ,  $4 \cdot 10^7$  and  $8 \cdot 10^7$  MB/mL) on spheroid  
152 permeabilization. This molecule is a non-permeant and fluorescent DNA intercalating agent,  
153 which is commonly used as a membrane integrity marker to investigate the membrane  
154 permeabilization [24–27]. This fluorescent dye has a very low fluorescent intensity in an aqueous  
155 solution, while its quantum yield is 1000-fold enhanced after its intracellular uptake and its binding  
156 to DNA. A suspension of 5 spheroids in 1.5 mL of McCoy’s 5A medium supplemented with 1 %  
157 FCS was placed in a dedicated plastic cuvette. Then, a solution of PI (100  $\mu$ M final concentration)  
158 and MBs was added to the spheroid suspension just before US exposure. The center of the cuvette  
159 was immersed in a deionized and degassed water tank at 37°C, and its center was positioned at the  
160 focal distance of the transducer (**Figure 1**). Subsequently, the spheroids were exposed to 1 MHz  
161 sinusoid US waves with a pulse repetition period (PRP) of 100  $\mu$ s, 40 cycles per pulse (*i.e.*, 40 %  
162 duty cycle) and for 1 min. Ten minutes later, the penetration and intracellular accumulation of PI  
163 into spheroids were assessed using a fluorescence microscope (EVOS, M5000, Thermo Fisher  
164 Scientific). A semi-quantitative analysis of microscopic images was performed with ImageJ  
165 software (NIH, Bethesda, MA) in order to determine the integrated intensity of PI (a.u./ $\mu$ m<sup>2</sup>).  
166 Representative confocal images of permeabilized spheroids were obtained using LEICA SP8  
167 gSTED confocal microscope (Leica, Wetzlar, Germany).

168 The spheroids were exposed to MB-assisted US in the absence of PI in order to assess the  
169 effects of acoustically mediated membrane permeabilization on the spheroid growth and viability  
170 following the protocols described below.



171  
 172 **Figure 1.** Set up and experimental timeline. (A) *In vitro* US setup. (B) Timeline of acoustically mediated drug delivery  
 173 experiments on CRC spheroids.

174 *Drug Delivery*

175 Three chemotherapeutic drugs, including bleomycin (0.1  $\mu\text{M}$  and 1  $\mu\text{M}$ ), doxorubicin (1  
 176  $\mu\text{M}$  and 10  $\mu\text{M}$ ) and irinotecan (0.1  $\mu\text{g/mL}$  and 1  $\mu\text{g/mL}$ ), were acoustically delivered (1 MHz,  
 177 100  $\mu\text{s}$  PRP, 40 cycles/pulse for 1 min in the presence of  $4 \cdot 10^7$  MB/mL) into the spheroids using  
 178 the same US protocol and setup described above for spheroid permeabilization. The concentrations  
 179 of drugs were selected based on our *in vitro* preliminary studies. Ten minutes after US exposure,  
 180 the spheroids were transferred to a 96-well ULA microplate. The final concentrations of FCS and  
 181 antibiotics were increased to 10% and 1%, respectively. Finally, the microplate was incubated at  
 182 37  $^{\circ}\text{C}$  in a humidified atmosphere in a 5%  $\text{CO}_2$  incubator for spheroid growth.

183 *Spheroid growth*

184 Spheroid growth was monitored by optical microscopy (EVOS, M5000) every two days  
185 for ten days after US exposure. Then, the spheroid area was measured using ImageJ software.

#### 186 *Spheroid viability*

187 On the 13<sup>th</sup> day post-US exposure, spheroid viability was assessed using the trypan blue  
188 exclusion assay. Briefly, the spheroids were harvested and washed with PBS. The spheroids were  
189 pooled in two groups and incubated with 50  $\mu$ L of Accumax (Thermo Fisher Scientific) for 15 min  
190 at 37 °C in a humidified atmosphere in a 5% CO<sub>2</sub> incubator for enzymatic dissociation. Then, they  
191 were dissociated mechanically (rapid pipetting) to generate a cell suspension. One volume of cells  
192 was mixed with an equal volume of trypan blue. Finally, the concentration of viable cells was  
193 measured using a Countess<sup>TM</sup> automated cell counter (Invitrogen, Waltham, MA).

#### 194 *Transmission electron microscopy*

195 As previously reported [28], the spheroids were fixed in 1% glutaraldehyde and 4%  
196 paraformaldehyde (Sigma–Aldrich) in 0.1 M phosphate buffer (pH 7.2) for 24 h. Then, the samples  
197 were washed in PBS and postfixed in 2% buffered osmium tetroxide (Agar Scientific, Stansted,  
198 UK) for 1 h. After dehydration in a graded series of ethanol solutions (70% and 90% v/v) and  
199 propylene oxide (100%), an impregnation step was performed with a mixture of propylene  
200 oxide/Epon resin (1:1; Sigma–Aldrich) and then left overnight in pure resin. The samples were  
201 then embedded in Epon resin (Sigma–Aldrich), which was allowed to polymerize for 48 h at 60  
202 °C. Thin (250 nm) and ultrathin sections (90 nm) of these blocks were generated with a Leica EM  
203 UC7 ultramicrotome (Leica, Wetzlar, Germany). Thin sections were stained with 0.5% toluidine  
204 blue (Sigma–Aldrich), and ultrathin sections were stained with 2% uranyl acetate (Agar Scientific  
205 Ltd, Stansted, UK) and 5% lead citrate (Sigma–Aldrich). The microscopic observations were made  
206 with a transmission electron microscope (JEOL 1011, Tokyo, Japan).

207 *Statistical analysis*

208 All quantitative data were analyzed using Kruskal–Wallis’s test and Dunn's multiple  
209 comparison test for spheroid permeabilization and viability and two-way ANOVA and Dunnett’s  
210 test for spheroid growth (significance was defined as  $p < 0.05$ ) with GraphPad Prism v.9.5.1  
211 (GraphPad Software Inc., La Jolla, CA).

## 212 RESULTS

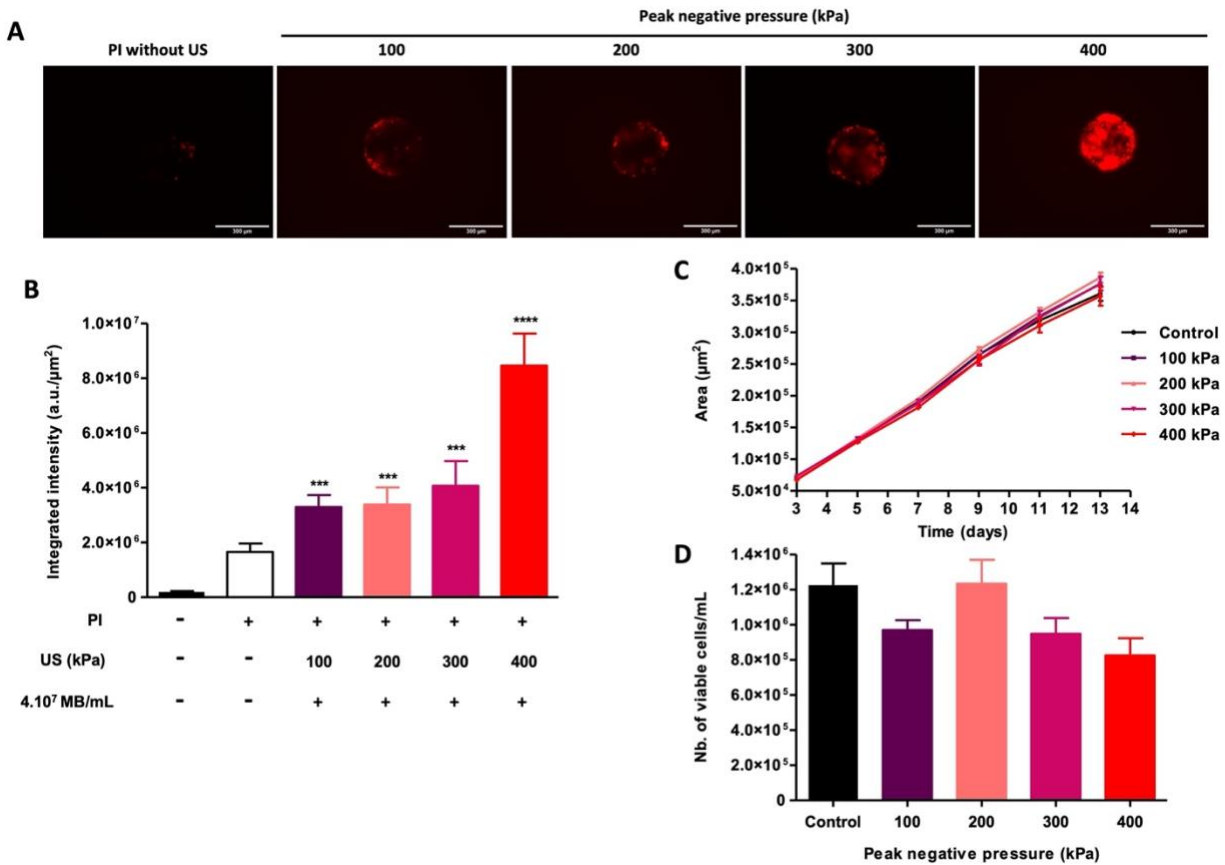
### 213 *CRC spheroid permeabilization to small molecules*

214 To evaluate the influence of PNP and MB concentration on the permeabilization level of  
215 CRC spheroids, the membrane permeabilization of tumor cells was monitored using a small and  
216 nonpermeant drug model, PI, and the permeabilization level was assessed by epifluorescence  
217 microscopy.

218 As depicted in **Figures 2A** and **2B**, the simple incubation of spheroids with PI revealed the  
219 presence of fluorescence labeling ( $1.7 \pm 0.3 \times 10^6$  a.u./ $\mu\text{m}^2$ ) inside the spheroids corresponding to  
220 the presence of dead cells. The exposure of spheroids to 100 kPa in the presence of MBs  
221 ( $4 \times 10^7$  MB/mL) significantly increased their permeabilization to PI compared to PI incubation  
222 alone ( $***p < 0.001$ ;  $3.3 \pm 0.4 \times 10^6$  versus  $1.7 \pm 0.3 \times 10^6$  a.u./ $\mu\text{m}^2$ ). The increase in the acoustic  
223 pressure from 200 to 300 kPa caused spheroid permeabilization similar to that at an acoustic  
224 pressure of 100 kPa ( $p > 0.05$ ;  $3.4 \pm 0.6 \times 10^6$  and  $4.1 \pm 0.9 \times 10^6$  a.u./ $\mu\text{m}^2$ , respectively).  
225 However, the exposure of spheroids to 400 kPa induced a 2.5-fold increase in their  
226 permeabilization in comparison to their exposure to 100 kPa ( $****p < 0.0001$ ;  $8.5 \pm 1.2 \times 10^6$   
227 versus  $3.3 \pm 0.4 \times 10^6$  a.u./ $\mu\text{m}^2$ ).

228 The influence of this range of PNP (from 100 to 400 kPa) on spheroid growth and viability  
229 was investigated by optical microscopy over 13 days and using a trypan blue exclusion assay on

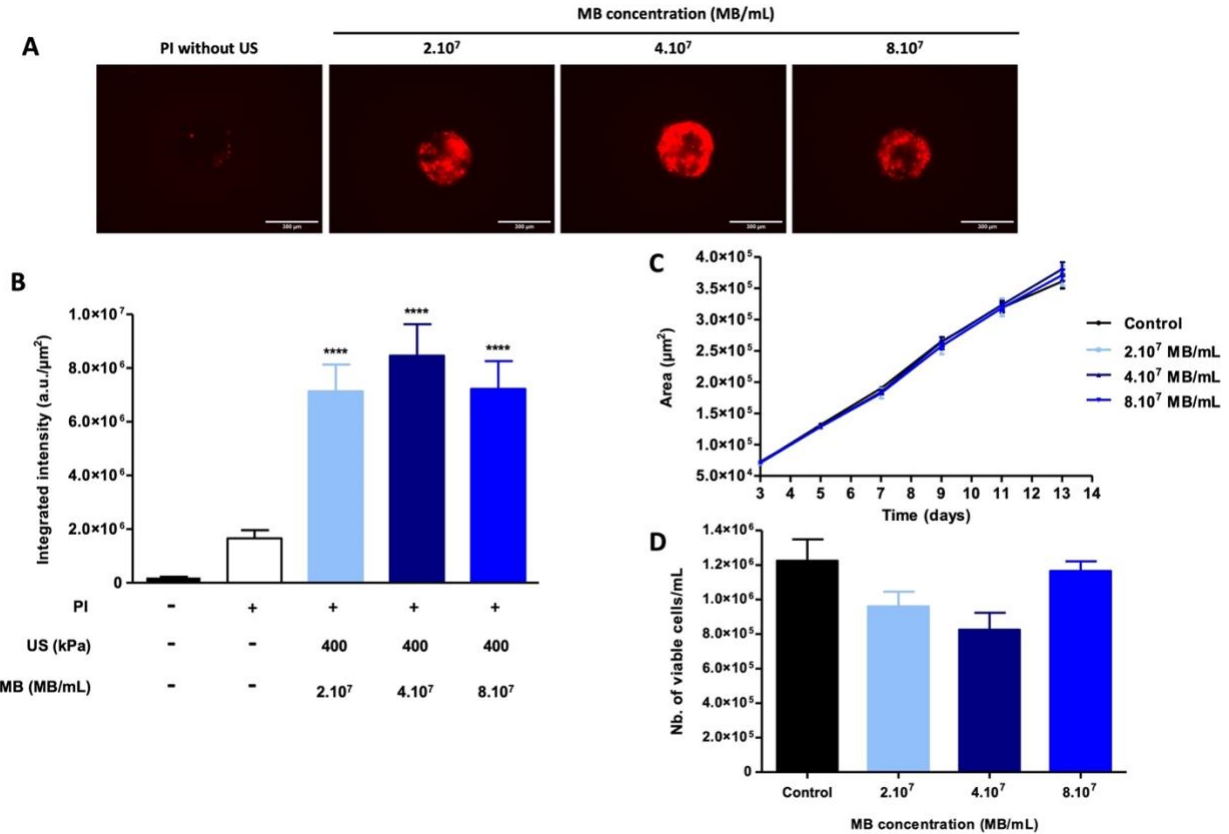
230 the 13<sup>th</sup> day of spheroid growth, respectively. The increase in PNP had no significant influence on  
 231 spheroid growth (**Figure 2C**) or viability (**Figure 2D**).



232  
 233 **Figure 2.** Influence of peak negative pressure (PNP) on CRC spheroid permeabilization, growth and viability. CRC  
 234 spheroids were incubated with 100  $\mu\text{M}$  PI alone or with MB-assisted US at 100 to 400 kPa for 1 min. (A)  
 235 Representative fluorescence images of permeabilized spheroids. The scale bar indicates 300  $\mu\text{m}$ . (B) Quantification  
 236 of PI fluorescence intensity of permeabilized spheroids. Data expressed as mean  $\pm$  SEM was calculated from 18 CRC  
 237 spheroids. Statistical analysis was performed using Kruskal–Wallis’s test and Dunn’s multiple comparison test.  
 238 Significance was defined as  $p < 0.05$  (\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). Then, CRC spheroids were exposed to MB-  
 239 assisted US at 100 to 400 kPa for 1 min. (C) The spheroid growth was assessed over time using optical imaging. Data  
 240 expressed as mean  $\pm$  SEM was calculated from 18 CRC spheroids. Statistical analysis was performed using Two-way  
 241 ANOVA test and Dunnett’s test. Significance was defined as  $p < 0.05$  (D) The spheroid viability was evaluated on the  
 242 13<sup>th</sup> day post-US exposure, using trypan blue dye exclusion assay. Data expressed as mean  $\pm$  SEM was calculated

243 from 18 CRC spheroids. Statistical analysis was performed using Kruskal–Wallis’s test and Dunn’s multiple  
244 comparison test. Significance was defined as  $p < 0.05$ .

245 Then, the influence of MB concentration ( $2 \cdot 10^7$ ,  $4 \cdot 10^7$  and  $8 \cdot 10^7$  MB/mL) on spheroid  
246 permeabilization to PI was investigated at the given PNP of 400 kPa using the same experimental  
247 strategy as shown in **Figures 3A** and **3B**. As described above, the coincubation of spheroids with  
248 PI stained the dead cells, thus causing an increase in the associated fluorescence intensity  
249 ( $1.7 \pm 0.3 \times 10^6$  a.u./ $\mu\text{m}^2$ ). The insonation of spheroids in the presence of MBs at  $2 \cdot 10^7$  MB/mL  
250 led to a significant permeabilization of spheroids compared to PI incubation alone  
251 ( $****p < 0.0001$ ;  $7.1 \pm 1 \times 10^6$  *versus*  $1.7 \pm 0.3 \times 10^6$  a.u./ $\mu\text{m}^2$ ). Surprisingly, the doubling or  
252 quadrupling of the MB concentration resulted in similar permeabilization of spheroids as for an  
253 MB concentration of  $2 \cdot 10^7$  MB/mL ( $p > 0.05$ ;  $8.5 \pm 1.1 \times 10^6$  and  $7.2 \pm 1 \times 10^6$  a.u./ $\mu\text{m}^2$ ,  
254 respectively). In addition, the increase in MB concentration from  $2 \cdot 10^7$  to  $8 \cdot 10^7$  MB/mL did not  
255 affect either spheroid growth over time (**Figure 3C**) or viability on the 13<sup>th</sup> day (**Figure 3D**).  
256 Altogether, these data show that MB-assisted US permeabilizes CRC spheroids without affecting  
257 their growth and viability under our experimental conditions.



258

259

**Figure 3.** Influence of MB concentration on CRC spheroid permeabilization, growth and viability. CRC spheroids

260

were incubated with 100 μM PI alone or with MB-assisted US at 400 kPa for 1 min in the presence of 2.10<sup>7</sup>, 4.10<sup>7</sup> or

261

8.10<sup>7</sup> MB/mL. (A) Representative fluorescence images of permeabilized spheroids. The scale bar indicates 300 μm.

262

(B) Quantification of PI fluorescence intensity of permeabilized spheroids. Data expressed as mean ± SEM was

263

calculated from 18 CRC spheroids. Statistical analysis was performed using Kruskal–Wallis’s test and Dunn’s

264

multiple comparison test. Significance was defined as  $p < 0.05$  (\*\*\*\* $p < 0.0001$ ). CRC spheroids were incubated with

265

100 μM PI alone or with MB-assisted US at 400 kPa for 1 min in the presence of 2.10<sup>7</sup>, 4.10<sup>7</sup> or 8.10<sup>7</sup> MB/mL. (C)

266

The spheroid growth was assessed over time using optical imaging. Data expressed as mean ± SEM was calculated

267

from 18 CRC spheroids. Statistical analysis was performed using Two-way ANOVA test and Dunnett’s test.

268

Significance was defined as  $p < 0.05$  (D) The spheroid viability was evaluated on the 13<sup>th</sup> day post-MB-assisted US

269

exposure, using trypan blue dye exclusion assay. Data expressed as mean ± SEM was calculated from 18 CRC

270

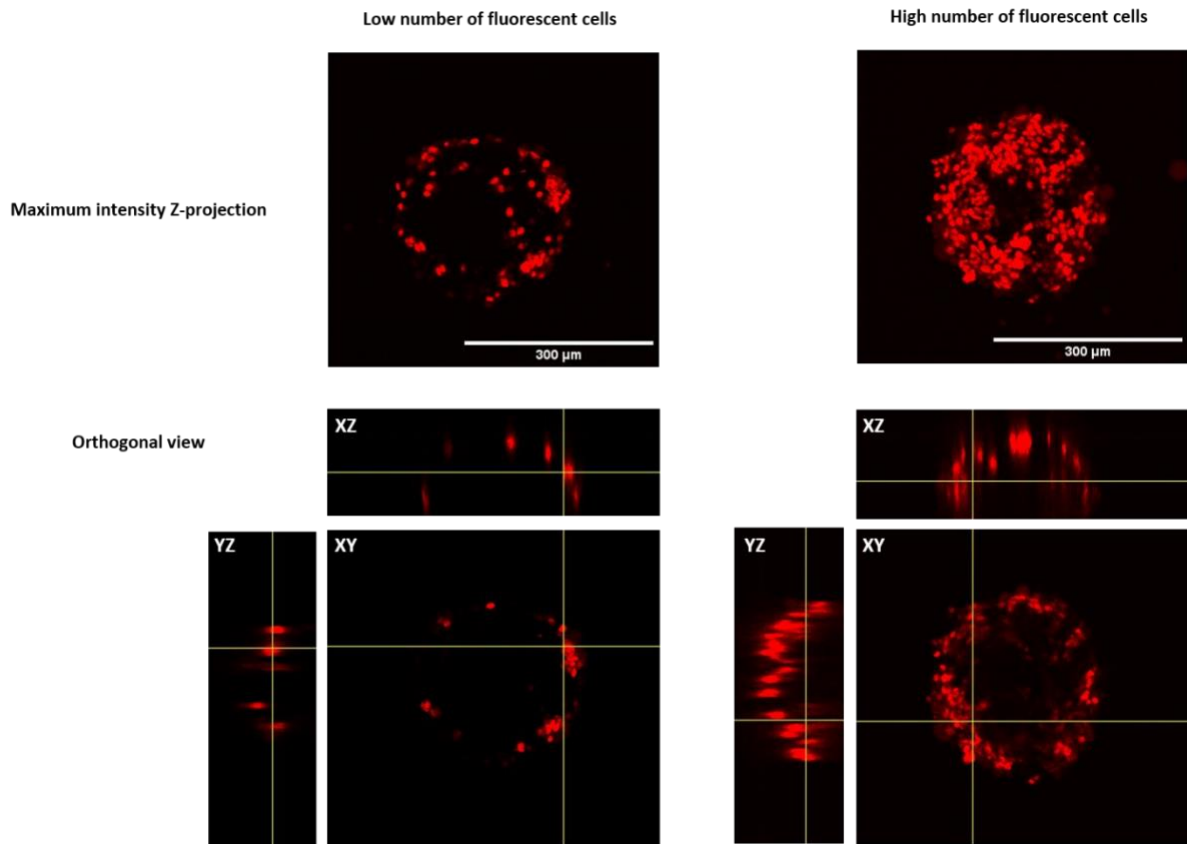
spheroids. Statistical analysis was performed using Kruskal–Wallis’s test and Dunn’s multiple comparison test.

271

Significance was defined as  $p < 0.05$ .



272 In addition, our confocal microscopic observations of permeabilized spheroids revealed  
273 heterogeneous permeabilization between the spheroids and within each spheroid. Indeed, the  
274 spheroids showed a low or high number of fluorescent cells predominantly located in the outer cell  
275 layer of spheroids (**Figure 4**).

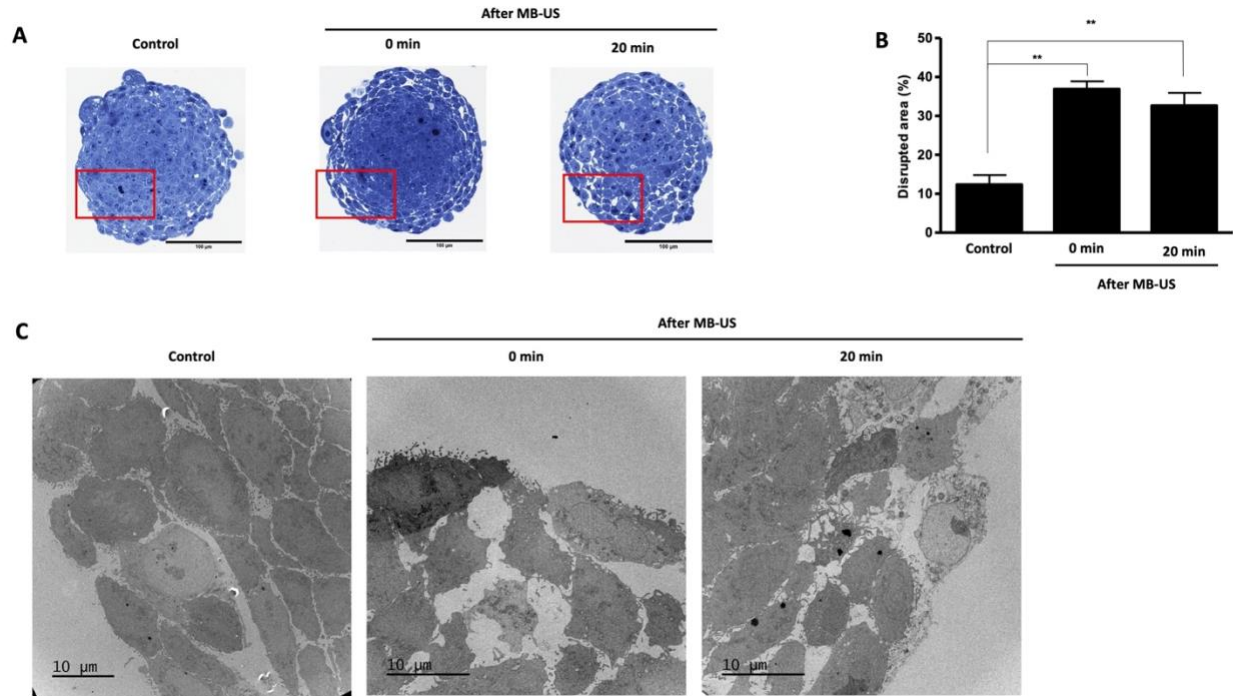


276  
277 **Figure 4. Representative confocal images of permeabilized spheroids.** The scale bar indicates 300 μm.

### 278 *Impact of US-MB on spheroid structure*

279 Using the same setup and experimental conditions (400 kPa PNP,  $4 \cdot 10^7$  MB/mL)  
280 previously described for spheroid permeabilization, ultrastructural modifications of CRC  
281 spheroids were investigated using both histology after toluidine blue staining (**Figures 5A and**  
282 **5B**) and transmission electron microscopy (TEM; **Figure 5C**) immediately and 20 min after MB-  
283 assisted US (in the absence of PI). Optical images of control spheroids (no MB-assisted US

284 exposure) revealed no visually apparent effects on spheroid morphology (**Figure 5A**). In the  
285 control condition, few intercellular disruptions were observed in the peripheral layers of tumor  
286 cells, which could be attributed to the putative artifactual effect of sample preparation and handling  
287 (**Figures 5A and 5B**). However, the spheroids appeared less cohesive in structure immediately  
288 after MB-assisted US exposure compared to the control spheroids (**Figure 5A**). The intercellular  
289 junctions of tumor cells located in the peripheral layers of spheroids were significantly disrupted  
290 in comparison to the control spheroids (\*\* $p < 0.01$ ;  $37 \pm 2\%$  versus  $12 \pm 2\%$ ; **Figure 5B**). A  
291 slight but nonsignificant decrease in this acoustically disrupted area was observed 20 min after  
292 US-assisted MB exposure ( $p > 0.05$ ;  $32 \pm 3\%$  versus  $37 \pm 2\%$ ; **Figure 5B**). One noticed that MB-  
293 assisted US did not seem to affect the structure of the intermediate layer or the core of spheroids  
294 under our experimental conditions. Moreover, TEM analysis confirmed these histological  
295 observations. The sonicated spheroids exhibited significant changes in cell morphology and  
296 organization (*i.e.*, wide intercellular gaps) in the peripheral layer of spheroids, which resulted in  
297 loss of spheroid cohesion in this layer compared to the control (**Figure 5C**). These results  
298 demonstrate that MB-assisted US affects only the peripheral layer of CRC spheroids.



299

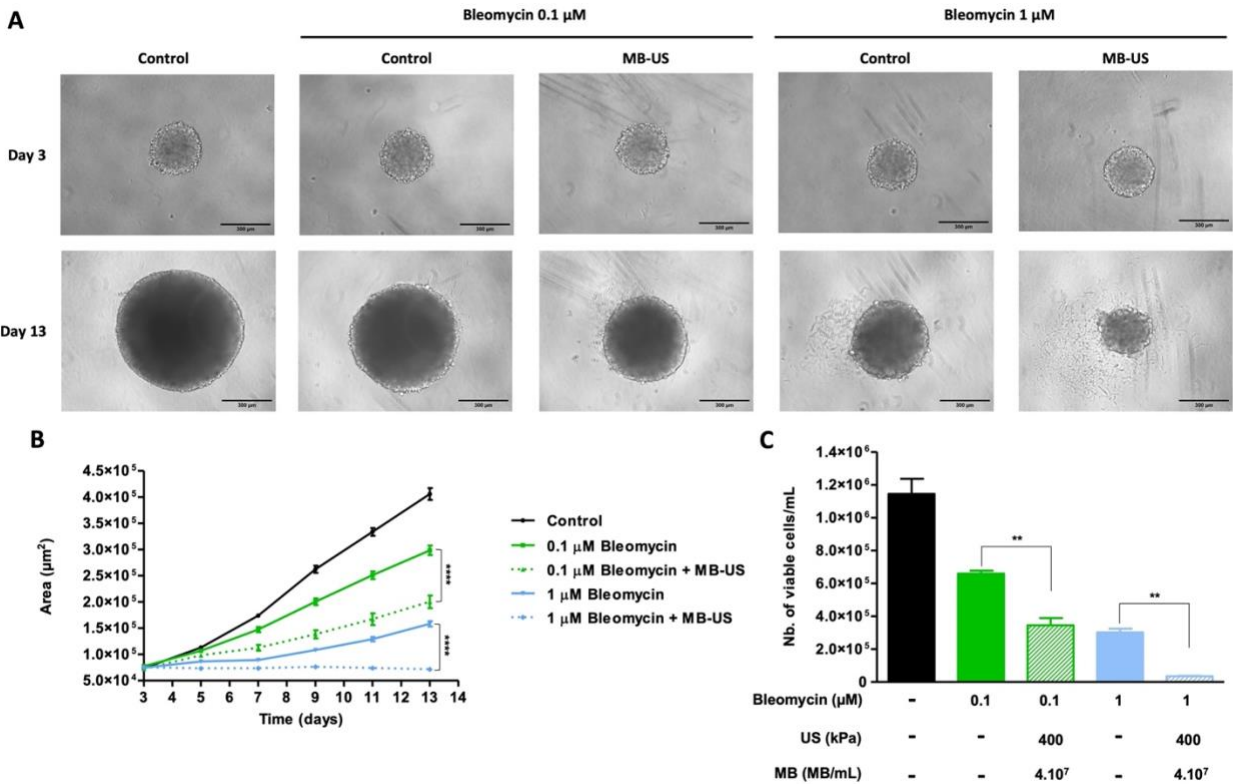
300 **Figure 5.** Effects of MB-assisted US on spheroid structure. (A) Representative optical images of CRC spheroid slices  
 301 stained with toluidine blue, immediately or 20 min after MB-assisted US exposure (MB concentration:  $4.10^7$  MB/mL,  
 302 US parameters: 400 kPa for 1 min). The scale bar indicates 100  $\mu$ m. The red square shows peripheral layer of CRC  
 303 spheroids. (B) Percentage of the disrupted spheroid area after MB-assisted US exposure. Data expressed as mean  $\pm$   
 304 SEM was calculated from 12 CRC spheroids. Statistical analysis was performed using Kruskal–Wallis’s test and  
 305 Dunn’s multiple comparison test. Significance was defined as  $p < 0.05$  (\*\* $p < 0.01$ ). (C) Representative TEM images  
 306 of disrupted area of CRC spheroids. The scale bar indicates 10  $\mu$ m.

### 307 *Drug delivery using MB-assisted US*

308 Three anticancer drugs, bleomycin, doxorubicin and irinotecan, were acoustically delivered  
 309 at two different concentrations inside CRC spheroids using the US setup and parameters (400 kPa  
 310 PNP,  $4.10^7$  MB/mL). The cytotoxic effects of such drugs were investigated by monitoring spheroid  
 311 growth over 13 days under optical microscopy and by assessing spheroid viability on the 13<sup>th</sup> day  
 312 using a trypan blue exclusion assay.

313 **Figure 6A** displays microscopy images of control spheroids (*i.e.*, no treatment) and  
314 spheroids treated once either with bleomycin alone or with bleomycin delivered using MB-assisted  
315 US (*i.e.*, 0.1 and 1  $\mu\text{M}$  bleomycin). As shown in this figure, the spheroid size and morphology  
316 were highly uniform between all treatment groups on the 3<sup>rd</sup> day (*i.e.*, images acquired before the  
317 treatment). This good reproducibility of our spheroid model enabled us to clearly assess their  
318 cytotoxic effects over time; here, only the last day of follow-up is shown. Control spheroids  
319 exhibited linear growth, and the spheroid-doubling time was close to 5 days (**Figure 6B**). The  
320 incubation of spheroids with 0.1  $\mu\text{M}$  bleomycin significantly reduced spheroid growth over 13  
321 days without inducing spheroid destruction compared to the control condition ( $****p < 0.0001$ ).  
322 Consequently, the spheroid-doubling time increased to 6 days. The acoustically mediated delivery  
323 of bleomycin at 0.1  $\mu\text{M}$  led to an additional and significant reduction in spheroid growth (without  
324 inducing spheroid destruction) in comparison with bleomycin treatment alone ( $****p < 0.0001$ ).  
325 This reduction in spheroid growth was associated with the prolongation of spheroid-doubling time,  
326 *i.e.*, 7 days. The increase in bleomycin concentration from 0.1 to 1  $\mu\text{M}$  significantly accentuated  
327 the slowdown of spheroid growth ( $****p < 0.0001$ ; **Figure 6B**) without causing spheroid  
328 destruction and increased the spheroid-doubling time to 9 days. One noticed that the therapeutic  
329 efficacy of acoustically mediated delivery of bleomycin at 0.1  $\mu\text{M}$  is close to that obtained with  
330 bleomycin treatment alone at 1  $\mu\text{M}$  ( $p > 0.05$ ). The exposure of spheroids to MB-assisted US in  
331 the presence of 1  $\mu\text{M}$  bleomycin completely inhibited their growth compared to the bleomycin  
332 treatment alone ( $****p < 0.0001$ ). On the 13<sup>th</sup> day, the trypan blue exclusion assay confirmed these  
333 results (**Figure 6C**). The acoustically mediated delivery of 0.1  $\mu\text{M}$  bleomycin induced a 2-fold  
334 decrease in spheroid viability compared to bleomycin treatment alone ( $**p < 0.01$ ). This reduction  
335 in spheroid viability was similar to that caused by the treatment of spheroids with 1  $\mu\text{M}$  bleomycin

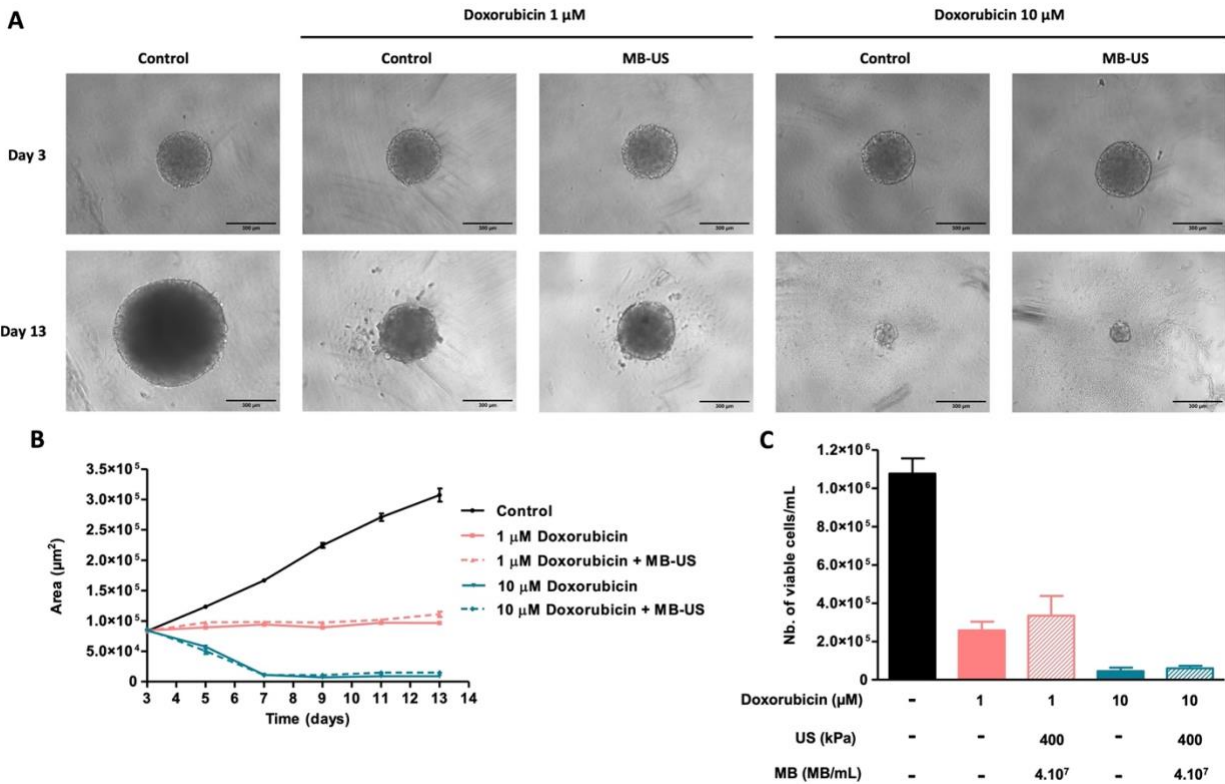
336 alone ( $p > 0.05$ ). As expected, the delivery of 1  $\mu\text{M}$  bleomycin using MB-assisted US significantly  
 337 decreased spheroid viability compared to bleomycin treatment alone at 1  $\mu\text{M}$  (\*\* $p < 0.01$ ). These  
 338 results show that MB-assisted US potentiates the cytotoxic effect of bleomycin on CRC spheroids.



339  
 340 **Figure 6.** Bleomycin delivery using MB-assisted US. CRC spheroids were incubated with 0.1 or 1  $\mu\text{M}$  bleomycin  
 341 alone or with MB-assisted US at 400 kPa for 1 min in the presence of  $4.10^7$  MB/mL. (A) Representative optical images  
 342 of CRC spheroids before (Day 3) and 10 days after treatment (Day 13). The scale bar indicates 300  $\mu\text{m}$ . (B) The  
 343 spheroid growth was assessed over time using optical imaging. Data expressed as mean  $\pm$  SEM was calculated from  
 344 8 CRC spheroids. Statistical analysis was performed using Two-way ANOVA test and Dunnett's test. Significance  
 345 was defined as  $p < 0.05$  (\*\*\*\* $p < 0.0001$ ). (C) The spheroid viability was evaluated on the 13<sup>th</sup> day post- MB-assisted  
 346 US exposure, using trypan blue dye exclusion assay. Statistical analysis was performed using Kruskal–Wallis's test  
 347 and Dunn's multiple comparison test. Significance was defined as  $p < 0.05$  (\*\* $p < 0.01$ ).

348 As shown in **Figure 7**, the cytotoxic effect of the acoustically mediated delivery of  
 349 doxorubicin (*i.e.*, 1 and 10  $\mu\text{M}$ ) was investigated following the same protocol reported above. As

350 previously described, control spheroids displayed linear growth with a doubling time close to 5  
351 days (**Figures 7A and 7B**). Doxorubicin treatment alone at 1  $\mu\text{M}$  induced complete inhibition of  
352 spheroid growth ( $****p < 0.0001$ ; **Figures 7A and 7B**), while treatment with a 10-fold higher  
353 concentration induced a significant reduction in spheroid area related to an almost complete  
354 destruction of the spheroid compared to control spheroids ( $****p < 0.0001$ ; **Figures 7A and 7B**).  
355 Regardless of the doxorubicin concentration, its cytotoxic effect was not potentiated when this  
356 anticancer drug was delivered using MB-assisted US inside the spheroids ( $p > 0.05$ ). These data  
357 were also confirmed by trypan blue exclusion assay on Day 13 (**Figure 7C**). Doxorubicin  
358 treatment alone at 1  $\mu\text{M}$  led to a 3.8-fold decrease in spheroid viability compared to the control  
359 condition ( $***p < 0.001$ ;  $2.6 \pm 0.4 \times 10^5$  versus  $1.0 \pm 0.08 \times 10^6$  viable cells/mL). The acoustically  
360 mediated delivery of 1  $\mu\text{M}$  doxorubicin did not result in an additional reduction in spheroid  
361 viability in comparison to doxorubicin treatment alone ( $p > 0.05$ ). Similarly, 10  $\mu\text{M}$  doxorubicin  
362 delivered or not using MB-assisted US induced a comparable decrease in spheroid viability  
363 ( $p > 0.05$ ;  $6.0 \pm 1.2 \times 10^4$  versus  $4.5 \pm 1.8 \times 10^4$  viable cells/mL). Altogether, these data reveal that  
364 under our experimental conditions, MB-assisted US does not increase the cytotoxic effect of  
365 doxorubicin on CRC spheroids at either selected concentration.

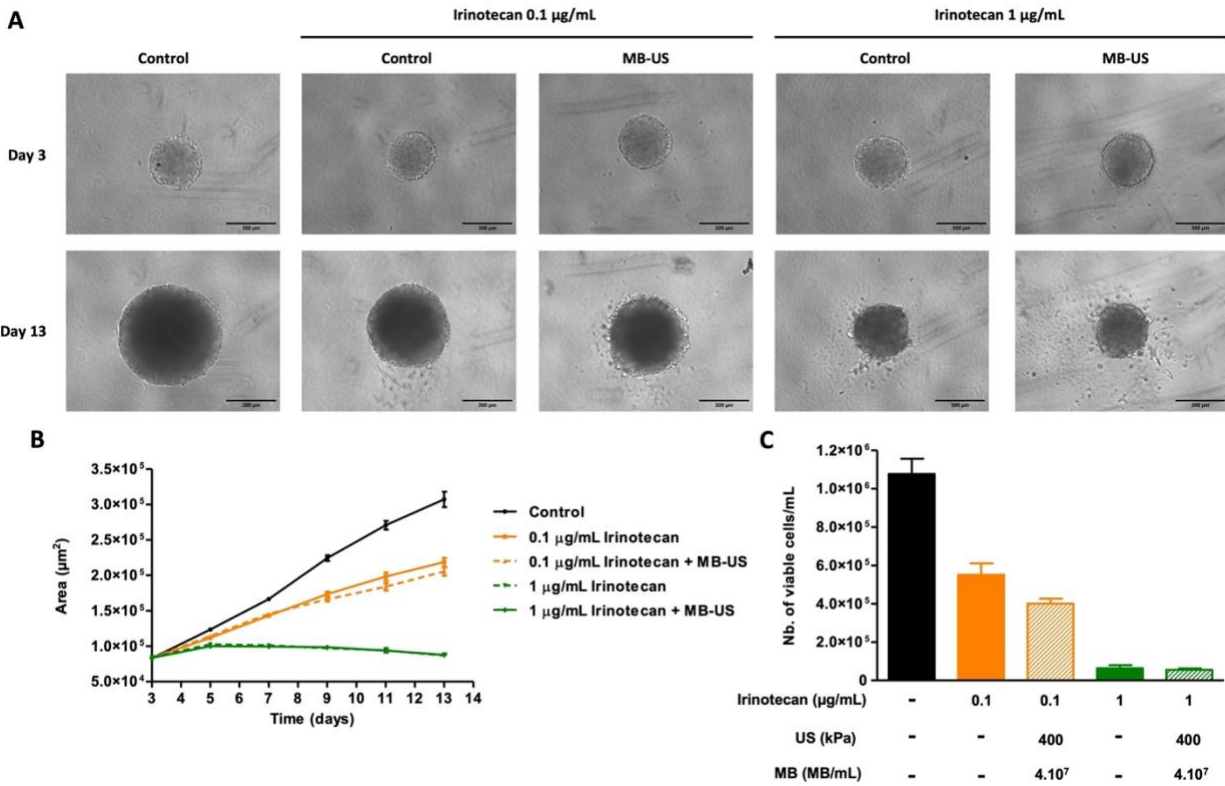


366  
 367 **Figure 7.** Doxorubicin delivery using MB-assisted US. CRC spheroids were incubated with 1 or 10  $\mu\text{M}$  doxorubicin  
 368 alone or with MB-assisted US at 400 kPa for 1 min in the presence of  $4.10^7$  MB/mL. (A) Representative optical images  
 369 of CRC spheroids before (Day 3) and 10 days after treatment (Day 13). The scale bar indicates 300  $\mu\text{m}$ . (B) The  
 370 spheroid growth was assessed over time using optical imaging. Data expressed as mean  $\pm$  SEM was calculated from  
 371 8 CRC spheroids. Statistical analysis was performed using Two-way ANOVA test and Dunnett's test. Significance  
 372 was defined as  $p < 0.05$ . (C) The spheroid viability was evaluated on the 13<sup>th</sup> day post- MB-assisted US exposure,  
 373 using trypan blue dye exclusion assay. Statistical analysis was performed using Kruskal–Wallis's test and Dunn's  
 374 multiple comparison test. Significance was defined as  $p < 0.05$ .

375 Moreover, the CRC spheroids were treated with irinotecan at 0.1  $\mu\text{g/mL}$  or 1  $\mu\text{g/mL}$  alone  
 376 or delivered using MB-assisted US. Once again, the spheroids exhibited linear growth with a  
 377 doubling time close to 5 days under control conditions (**Figures 8A** and **8B**). At a 0.1  $\mu\text{g/mL}$   
 378 concentration, slower spheroid growth was observed compared to the control condition  
 379 ( $****p < 0.0001$ ; **Figures 8A** and **8B**). The acoustically mediated delivery of such irinotecan

380 concentration induced a slightly more important reduction of spheroid growth compared to the  
381 0.1 µg/mL irinotecan treatment alone, but this result was not significant ( $p > 0.05$ ). The incubation  
382 of spheroids with 1 µg/mL irinotecan alone resulted in a complete inhibition of spheroid growth  
383 without generating their destruction in comparison to the control condition (\*\*\*\* $p < 0.0001$ ;  
384 **Figures 8A** and **8B**). Unfortunately, the delivery of such irinotecan concentrations using MB-  
385 assisted US did not lead to an additional and significant decrease in spheroid growth compared to  
386 irinotecan treatment alone ( $p > 0.05$ ). The trypan blue exclusion assay supported these results  
387 (**Figure 8C**). The acoustically mediated delivery of 0.1 µg/mL irinotecan caused a slight but  
388 nonsignificant decrease in spheroid viability in comparison to 0.1 µg/mL irinotecan treatment  
389 alone ( $p > 0.05$ ;  $4.0 \pm 0.3 \times 10^5$  versus  $5.5 \pm 0.6 \times 10^5$  viable cells/mL). No significant decrease in  
390 spheroid viability was observed between their treatment with 1 µg/mL irinotecan alone or  
391 delivered using MB-assisted US ( $p > 0.05$ ;  $6.0 \pm 1.5 \times 10^4$  versus  $5.5 \pm 0.7 \times 10^4$  viable cells/mL).  
392 These results demonstrate that under our experimental conditions, MB-assisted US does not  
393 potentiate the cytotoxic effect of irinotecan on CRC spheroids regardless of its selected  
394 concentration.





395  
 396 **Figure 8.** Irinotecan delivery using MB-assisted US. CRC spheroids were incubated with 0.1 or 1 µg/mL irinotecan  
 397 alone or with MB-assisted US at 400 kPa for 1 min in the presence of 4.10<sup>7</sup> MB/mL. (A) Representative optical images  
 398 of CRC spheroids before (Day 3) and 10 days after treatment (Day 13). The scale bar indicates 300 µm. (B) The  
 399 spheroid growth was assessed over time using optical imaging. Data expressed as mean ± SEM was calculated from  
 400 8 CRC spheroids. Statistical analysis was performed using Two-way ANOVA test and Dunnett’s test. Significance  
 401 was defined as p < 0.05. (C) The spheroid viability was evaluated on the 13<sup>th</sup> day post-MB-assisted US exposure,  
 402 using trypan blue dye exclusion assay. Statistical analysis was performed using Kruskal–Wallis’s test and Dunn’s  
 403 multiple comparison test. Significance was defined as p < 0.05.

404 **DISCUSSION**

405        The aim of the present study was to evaluate the efficacy of MB-assisted US for the delivery  
 406 of anticancer drugs to CRC spheroids. First, the influence of PNP and MB concentration on the  
 407 permeabilization of these spheroids to small molecules was assessed using PI as a drug model. Our  
 408 results show that MB-assisted US significantly increases the delivery of this drug model into CRC

409 spheroids (**Figures 2A and 2B**). As previously reported for cell suspensions or *in vivo*, the  
410 permeabilization efficiency depends on PNP [29–31]. The increase in PNP induces a gradual  
411 permeabilization of spheroids, and a maximum permeabilization efficiency is reached at a PNP of  
412 400 kPa. In future experiments, it could be relevant to investigate in depth the influence of other  
413 US parameters on the permeabilization of spheroids. Surprisingly, the increase in MB  
414 concentration did not affect the permeabilization efficiency of spheroids (**Figures 3A and 3B**).  
415 The data available in the literature showed that the permeabilization of cells and tissues is  
416 dependent on the MB concentration [32–34]. In the near future, the influence of a wider range of  
417 MB concentrations as well as the type of MBs (*i.e.*, soft- vs. hard-shelled MBs, bare vs. targeted  
418 MBs, poly- vs. mono-disperse MBs, *etc.*) on spheroid permeabilization will be evaluated with our  
419 US setup. The 3D view of permeabilized spheroids using confocal fluorescence microscopy  
420 allowed accurate location of permeabilized cells in the peripheral layer of spheroids (**Figure 4**).  
421 Such spheroid phenotype with more or less significant number of permeabilized cells is the most  
422 frequently observed phenotype in previous studies. Nevertheless, Paškevičiūtė et al.[35] reported  
423 that MB-assisted US enhanced the permeabilization of peripheral and middle layers to doxorubicin  
424 (anticancer fluorescent drug, 544 Da) in lung and breast cancer spheroids. This difference observed  
425 between our study and those published may be related to the US setup and parameters, MBs (*e.g.*,  
426 type, dose, *etc.*), tumor cell line and the dye used as the model drug.

427         Subsequently, we investigated the bioeffects of MB-mediated US on the architecture of  
428 spheroids using TEM and histology after toluidine blue staining. MB-assisted US strongly altered  
429 the peripheral cell layers of spheroids. The shape of the tumor cells was modified, and the  
430 intercellular junctions were disrupted from the spheroid surface to a depth of 70 nm immediately  
431 after US exposure as well as 20 min later (**Figure 5**). These results suggest that the mechanical

432 actions of cavitating MBs take place on the surface and in the outer layers of the spheroids. Due  
433 to their micrometric size, the MBs would not diffuse inside the spheroids. To confirm this  
434 hypothesis, spheroid permeabilization in the presence of fluorescent MBs and PI could be  
435 conducted in real time under a confocal microscope. In addition, our TEM results confirmed our  
436 confocal microscopic observations of permeabilized spheroids. This preliminary electron  
437 microscopy study is, to our knowledge, the first of its kind in the field of acoustically mediated  
438 drug delivery into spheroids.

439         Moreover, we assessed the ability of MB-assisted US to deliver chemotherapeutic drugs,  
440 including bleomycin, doxorubicin and irinotecan, into CRC spheroids. These spheroids were  
441 treated with two different drug concentrations and then exposed or not to MB-assisted US. The  
442 results showed that the cytotoxic effect of bleomycin was potentiated when this drug was delivered  
443 using MB-assisted US (**Figure 6**). In agreement with published data, these results indirectly  
444 demonstrated that the increased cytotoxicity of bleomycin could be ascribed to an enhancement in  
445 its intracellular uptake through acoustically induced hydrophilic pores [36,37]. The transport of  
446 bleomycin across the plasma membrane of tumor cells is governed by the receptor-mediated  
447 endocytosis process [38]. The low number of these membrane receptors exposed at the cell surface  
448 limits the intracellular accumulation of bleomycin and therefore its cytotoxicity. The exposure of  
449 spheroids to MB-assisted US leads to an increased membrane permeability of tumor cells and  
450 enables the direct access of bleomycin to their cytoplasm, where its molecular targets are located.  
451 These results are in agreement with previous studies conducted *in vitro* on different human and  
452 murine tumor cell lines (*i.e.*, gingival squamous carcinoma, melanoma, glioblastoma and  
453 colorectal cancer) [39–41] and *in vivo* (*i.e.*, gingival squamous carcinoma and melanoma) [39,40].  
454 The acoustically mediated delivery of bleomycin induces a 50% to 80% decrease in cell viability

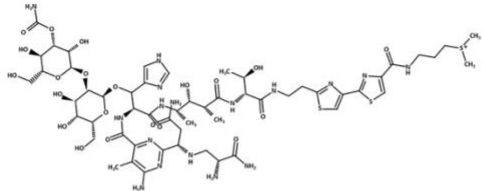
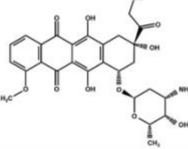
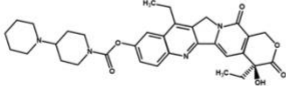
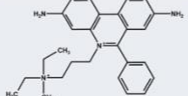
455 regardless of the cell line used and experimental conditions (*i.e.*, cell suspension *versus* adherent  
456 cells, type and dose of MBs, bleomycin concentration and US parameters). *In vivo*, Iwanaga et al.  
457 reported that the repeated delivery of a low bleomycin dose (10 µg) using MB-assisted US led to  
458 a near disappearance of the tumor in a murine squamous carcinoma model without inducing severe  
459 side effects [39]. Altogether, these results suggest that spheroids could be a suitable predictive and  
460 complementary tumor model between *in vitro* and *in vivo* models.

461 Surprisingly, MB-assisted US did not potentiate the cytotoxic effects of doxorubicin and  
462 irinotecan on CRC spheroids under our experimental conditions (**Figures 7 and 8**). The first  
463 hypothesis, which could explain these results, relies on the intrinsic properties of these anticancer  
464 drugs (**Table 1**). Doxorubicin and irinotecan are low molecular weight molecules (< 600 Da) that  
465 can passively cross the plasma membrane of tumor cells, while bleomycin (> 1 400 Da) requires  
466 a receptor-mediated endocytosis process. In addition, the lipophilicity of these anticancer  
467 molecules is also a physico-chemical parameter, which must be considered because it plays a major  
468 role in their pharmacological properties (pharmacokinetics, pharmacodynamics, tissue  
469 biodistribution, *etc.*) [42]. Doxorubicin and irinotecan are more lipophilic than bleomycin [43,44].  
470 As a result, MB-assisted US would not significantly enhance the intracellular concentrations of  
471 doxorubicin and irinotecan under our experimental conditions. However, previous investigations  
472 reported that this US modality significantly increased the intracellular uptake of both drugs and  
473 their cytotoxic effects *in vitro* and *in vivo* [20,22,45,46]. If we consider doxorubicin first, the  
474 acoustically mediated delivery of free doxorubicin (as opposed to doxorubicin loaded in MBs or  
475 liposomes) enhances the membrane permeability of tumor cells to this drug, thus resulting in a  
476 significant increase in its cytotoxic effects on cell suspensions or monolayers [47,48]. The *in vitro*  
477 studies conducted on the tumor spheroids depicted contradictory results [15,35,49]. Indeed, Misra

478 et al. showed that this US modality increased the penetration of doxorubicin (50  $\mu$ M) inside  
479 mammary tumor (MDA-MB-231) spheroids and its intracellular uptake. Surprisingly, these results  
480 were not correlated with a significant inhibition of spheroid growth compared to doxorubicin  
481 treatment alone. The use of a high concentration of doxorubicin, *i.e.*, 50  $\mu$ M, could explain these  
482 results. Bourn et al. described a positive correlation between an increase in the intracellular uptake  
483 of doxorubicin and a decrease in the viability of CRC/fibroblast (HCT-116/HFFF2) spheroids after  
484 acoustically mediated delivery of doxorubicin at 3  $\mu$ M. This last study demonstrates that MB-  
485 assisted US can potentiate the therapeutic efficacy of low concentrations of doxorubicin (*i.e.*, < 5  
486  $\mu$ M) on spheroids, thus partly explaining our results obtained at 10  $\mu$ M doxorubicin. Indeed, we  
487 failed to improve the cytotoxic effects of low concentrations of doxorubicin (< 1  $\mu$ M) on CRC  
488 spheroids in the present study but also in preliminary studies (data not shown), suggesting that  
489 HCT-116 cells would be highly sensitive to doxorubicin. In their study, Bourn et al. exploited CRC  
490 spheroids made up of human colorectal cancer HCT-116 cells (as reported in the present study)  
491 and human HFFF2 fibroblasts. It is now widely known that such coculture decreases the sensitivity  
492 of tumor cells to anticancer drugs [16,49–51], thus explaining why Bourn et al. succeeded in  
493 potentiating the therapeutic efficacy of low concentrations of doxorubicin on their spheroids and  
494 not us. In the present study, we can only conclude that MB-assisted US did not potentiate the  
495 cytotoxic effects of doxorubicin on HCT-116 spheroids. In the near future, these experiences will  
496 have to be reproduced on spheroids made-up of different types of cancer cells cocultured with or  
497 without other cell lines such as fibroblast, in order to conclude on the efficacy of MB-assisted US  
498 to enhance the therapeutic efficacy of doxorubicin on tumor spheroids. We note that the efficacy  
499 of acoustically mediated delivery of free doxorubicin has only been described in a few *in vivo*  
500 tumor models [46,52,53]. Indeed, it is mainly the delivery of its liposomal formulation (Doxil<sup>®</sup> or

501 Caelyx<sup>®</sup>) as well as doxorubicin-loaded MBs, which has been reported *in vivo* due to the strong  
 502 cardiotoxicity of free doxorubicin [54–56]. The use of liposomal doxorubicin will be more relevant  
 503 in our further investigations on tumor spheroids.

504 **Table 1.** Description of chemotherapeutic drugs and propidium iodide.

	Molecule	Structure	Molecular weight (g/mol)	Mechanisms of action
THERAPEUTIC MOLECULES	Bleomycin		1 415.551	<ul style="list-style-type: none"> <li>• ROS generation</li> <li>• Intercalation into DNA</li> <li>• Topoisomerase II inhibition</li> </ul>
	Doxorubicin		543.52	<ul style="list-style-type: none"> <li>• ROS generation</li> <li>• Intercalation into DNA</li> <li>• Topoisomerase II inhibition</li> </ul>
	Irinotecan		586.678	Topoisomerase I inhibition
DRUG MODEL	Propidium iodide		668.403	N/A

505

506 Regarding the acoustically mediated delivery of irinotecan, several proofs of concept  
 507 showed that MB-assisted US efficiently delivered free irinotecan *in vitro* [20] and *in vivo* in healthy  
 508 and tumor brain tissues [20,45,57]. We demonstrated that the therapeutic efficacy of a 500 µg/mL  
 509 irinotecan concentration can be achieved by delivering a 1000 times lower concentration by MB-  
 510 assisted US in human glioblastoma (U-87 MG) cells [20]. *In vivo*, the acoustically mediated  
 511 delivery of free irinotecan in a glioblastoma (U-87 MG) murine model resulted in a significant  
 512 decrease in tumor growth compared to irinotecan treatment alone. To the best of our knowledge,  
 513 spheroids have never been exploited to evaluate the acoustically mediated delivery of free  
 514 irinotecan. However, they are used to assess new formulations of therapeutic MBs, in which  
 515 irinotecan is loaded alone [58,59] or with other anticancer drugs [59,60]. Indeed, Gao et al.

516 demonstrated that MB-assisted US using irinotecan-loaded MBs (50  $\mu$ M) induced a significant  
517 decrease in the viability of pancreatic tumor spheroids compared to irinotecan-loaded MB  
518 treatment alone. As opposed to our irinotecan delivery strategy, the encapsulation of irinotecan  
519 inside MBs limits its intracellular uptake and therefore its cytotoxic effects. The exposure of tumor  
520 cells and irinotecan-loaded MBs to US induces the release of irinotecan from MBs and increases  
521 the membrane permeability of tumor cells to this drug, thus potentiating its therapeutic efficacy.  
522 Regardless of the type of tumor cells and drug delivery protocol, the loading of irinotecan inside  
523 the MBs is the main reason why these authors observed a significant difference in spheroid  
524 viability between their experimental and control conditions, contrary to our present study.

525 In addition to the further improvements described above, future developments would  
526 consist of the use of vascularized tumor spheroid-on chip where the spheroids will be formed from  
527 patient-derived cancer cells and have different sizes [61–63]. This strategy will be more relevant  
528 to design and to assess protocols (*e.g.*, US parameters, MB and drug-related parameters,  
529 therapeutic schemes, *etc.*) for acoustically mediated delivery of therapeutic molecules of different  
530 molecular weights in a pathophysiological context close to that encountered *in vivo*.

## 531 CONCLUSION

532 In summary, the present results suggest that MB-assisted US enhanced the therapeutic  
533 efficacy of bleomycin on CRC spheroids but not that of doxorubicin and irinotecan. Nevertheless,  
534 further improvements are required to potentiate their therapeutic efficacies. The exploitation of  
535 tumor spheroids should be a complementary approach to design and validate innovative protocols  
536 for acoustically mediated drug delivery.

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#### 547 *ABBREVIATIONS*

548 2D, two-dimensional; 3D, three-dimensional; CRC spheroid, colorectal cancer spheroid;  
549 DPBS, Dulbecco's phosphate-buffered saline solution; FCS, fetal calf serum; MB-assisted US,  
550 microbubble-assisted ultrasound; MBs, microbubbles; PI, propidium iodide; PNP, peak negative  
551 pressure; PRP, pulse repetition period; SEM, mean standard deviation; TEM, transmission electron  
552 microscopy; ULA, ultra-low attachment; US, ultrasound.

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