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Induction of Apoptosis in U937 Cells by Using a Combination of Bortezomib and Low-Intensity Ultrasound

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: We scrutinized the feasibility of apoptosis induction in blood cancer cells by means of low-intensity ultrasound and the proteasome inhibitor bortezomib (Velcade).

Material/Methods: Human leukemic monocyte lymphoma U937 cells were subjected to ultrasound in the presence of bortezomib and the echo contrast agent Sonazoid. Two types of acoustic intensity (0.18 W/cm^2 and 0.05 W/cm^2) were used for the experiments. Treated U937 cells were analyzed for viability and levels of early and late apoptosis. In addition, scanning electron microscopy analysis of treated cells was performed.

Results: The percentage of cells that underwent early apoptosis in the group treated with ultrasound and Sonazoid was 8.0±1.31% (intensity 0.18 W/cm^2) and 7.0±1.69% (0.05 W/cm^2). However, coupling of bortezomib and Sonazoid resulted in an increase in the percentage of cells in the early apoptosis phase, up to 32.50±3.59% (intensity 0.18 W/cm^2) and 33.0±4.90% (0.05 W/cm^2). The percentage of U937 cells in the late apoptosis stage was not significantly different from that in the group treated with bortezomib only.

Conclusions: Our findings indicate the feasibility of apoptosis induction in blood cancer cells by using a combination of bortezomib, ultrasound contrast agents, and low-intensity ultrasound.

MeSH Keywords: Antineoplastic Protocols • Apoptosis • Lymphoma • Sound

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Background

One of the main characteristics of tumors is a low level of apoptosis, which plays an important role in pathogenesis of cancer [1,2]. Furthermore, defects in apoptosis machinery protect malignant cells from oxidative stress and hypoxia, resulting in deregulation of cell proliferation and differentiation along with promoting of angiogenesis [3]. Given the fact that suppression of apoptosis is a hallmark of tumors, induction of apoptosis in cancer cells is a great interest for clinicians and biochemists [4]. It has been found that apoptosis can be triggered by using various chemical, biological, and physical stimuli [5–7]. However, modern clinical strategies mainly rely on the application of pharmacological apoptotic modulators of different origins and mechanisms of action. Most of current pharmacological agents were designed to control apoptotic pathways through blocking or down-regulating the anti-apoptotic signaling [8,9]. One such apoptotic modulator is known as bortezomib (Velcade, Janssen-Cilag Pty Ltd.). Bortezomib was approved by the U.S. Food and Drug Administration (FDA) for the treatment of multiple myeloma and mantle-cell lymphoma in the USA. The mechanism of action is based on blocking 26S proteasome, which results in induction of Bcl-2 phosphorylation, and consequently, in triggering of apoptosis [10]. Particularly, bortezomib decreases the level of nuclear factor-κB, which plays an important role in resistance of cancer cells to apoptosis [11]. At the same time bortezomib increases the levels of FAS1, P53, and BAX responsible for the initiation of intrinsic apoptotic pathway [12,13]. As a result, the apoptotic process is launched, leading to arrest of growth and differentiation of malignant cells. However, despite the proven effectiveness, many apoptotic modulators, including bortezomib, can cause a range of serious side effects.

Apart from the pharmacological approach, the induction of apoptosis can be achieved by using various physical methods, including electromagnetic fields [14], high temperature [15], ultraviolet radiation [16,17], ionizing radiation [18], and ultrasound [19,20]. It has been thought that coupling of anticancer drugs with biophysical methods could provide a possibility of lowering the dosage of these agents and reducing their adverse effects. It is important to note that the application of ultrasound for apoptosis induction is safer than ionizing radiation due to the nonionizing nature of acoustic exposure [21]. It has been already demonstrated that low-intensity ultrasound [22] and high-intensity ultrasound [23] are capable of initiating apoptosis in malignant cells.

There were some attempts to exploit bortezomib in combination with ultrasound for apoptosis induction. Poff et al. have already shown that high-intensity focused ultrasound (HIFU) coupled with bortezomib could be used for tumor growth inhibition through initiation of apoptosis [24]. During the study, the squamous cell carcinoma tumors (in mice) were subjected to pulsed HIFU (image guided) with and without administration of bortezomib. The application of HIFU and the anticancer agent resulted in an increased level of apoptosis and tumor growth inhibition. Poff et al. used bortezomib in a low concentration compared with the standard administration [24]. The obtained results indicate a great potential for using HIFU in combination with special chemical agents for induction of apoptosis. Poff and co-workers suggested that the coupling capacities of magnetic resonance imaging (MRI) and HIFU would enhance the therapeutic potential of ultrasound. In a comprehensive review, Dr. Nahum Goldberg highlighted the importance of Poff and colleagues' work in finding new approaches for reduction of tumor growth along with decreasing toxic chemotherapeutic side effects [25].

HIFU has two aspects that could be exploited for apoptosis induction: thermal and mechanical [21]. In addition, HIFU can be effectively coupled with MRI to improve ultrasound beam navigation and organ targeting [21,24]. However, despite definite advantages, HIFU therapy might lead to some systemic and local complications such as skin burns and local pain [26,27]. Moreover, HIFU application requires special equipment for cooling the ultrasound transducer to avoid its overheating.

Those facts have encouraged us to validate the effectiveness of low-intensity ultrasound in combination with a pro-apoptotic agent (bortezomib) for blood cancer management. The main benefit of using a low-intensity acoustic field is the possibility of avoiding problems such as excessive cavitation and heating of adjacent normal tissues. If successful, new findings could provide essential information for developing a novel type of device for extracorporeal blood sonication. Such a device would allow selective triggering of apoptosis in blood cancer cells in a noninvasive manner.

In addition, we intended to scrutinize the impact of the ultrasound contrast agent Sonazoid on apoptosis induction alone and in the combination with bortezomib. The bio-effects of ultrasound contrast agents have been previously reported and demonstrated in a range of studies [28–30], including studies of bio-effects induced by Sonazoid [31,32]. However, there are no data in the literature reporting the use of Sonazoid in combination with anticancer pharmacological agents.

Material and Methods

Study design

To validate the impact of ultrasound, bortezomib, and ultrasound contrast agents on apoptosis induction, suspensions of U937 blood cancer cells were subjected to acoustic fields of

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two different intensities (0.05 W/cm^2 and 0.18 W/cm^2). U937 cells at a final concentration of 200×10^3 cells/mL (in Roswell Park Memorial Institute [RPMI] medium +10% fetal bovine serum [FBS]) were sonicated in the chamber with polyethylene film wall (passable for ultrasound). (For ultrasound parameters, please see the section below titled Experimental set-up.) The samples containing cells from the sham group were kept at 37.0°C during the duration of the sonication experiment. The same medium was used for all experimental samples.

Cells were subdivided into following groups: (1) control (no ultrasound exposure); (2) bortezomib group (no ultrasound exposure); (3) group treated with ultrasound only (intensity 0.05 W/cm^2); (4) group treated with ultrasound only (intensity 0.18 W/cm^2); (5) Sonazoid + ultrasound (intensity 0.05 W/cm^2) group; (6) Sonazoid + bortezomib + ultrasound (intensity 0.05 W/cm^2) group; (7) Sonazoid + ultrasound (intensity 0.18 W/cm^2) group; and (8) Sonazoid + bortezomib + ultrasound (intensity 0.18 W/cm^2) group.

Experimental set-up

The system SonoPore KTAC-4000 (NepaGene, Chiba, Japan) was utilized for exposing cells to ultrasound. The ultrasound transducer (20 mm in diameter, single array) was positioned under the sonication chamber in close contact with the wall. For sonication of cell culture, we used the following ultrasound parameters: frequency 1 MHz; intensities (spatial peak temporal average intensity) 0.18 W/cm^2 and 0.05 W/cm^2; duty cycle 25%; burst rate 0.5 Hz; and sinusoidal wave shape. Duration of sonication was 10 sec. The setup was previously described elsewhere by Yamaguchi et al. [33].

Cell culture

A human leukemic monocyte lymphoma cell line (U937) was used for the experiments. The U937 cells were obtained from Japanese Cancer Research Resource Bank. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Gibco, USA) at 37.0°C in an incubator with humidified air (5% CO_2). Cell viability was always more than 95% prior to the treatment.

Preparation of bortezomib

Bortezomib (Velcade, Janssen-Cilag Pty Ltd, Australia) was reconstituted in 0.9% sodium chloride according to the manufacturer's instructions.

Tests for cytotoxicity

Prior to ultrasound experiments, tests were conducted to identify bortezomib cytotoxicity under *in vitro* conditions. Results showed that bortezomib was highly toxic to U937 cells. Maximum toxicity was detected when 10 ng/mL was used; therefore, bortezomib 5 ng/mL was chosen for the experiments.

Ultrasound contrast agents

Sonazoid (GE HealthCare, Japan) solution was prepared according to the manufacturer's instructions [34]. Reconstituted Sonazoid solution at 10 µL/mL (equivalent to 1.2×10^7 microspheres per milliliter) was added to the sonication chamber prior to sonication and then gently mixed.

Determination of cell viability

Viability of U937 cells was determined before and immediately after exposure to ultrasound using the LUNA automated cell counter (Logos Biosystems, Korea). We performed the trypan blue exclusion test by mixing 100 µL of cell suspension with an equal amount of 0.3% trypan blue solution (Sigma, St. Louis, Missouri, USA) in phosphate-buffered saline. After a 5-minute incubation at room temperature, unstained cells were counted using a hemocytometer to determine cell viability (percentage).

Detection of apoptosis

Bortezomib 5 ng/mL was added to cell culture. Then U937 cells were incubated for 24 hours prior to sonication. Cells exposed to ultrasound were collected after 6 hours of incubation and analyzed for levels of early and late apoptosis. Apoptosis detection was performed using the Tali Image-Based Cytometer (Life Technologies, USA). For this purpose, Annexin V Alexa Fluor 488 (component A) and Tali propidium iodide (component B) were added to each sample step by step following the manufacturer's instructions. Annexin V is a fluorescent agent routinely exploited for detection of cells in apoptosis phase, while propidium iodide is usually utilized for the detection of apoptotic cells with damaged membranes (late stage of apoptosis) [28].

Scanning electron microscopy (SEM)

For SEM analysis the U937 cells (from sham and treated groups) were collected and then washed in 0.1 M phosphate buffer. Afterwards, cells were fixed in suspension with 2.5% glutaraldehyde in the same buffer for 1 hour; then, they were positioned on coverslips for 24 hours at 4°C. All samples were post-fixed in 2% osmium tetroxide solution for 2 hours at 4°C. After fixation, cells were dried in an Eiko ID-2 critical-point dryer, followed by coating with gold using the BIO-RAD-SEM Coating System (Japan). Cells then were examined with a high-resolution SEM (HRSEM, Hitachi, S-900, Japan) at 10 kV [35].

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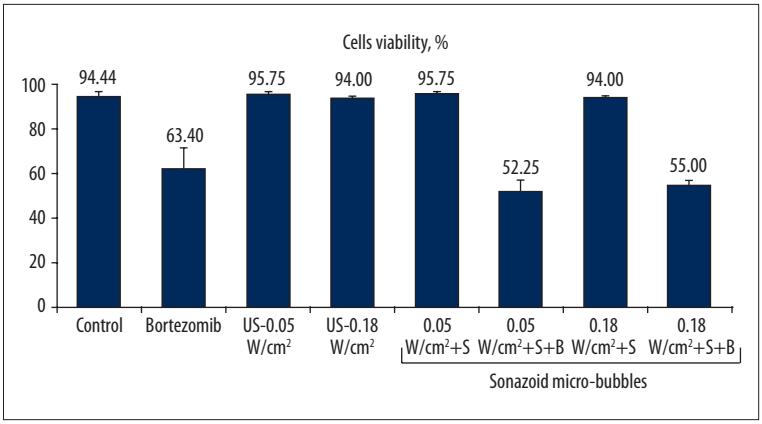

Figure 1. The viability of U937 cells subjected to ultrasound exposure (percentage). S stands for Sonazoid; B stands for bortezomib.

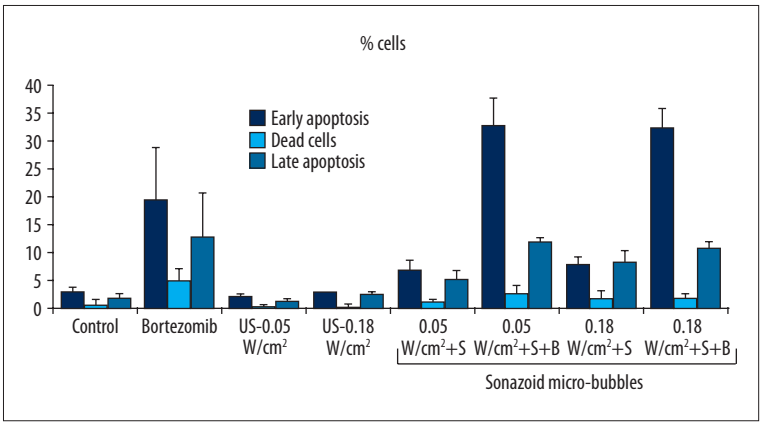

Figure 2. The effect of ultrasound exposure on apoptosis induction in U937 cells (percentage). S stands for Sonazoid; B stands for bortezomib.

Statistical analysis

A two-way factorial analysis of variance (two-way ANOVA with replication) was used to analyze the impact of ultrasound, bortezomib, and the echo contrast agent Sonazoid on apoptosis induction and cell viability of U937 cells.

Results

Data from the viability and apoptosis studies

The data from the viability studies indicated that the combined use of bortezomib, ultrasound, and echo-contrast agent led to a significant decrease in viability: the cell survival rates were 52.25±4.98% (for 0.05 W/cm^2 intensity) and 55.0±2.0% (0.18 W/cm^2) (Figure 1). However, bortezomib treatment alone was almost as effective as the combined treatment (62.4±9.08% survival rate). Although the mean values for the group treated with bortezomib and the groups exposed to a combination of ultrasound (two intensities), Sonazoid, and bortezomib look similar, ANOVA analysis showed that there was no statistical significance between them (F-statistic <F-critical [2.6703041 <4.102821]).

Results of apoptosis studies revealed that bortezomib stimulated the induction of apoptosis in U937 cells without ultrasound exposure (Figure 2). For this group, 19.6±9.49% of cells underwent early apoptosis, while 13±7.75% of cells were found in the late apoptosis stage. Exposure to ultrasound alone with intensities of 0.18 W/cm^2 and 0.05 W/cm^2 did not result in considerable elevation of apoptosis. At the same time, U937 cells exposed to ultrasound in the presence of bortezomib demonstrated the highest level of early apoptosis: 32.5±3.59% (intensity 0.18 W/cm^2) and 33±4.9% (0.05 W/cm^2). The proportion of cells in the late apoptosis phase was also elevated for both groups: 11.0±1.07% (0.18 W/cm^2) and 12.0±0.76% (0.05 W/cm^2), respectively.

ANOVA analysis of data on early apoptosis demonstrated that there were statistical differences between the three experimental groups: (1) bortezomib, (2) ultrasound 0.05 W/cm^2 + Sonazoid + bortezomib, and (3) ultrasound 0.18 W/cm^2 + Sonazoid + bortezomib; F-statistic >F-critical (4.984736 >4.102821) and P=0.0315 (<0.05). In addition, pairs comparison analysis showed that there was a significant difference between the groups: bortezomib, ultrasound 0.05 W/cm^2 + Sonazoid + bortezomib (P=0.048), and ultrasound 0.18 W/cm^2 + Sonazoid + bortezomib (P=0.0476). However, no difference

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was found between the two groups exposed to ultrasound of both intensities (ultrasound + Sonazoid + bortezomib).

Statistical analysis of the results of late apoptosis detection demonstrated that the mean values of the group treated with bortezomib and the groups exposed to combinations of ultrasound (two intensities), Sonazoid, and bortezomib were not statistically different. F-statistic <F-critical (0.16165 <4.102821) and *P*=0.852918 (>0.05).

Notably, ultrasound in combination with Sonazoid was capable of triggering apoptotic processes without adding bortezomib. The percentage of cells in early apoptosis was 8.0±1.31% (for 0.18 W/cm^2) and 7.0±1.69% (0.05 W/cm^2). Similar results were observed in populations in the late apoptosis phase: 8.50±1.93% (0.18 W/cm^2) and 5.25±1.58% (0.05 W/cm^2). The detected enhancement of bio-effects can be attributed to the phenomenon of acoustic cavitation, which is responsible for the formation and rapid collapse of microbubbles [36–38].

Analysis of SEM images

The analysis of SEM images showed a good viability of U937 cells in the control group (Figure 3A). At SEM, the cell culture treated with bortezomib demonstrated various stages of apoptosis and lysis (Figure 3B). In this group, all morphological features of intensive apoptotic processes, including changing membrane morphology and integrity, losing microvilli, budding, swelling, formation of apoptotic body, and total cell rupture, were presented. In the groups subjected to ultrasound only (0.05 W/cm^2 and 0.18 W/cm^2 intensities) we did not observe any significant signs of apoptosis processes (Figure 3C, 3D). We also found some attributes of early and late apoptosis in the cells + Sonazoid + ultrasound (intensity 0.05 W/cm^2) group (Figure 3E) and the cells + Sonazoid + ultrasound (intensity 0.18 W/cm^2) group (Figure 3G). However, in the groups treated with the combination of ultrasound (0.05 W/cm^2 and 0.18 W/cm^2), bortezomib and Sonazoid, we detected all signs of late and early apoptosis as well as a huge number of remains of necrotic cells (Figure 3F, 3H).

Results of optical microscopy studies

The optical microscopy data conform to the data from SEM analysis (Figure 4). There were a few cells in early or late apoptosis stage in the control group (Figure 4A), while in the group treated with bortezomib we observed a population of cells stained in red (signature of late apoptosis) and green (early apoptosis) colors (Figure 4B). In the groups treated with ultrasound only (intensities 0.05 W/cm^2 and 0.18 W/cm^2), some cells were stained in green (early apoptosis stage) and red (late phase of apoptosis) colors (Figure 4C, 4D). We observed a number of red-colored cells (stained with propidium iodide, indicating the late apoptosis stage) in the cells + Sonazoid + ultrasound groups (intensities 0.05 W/cm^2 and 0.18 W/cm^2) (Figure 4E, 4G). In the groups exposed to the combination of ultrasound (two intensities), Sonazoid, and bortezomib, microscopic analysis demonstrated the remains of destroyed cells along with cells in different phases of apoptosis (Figure 4F, 4H). The visual analysis demonstrated that the most effective intensity in terms of apoptosis induction was 0.05 W/cm^2 (Figure 4F).

Discussion

The current strategies for the treatment of blood cancers, including mantle cell lymphoma, Hodgkin lymphoma, and myeloid leukemia, heavily rely on chemotherapy [39–41]. Apart from this standard approach, novel treatment methods have been also proposed in the recent decade, including monoclonal antibodies [42], immuno-chemotherapy [43], interferon-α-maintenance therapy [44], radio-immunotherapy [45], and stem cell transplantation [46]. Despite promising results, most of these methods are in preclinical or clinical trials.

Among current approaches for blood cancer management, administration of various apoptotic modulators such as bortezomib has been approved by the FDA (USA) for the treatment of some blood cancer types, particularly multiple myeloma and mantle cell lymphoma. In this work we validated the apoptotic effect of low-intensity ultrasound in combination with bortezomib, and its correlation with acoustic intensity.

Our data suggest that combined use of low-intensity ultrasound, echo contrast agents, and bortezomib results in a significant decrease in the viability of blood cancer cells (Figure 1). At the same time, we observed a high level of early apoptosis (Figure 2) in the groups exposed to the same combination (ultrasound + Sonazoid + bortezomib). The detected enhancement of apoptosis can be interpreted as a cumulative effect originating from 26S proteasome blockade by bortezomib and acoustic cavitation. The cavitation has been associated with application of ultrasound contrast agents [47,48]. It was reported that echo contrast agents filled with a gas are able to provide nuclei for inertial cavitation [49,50], which in turn is thought to be responsible for triggering the bio-effects [51–53]. Notably, our findings suggest that the application of the contrast agent Sonazoid alone can amplify the pro-apoptotic effect elicited by low-intensity ultrasound. However, the detected impact was considerably lower than that in the group treated with bortezomib only. A combination of ultrasound, echo contrast agent, and bortezomib resulted in the maximal apoptotic rate. It indicates a great potential for using such a combination for blood cancer therapy in the future.

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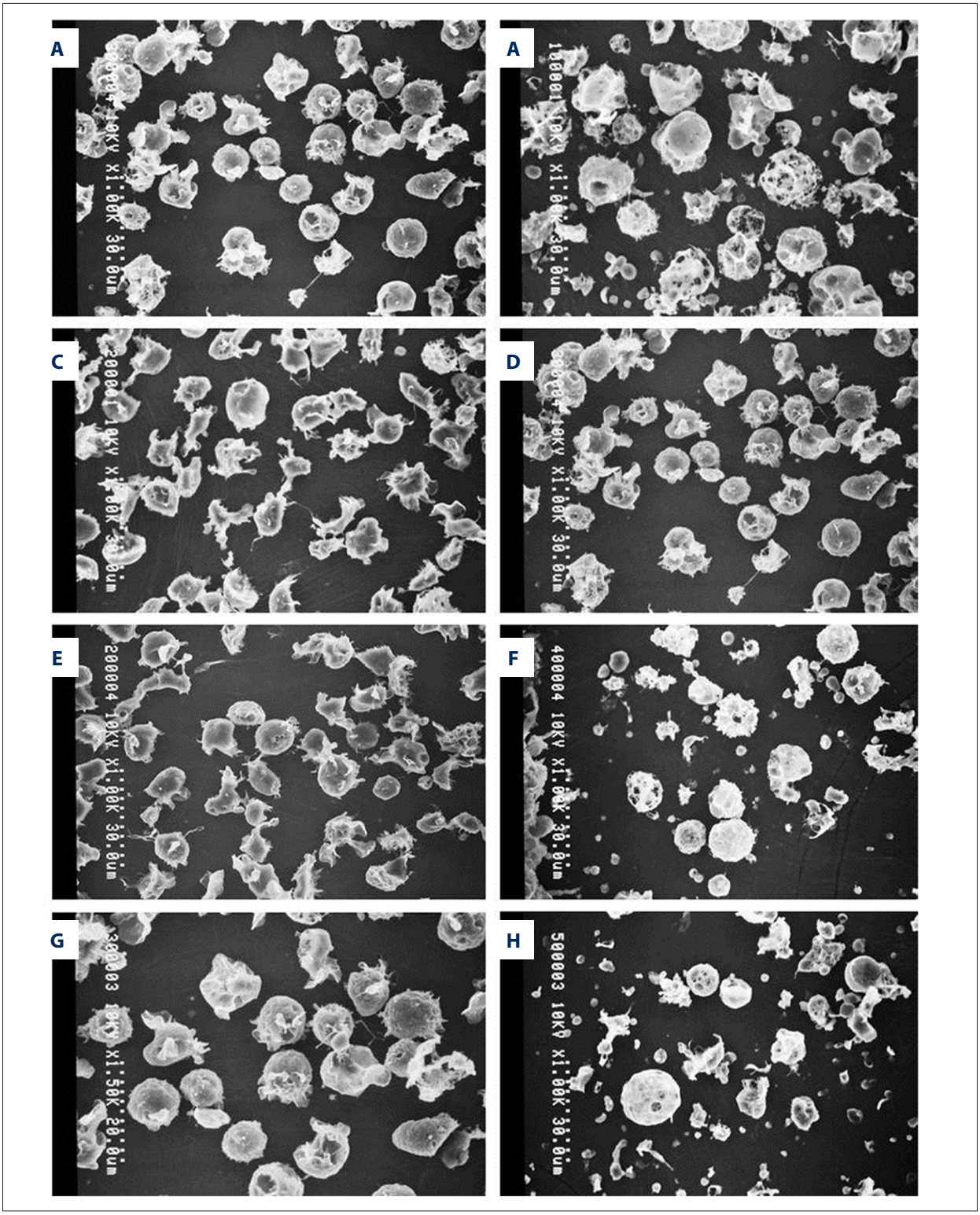

Figure 3. The SEM images of U937 cells. (**A**) Sham group of exposed U937 cells. (**B**) Cells treated with bortezomib (5 ng/mL). (**C**) Cells treated with ultrasound only (intensity 0.05 W/cm^2). (**D**) Cells treated with ultrasound only (intensity 0.18 W/cm^2). (**E**) Cells + Sonazoid + ultrasound (intensity 0.05 W/cm^2) group. (**F**) Cells + Sonazoid + bortezomib + ultrasound (intensity 0.05 W/cm^2) group. (**G**) Cells + Sonazoid + ultrasound (intensity 0.18 W/cm^2) group. (**H**) Cells + Sonazoid+ bortezomib + ultrasound (intensity 0.18 W/cm^2) group.

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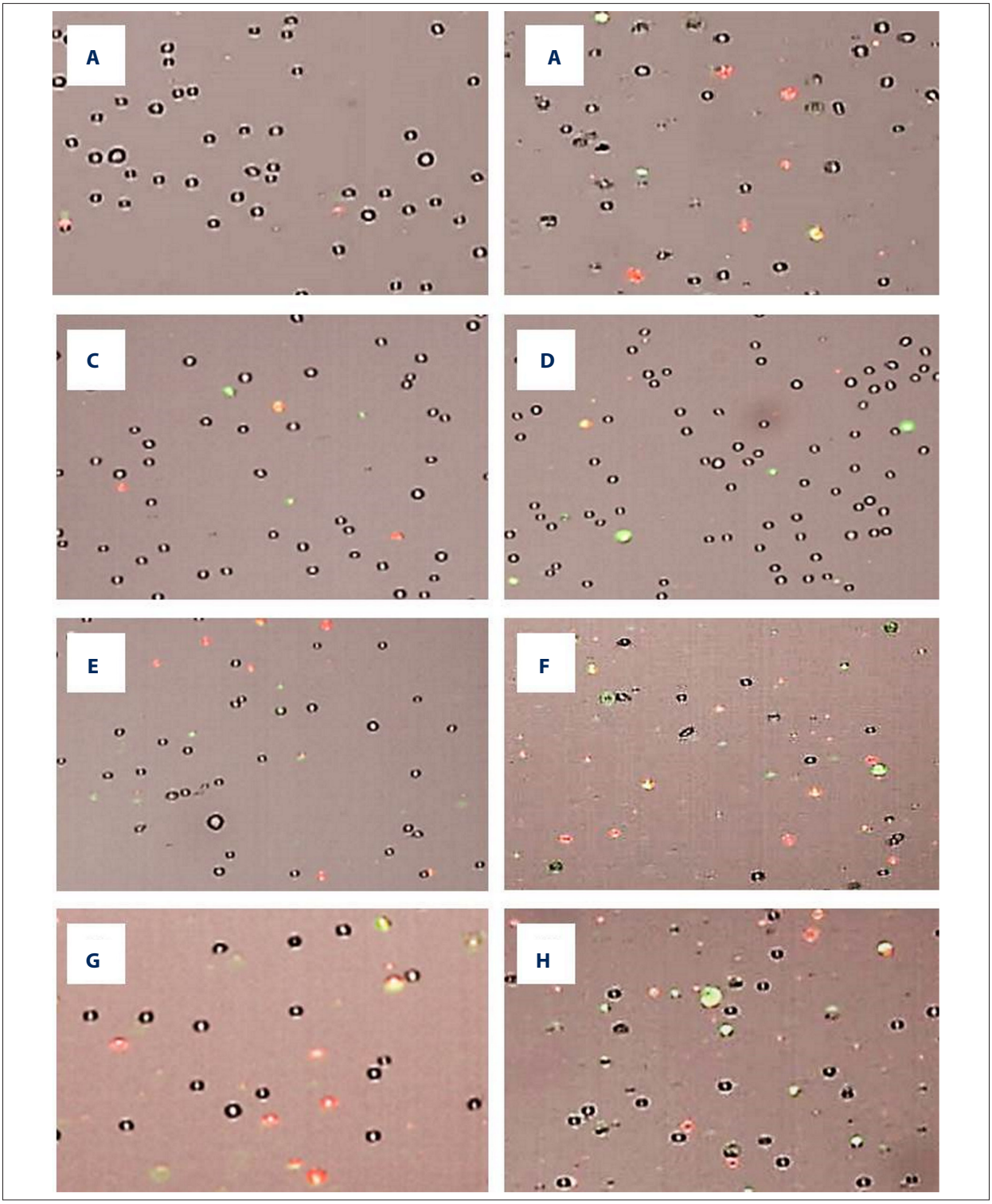

Figure 4. Microscopic images of U937 cells (×600). (**A**) Sham group of exposed U937 cells. (**B**) Cells treated with bortezomib (5 ng/mL). (**C**) Cells treated with ultrasound only (intensity 0.05 W/cm^2). (**D**) Cells treated with ultrasound only (intensity 0.18 W/cm^2). (**E**) Cells + Sonazoid + ultrasound (intensity 0.05 W/cm^2) group. (**F**) Cells + Sonazoid + bortezomib + ultrasound (intensity 0.05 W/cm^2) group. (**G**) Cells + Sonazoid + ultrasound (intensity 0.18 W/cm^2) group. (**H**) Cells + Sonazoid + bortezomib + ultrasound (intensity 0.18 W/cm^2) group. Green staining indicates early apoptosis stage (Annexin V dye), while red staining reflects the late phase of apoptosis (propidium iodide dye).

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It must be noted that in our experiments we used bortezomib in a very low final concentration of 5 ng/mL. According to Bross et al., the median maximum plasma concentration of bortezomib is approximately 509 ng/mL (after i.v. administration of the 1.3-mg/m^2 dose to patients with multiple myeloma) [54]. It means that the concentration of bortezomib we used was at least 100 times lower than the known concentrations of the drug. Nevertheless, we observed a considerable enhancement of apoptosis induction by means of ultrasound of two intensities (0.05 W/cm^2 0.18 W/cm^2) and an echo contrast agent. These findings suggest that reduced doses of bortezomib might have an effect similar to that of higher doses when used in the combination with low-intensity ultrasound. Importantly, lowering the dose could provide a considerable decrease of side effects such as neuropathy.

From a therapeutic perspective, bortezomib or other pharmacological apoptosis modulators might be incorporated into a microbubbles shell for further release at the affected site by external application of ultrasound [55,56]. Such a technology would provide an opportunity to significantly decrease the dosage of anticancer drugs, thus reducing the risks of adverse effects. Moreover, Unger et al. proposed the incorporation of targeting ligands onto the head of the lipids of microbubble with the shell composed of fluorocarbon and phospholipids [57]. This technology would allow an attachment of site-specific ligands to desired regions of the body, leading to local high concentration of the drug.

In the context of clinical application, two options for sonicating blood cells might be considered. One of them would be exposing the blood to low-intensity ultrasound in an extracorporeal device. According to this scenario, the blood taken from the vein will be pumped through the device, where it will be subjected to the acoustic field, and then it will be sent back to the patient. Potentially, some of currently available devices for extracorporeal CO_2 removal and/or oxygen blood saturation could be coequipped with portable ultrasound transducers.

Another option is targeted sonication of affected organs in order to stimulate apoptosis among malignant cells. By using a focused ultrasound beam, the cancer blood cells can be exposed to a low-intensity acoustic field at the places of main agglomeration (spleen, lymph nodes, etc.). In addition, the novel methods of image-controlled interventions could be used for precisely directing the beam, such as magnetic resonance–guided focused ultrasound (MRgFUS).

Conclusions

Despite the encouraging results, it must be noted that our study was limited to *in vitro* conditions. There is a need to conduct more research with various ultrasound parameters and experimental conditions. In particular, *in vivo* tests could help to validate the effectiveness of low-intensity ultrasound application for apoptosis induction in combination with a range of apoptosis modulators.

Conflict of interest

The authors declare no conflict of interest.

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