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Original Articles

Preferential enlargement of leukemia cells using cytoskeletal-directed agents and cell cycle growth control parameters to induce sensitivity to low frequency ultrasound

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ABSTRACT

Sonodynamic therapy (SDT) is a form of ultrasound therapy that has been shown to preferentially damage malignant cells based on the relatively enlarged size and altered cytology of neoplastic cells in comparison to normal cells. This study sought to determine whether cytoskeletal-directed agents that either disrupt (cytochalasin B and vincristine) or rigidify (jasplakinolide and paclitaxel) microfilaments and microtubules, respectively, affect ultrasonic sensitivity. U937 human monocytic leukemia cell populations were treated with each cytoskeletal-directed agent alone, and then sonicated at 23.5 kHz under relatively low power and intensity (20–40 W; 10–20 W/cm^2), or at 20 kHz using moderate power and intensity (60 W; 80 W/cm^2). In addition, human leukemia lines U937, THP1, K562, and Molt-4, and the murine leukemia line L1210 were sonicated using pulsed 20 kHz ultrasound (80.6 W; 107.5 W/cm^2) both with and without the addition of cytoskeletal-directed agents to assess whether cytoskeletal-directed agents can potentiate ultrasonic sensitivity in different leukemia lines. Human hematopoietic stem cells (hHSCs) and leukocytes were sonicated with continuous 23.5 kHz ultrasound (20 W; 10 W/cm^2) to determine whether this approach elicited the preferential damage of neoplastic cells over normal blood components. To determine whether ultrasonic sensitivity is exclusively dependent on cell size, leukemia cells were also enlarged via alteration of cell growth parameters including serum deprivation and re-addition, and plateau-phase subculturing. Results indicated that cytochalasin B/ultrasound treatments had the highest rates of initial U937 cell damage. The cells enlarged and partially synchronized, either by serum deprivation and re-addition or by plateau-phase subculturing and synchronous release, were not comparably sensitive to ultrasonic destruction based solely on their cell size. In addition, cytochalasin B significantly potentiated the ultrasonic sensitivity of all neoplastic cell lines, but not in normal blood cells, suggesting that preferential damage is attainable with this treatment protocol. Therefore, it is likely that ultrasonic cell lysis depends not only on cell size and type, but also on the specific molecular mechanisms used to induce cell enlargement and their effects on cell integrity. This is supported by the fact that either the microfilament-or microtubule-disrupting agent produced a higher rate of lysis for cells of a given size than the corresponding stabilizing agents.

Introduction

Sonodynamic therapy (SDT) is a form of ultrasound therapy in which specialized chemotherapeutic agents known as sonosensitizers are administered to increase the ability of ultrasound to preferential damage neoplastic cells [1–3]. It has been shown in numerous experiments that ultrasound preferentially damages malignant cells based on the size differential between such cells and those of normal histology [4–7]. Therefore, SDT provides a novel physiochemical approach to cancer therapy. Indeed, SDT has been shown to have both *in vitro* and *in vivo* efficacy with a variety of cell lines [7–10], and might eventually be used to supplement current chemotherapeutic protocols. SDT has also shown particular efficacy against drug resistant cell lines. Ultrasound combined with doxorubicin (DOX) significantly increased its efficacy on the human leukemia multidrug resistant cell line K562/A02, indicating that sonication can significantly increase DOX concentration within malignant cells to amplify the cytotoxicity of DOX [8]. Such observations were exhibited in a

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cell line shown to be resistant to DOX-alone, further substantiating the amplifying effect sonosensitizers have in SDT. Evidence of DOX-resistance reversal has also been shown *in vivo*, as athymic nude mice inoculated with HepG2 multidrug resistant hepatocellular carcinoma cells had an average 62% reduction in tumor volume a month later in ultrasound/DOX groups compared to DOX-only groups [11]. This ultrasound/DOX group significantly down-regulated the expression of multidrug resistance protein-1 (MDR1) and multidrug resistance-associated protein (MRP) at the mRNA level, while producing excess reactive oxygen species (ROS) levels. This substantial reduction of MDR1 and MRP indicates a reversal of drug resistance, and provides evidence of the potential clinical utility of SDT.

In addition to its ability to reverse drug resistance, SDT is also able to preferentially lyse cells based on size. This observation suggests the possible therapeutic benefit of enlarging neoplastic cells to further increase their size difference compared to normal cells. Cell enlarging agents act during mitotic events, such as the formation of the spindle fiber and chromosome separation (microtubule-directed agents), or during cytokinesis (microfilament-directed agents). Agents that act during mitotic events in leukemia are likely to affect the poorly differentiated blasts that are responsible for observed pathological features, leukemic stem cells (LSCs) that perpetuate leukemic blast formation, and hematopoietic stem cells (HSCs) which give rise to normal blood cell lineages. However, intravenous administration of vincristine typically acts preferentially toward leukemia cells in circulation, and it is likely that other cell enlarging agents have a similar affinity for neoplastic cells. Indeed, cytochalasin B has been shown to induce enlargement and multinucleation in neoplastic cells while driving normal cells out of the cell cycle rendering them small and mononucleated [10,12]. Although normal HSCs are damaged by high dose chemotherapy, the cell population can sometimes be rescued through autologous stem cell rescue (ASCR), or donor bone marrow transplants [13,14]. Moreover, since normal cells remain small and mononucleated, it is likely that cell enlarging agents can be used concurrently with ultrasound in a physicochemical approach to preferentially damage leukemia cells, while producing minimal damage to smaller normal cells.

Microtubule-directed agents have long been used in chemotherapy, and are a component of many current treatment protocols for hematological malignancies, such as vinblastine in ABVD (adriamycin/doxorubicin, bleomycin, vinblastine, and dacarbazine) for the treatment of Hodgkin's Lymphoma, and vincristine in acute lymphoid leukemia (ALL) [15,16]. By contrast, there has yet to be a microfilament-directed agent approved for clinical use. The concomitant use of both classes of cytoskeletal-directed agents appears to be a viable chemotherapeutic approach. It is well known that malignant cells have a perturbed cytoskeleton which produces or is a cause of dysplasia and subsequent anaplasia [17]. These cytoskeletal alterations in neoplastic cells provide an ideal opportunity to obtain preferential enlargement and enhanced ultrasonic damage. Since microfilament-directed agents are cytokinesis inhibitors, it seems likely that using such agents in combination with known microtubule-directed mitotic inhibitors could elicit a synergistic or at least additive effect. In theory, this combination would limit the opportunity for malignant cells to carry out a successful mitosis as the microtubule-directed agents would prevent proper formation of a spindle fiber, while any cells that managed to evade this microtubule-directed mechanism and replicate their nuclei would be unable to undergo cytokinesis in the absence of functioning microfilaments. This weakened cytoskeleton can be further exploited when a physical modality such as ultrasound is used concurrently with cytoskeletal-directed agents, especially because neoplastic cells are fundamentally more susceptible to damage by ultrasound [1,2,18–20].

We have previously demonstrated that leukemia cells are profoundly sensitive to cytochalasin B in conjunction with ultrasound [10]. Therefore, this study seeks to determine whether microtubule-directed agents elicit the same response. Since vincristine is commonly used in the treatment of hematological malignancies, it is an ideal microtubule-directed agent to compare with cytochalasin B. In addition, microfilament-stabilizing jasplakinolide and microtubule-stabilizing paclitaxel are also assessed to determine whether the mechanism of cell enlargement (disruption vs. rigidification) has any bearing on ultrasonic sensitivity. However, it may be the case that ultrasonic sensitivity is exclusively dependent on cell size, with other cytotoxic effects potentiated by chemotherapeutic agents having almost no influence on lysis rates. We have previously demonstrated that untreated log-phase U937 monocytic leukemia cells ranging from 10 to 21 μm in diameter are sensitive to sonication, and that U937 cells enlarged by treatment with cytochalasin B show increased ultrasonic sensitivity because the cells are enlarged compared to untreated U937 cells [10]. In order to determine whether untreated leukemia cells of a given diameter exhibit the same ultrasonic sensitivity as treated cells of the same diameter, we examined leukemia cells enlarged via alteration of cell growth parameters such as serum deprivation and re-addition, and plateau-phase subculturing. These cells enlarged by alteration of cell growth conditions rather than by treatment with cytoskeletal-directed agents were then sonicated to determine whether their sensitivity at a given diameter is comparable to that of cells enlarged via treatment with chemotherapeutic agents. It is crucial to determine whether cell size alone potentiates sensitivity to low frequency ultrasound, or whether treatments affecting cytoskeletal elements and/or membrane composition and fluidity can enhance or antagonize ultrasonic lysis of enlarged neoplastic cells.

Materials and methods

Cell preparation and size analysis

U937 human monocytic leukemia cells (ATCC® CRL-1593.2) were placed at 5.2×10^4 viable cells/ml in 20% fetal bovine serum (FBS) in Iscove's medium without glutamine, with the following added: 200 units/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamicin sulfate, 40 μM glutamine (50 μl of 2 mM glutamine per 5 ml medium), and 50 μl of amphotericin B (2.5 μg/ml concentration) per 5 ml of medium. K562, Molt-4, and THP1 human leukemia (ATCC® CCL-243, CRL-1582, TIB-202), as well as L1210 murine leukemia (ATCC® CCL-219) were cultured under the same conditions. Human hematopoietic stem cells (hHSCs) and human leukocytes acquired from the State University of New York Upstate Medical University (Syracuse, NY, USA) were also cultured under these conditions. Cells were stained with Wright–Giemsa and DAPI (4′,6-diamidino-2-phenylindole) to examine nuclear structure. A Z2 Beckman-Coulter® Particle Count and Size Analyzer (Beckman Coulter Inc., Brea, CA, USA) along with a Bio-Rad® TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used for size determination. Cell viability was determined with trypan blue staining and analysis with the TC20 cell counter.

Enlarging U937 cells by serum deprivation and re-addition

In order for U937 cells to be enlarged by serum deprivation and re-addition, the cells were kept in serum-free or low serum (1%) medium to produce a very low mitotic fraction (cells at the G_0 arrest phase). This required finding the optimal time for collection of the cells after re-addition of 20% FBS to yield the maximum percentage of enlarged cells. Cells were kept in serum-free conditions for 12, 22, 26 or 46 hours before 20% FBS was returned to the growth medium. Cell counts were then taken at various intervals to determine the optimal time of U937 cell population enlargement. Both plateau-phase and log-phase cells were treated under the same conditions to determine if initial mitotic stage had any influence on cell size.

Enlarging U937 cells through plateau-phase subculturing

Plateau-phase subculturing involves growing cells to very late confluent plateau-phase where the mitotic fraction is less than 4%; then achieving partial synchronization by subculturing the cells back to early log-phase, referred to as subculture release. Plateau-phase cells at 1.2×10^6 U937 cells/ml (about 4% mitosis) were subcultured to 1×10^5 total cells/ml. The full spectrum of cell sizes from 13 to 32 μm was determined at 0, 6, 12, 18, 24, 30, 31, 36, 46 and 52 hours after subculture to determine the optimal time for obtaining the highest possible percentage of enlarged cells ≥19 μm.

Enlarging leukemia cells using cytoskeletal-directed agents

Concentrations for each cytoskeletal-directed agent were chosen based on the 50% inhibitory concentration (IC_{50}) assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay or by cell counts with the TC20 automated cell counter using trypan blue to assess viability. Cells exposed to microtubule-directed agents were treated with either 4 nM vincristine or 6 nM paclitaxel for 12 hours prior to sonication. Cells exposed to microfilament-directed agents were treated with either 2 μM cytochalasin B or 0.5 μM jasplakinolide for 48 hours prior to sonication. The different durations of exposure prior to sonication reflect the fact that microtubule-directed agents exert their cell biological effects at lower doses and more rapidly than do the microfilament-directed agents.

Sonication of cell populations

U937 cells were put into 2.4 cm diameter glass vials with Mylar bottoms. The cells were seeded in 1.0 ml of 20% FBS medium with 1% Gibco® Fungizone (Life Technologies, Grand Island, NY, USA). Each vial contained 3 ml of cells. Cells were sonicated using a Fisher Scientific® Sonic Probe (23.5 kHz) system (Fisher Scientific International Inc., Hampton, NH, USA), or a Fisher Scientific® Sonic Horn (20 kHz, 7.5 cm diameter cup) system (Fisher Scientific International Inc.). The target vial was moved in a circular path within an orbital plane with a Bellco® Orbital Shaker (Fisher Scientific International Inc.). Mylar-bottomed vials were sonicated in a 7.0 cm bath with deionized, distilled, and degassed water. The target vial was located 6.0 cm from the sonic horn. Cells were sonicated at 20–40 W (10–20 W/cm^2) for the 23.5 kHz system (2 mm diameter probe tip), or at 60 W (80 W/cm^2) for the 20 kHz system. In addition, cells were sonicated using thirty 1 second pulses of 20 kHz ultrasound (80.6 W; 107.5 W/cm^2) with 1 second spaced in between each pulse. Trypan blue staining was used to identify non-viable cells after sonications were performed. U937 cell populations exposed to serum deprivation and re-addition, as well as plateau phase subculturing were sonicated under the same conditions.

Cell cycle analysis and viability of leukemia cells

To confirm that U937 cells were either in log- or plateau-phase, cell populations were analyzed with a XTT Cell Proliferation Assay Kit (ATCC® 30-1011K), which measures whether cells are proliferating (log-phase) or confluent (plateau-phase). In addition, the XTT kit was used to assess the reduction in mitochondrial activity and cell viability following treatment of cytoskeletal-directed agents and/or ultrasound against the leukemia lines.

Results

Effects of varying cell growth parameters on cell size: serum deprivation and re-addition

Deliberately withholding serum from U937 cells to induce G_0 arrest before reintroduction of 20% FBS appeared to be a reliable method to enlarge cell populations. The optimal time to collect U937 cells was 6–12 hours after serum re-addition. This timing produced the highest proportion of large cells (17–20% ≥ 19 μm) compared to later times in the cell cycle after serum re-addition (Fig. 1A). This was after the log-phase cell population had been deprived of serum for 22 hours, and shows that cells synchronized in the G_0 arrest phase can be substantially enlarged through re-addition of 20% FBS at an optimal time. In addition, trypan blue staining indicated that cell populations maintained high viability after 24 hours in low serum, suggesting that this method is not significantly deleterious to U937 cells. The same method was then applied to mid-log and plateau-phase cells in order to determine whether initial cell cycle status had any bearing on cell enlargement. Results revealed that both cell populations were enlarged to the same extent, in the range of 17–20% (Fig. 1B), indicating that initial cell cycle status had minimal influence on enlarging U937 cell populations.

Effects of varying cell growth parameters on cell size: plateau-phase subculturing

Similar effects of cell enlargement were seen in plateau-phase subculturing 35 hours after confluent plateau-phase cells were subcultured back into early log-phase (Fig. 1C), as 19% of the cells were ≥19 μm. In addition, cell cycle analysis using the XTT kit indicated that log-phase cells readily reduced XTT to a soluble, brightly colored orange derivative by mitochondrial reduction, while the activity of XTT reduction in plateau-phase cells leveled off (Fig. 1D).

This confirmed that log-phase cells were indeed proliferating, while plateau-phase cells had become confluent and quiescent.

Effects of cytoskeletal-directed agents on cell size

Microtubule-directed agents were able to substantially increase the average cell size of U937 cells, as both 4 nM vincristine-treated and 6 nM paclitaxel-treated cells exhibited a 50% increase in cell size in comparison to untreated log-phase cells (Fig. 2A). Similar results were obtained with microfilament-directed agents when compared to serum deprivation and re-addition treated cells (Fig. 2B). The generation of cells ≥24 μm was apparent with 2 μM cytochalasin B after 2 days of treatment. Jasplakinolide at 0.5 μM for 2 days produced cell enlargement comparable to serum-synchronized cells. Visualization of cells enlarged by either cytochalasin B (microfilament-disrupting agent) or vincristine (microtubule-disrupting agent) is provided in Fig. 3, with multinucleation only being present in cells enlarged by cytochalasin B. We have previously characterized the extent of multinucleation in U937 cell populations treated with concentrations ranging from 0 to 4 μM cytochalasin B over a course of 8 days [21]. The present study involved treating U937 cells with 2 μM cytochalasin B for 48 hours, and a similar extent of multinucleation was characterized with these populations as in Ref. 21 (58% with 1 nucleus, 15% with 2 nuclei, 7% with 3 nuclei, 15% with 4 nuclei, and 5% with >4 nuclei).

Comparison of ultrasonic sensitivity potentiated by serum deprivation and re-addition, by microfilament-directed, or by microtubule-directed agents

As expected, U937 cells enlarged by serum deprivation and re-addition exhibited marked increases in % damage to larger cells after ultrasonic treatment. Cell populations enlarged by serum deprivation and re-addition had noticeable sensitivity to ultrasound, as the 50% inhibitory concentration (IC_{50}) value for cells ≥19 μm was reached by 8 min using 20 W (10 W/cm^2), 23.5 kHz ultrasound (Fig. 4A). Cells enlarged by plateau-phase subculturing demonstrated ultrasonic sensitivity comparable to those exposed to serum deprivation and re-addition (data not shown). Although cells enlarged by serum deprivation and re-addition demonstrate marked ultrasonic sensitivity, 4 nM vincristine-treated cells ≥19 μm were even more ultrasonic sensitive. Vincristine-treated cells ≥17 μm or ≥19 μm reached IC_{50} values by 4.2 or 3.8 min, respectively under the same conditions as employed in Fig. 5 (Fig. 4B). Interestingly, although 4 nM vincristine and 6 nM paclitaxel-treated U937 cells exhibited a similar increase in average cell size (Fig. 2A), paclitaxel cells ≥19 μm appeared to be more resistant to sonication, as it took ~6 min for an IC_{50} value to be reached using 40 W (20 W/cm^2), 23.5 kHz ultrasound (twice the intensity as used in Figs. 4A and B). This indicates that it took microtubule-stabilized (paclitaxel) cells ~2 min longer to reach an IC_{50} value than microtubule-disrupted (vincristine) cells, even though the paclitaxel-treated cells received higher power (20 W higher power) and intensity (10 W/cm^2 higher intensity) ultrasound at 23.5 kHz.

When microfilament-directed agents were compared, the difference between disrupting and stabilizing agents became more apparent. 2.0 μM cytochalasin B-treated cells ≥19 μm reached an IC_{50} value at ~1.7 min at 23.5 kHz, 20 W(10 W/cm^2), and the same size population was reduced to ~18% of the original cell population after only 1 min of sonication at 20 kHz, 60 W (80 W/cm^2) (Fig. 4D). By contrast, jasplakinolide-treated cells did not reach an IC_{50} value until well-after 4 min sonication (Fig. 4D). This is particularly telling, as cytochalasin B-treated cells were much more sensitive to

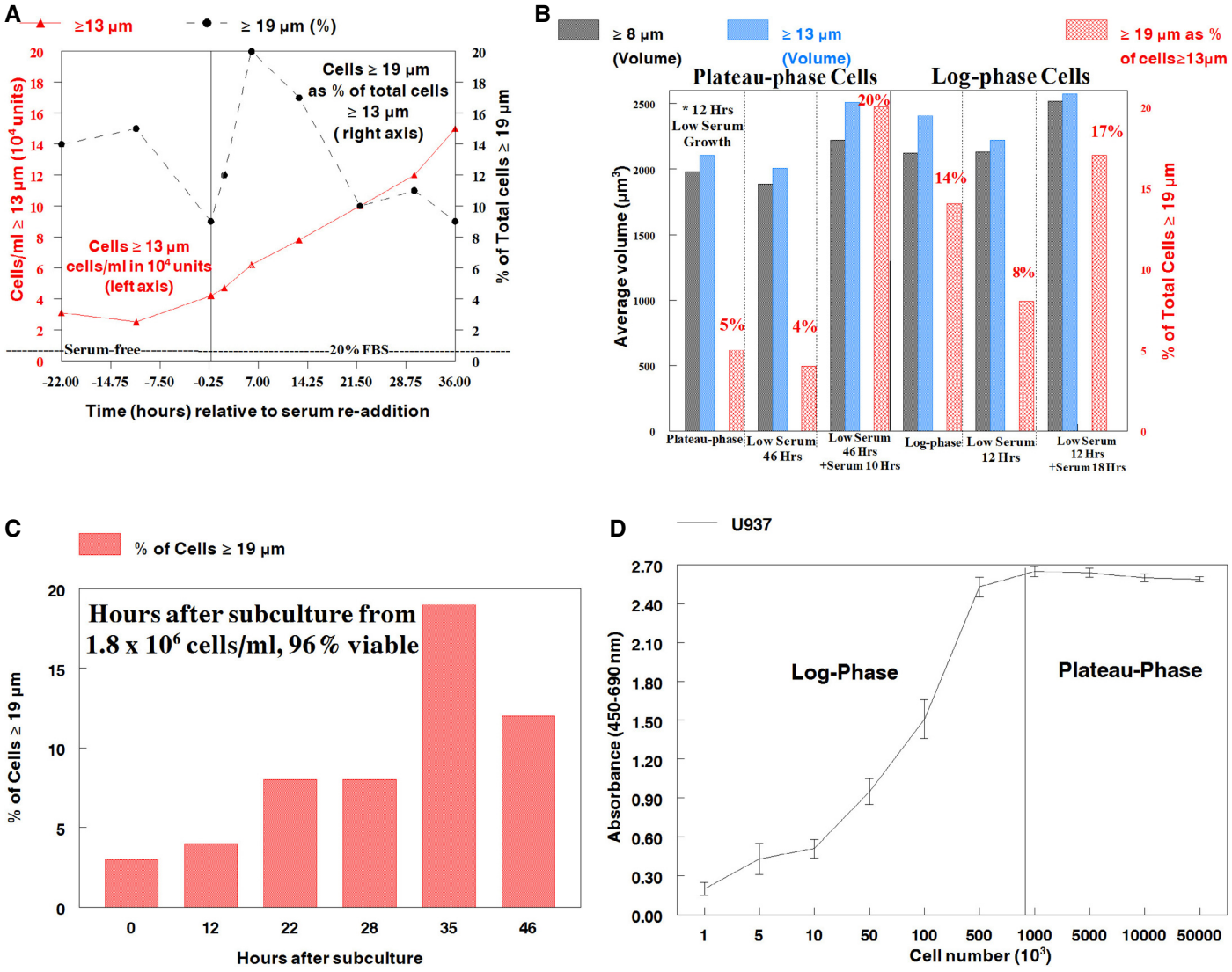

Fig. 1. Effects of serum deprivation and re-addition and subculture release on U937 cells. A) Influence of serum deprivation and re-addition on U937 cell size. Negative time reflects duration of serum deprivation. Left y axis (red) reflects the concentration of cells ≥13 µm, while the right y axis (black) shows the percentage of cells that are ≥19 µm and larger relative to the total population of cells ≥ 13 µm. Trypan blue staining revealed that U937 cell populations retain their viability (93% to 99%) at all points during the experiment. 91% of U937 cells were ≥ 13 µm at time 0, (22 hours of serum deprivation), while 9% were 8–12 µm. 20% of the cells ≥ 13 µm were ≥ 19 µm 6 hours after serum re-addition. B) Comparison of plateau- and log-phase U937 cell size after serum deprivation and re-addition. ≥ 8 µm (black); ≥ 13 µm (blue); ≥ 19 µm (red). A) Plateau-phase cells are shown in the left-hand panel and log-phase cells in the right-hand panel. The left y axis shows the average volume of the cells, while the right y axis corresponds to % of cells that are ≥ 19 µm from a total population that is ≥ 13 µm. After 46 hours serum deprivation, cells in plateau phase before serum re-addition had 4% of cells ≥ 19 µm. 10 hours after serum re-addition, 20% of cells were ≥ 19 µm. Log-phase cells that had 8% of the cells ≥ 19 µm initially, reached 17% ≥ 19 µm 10 hours after serum re-addition. C) Percentage of U937 cells ≥ 19 µm following subculture release. Cells were subcultured back into early log-phase at 0 hours. D) XTT proliferation assay of U937 cells in log- or plateau-phase. 100 µl cells were seeded per well into a flat-bottom 96-well microtiter plate in triplicate for each cell dilution. The plate was incubated for 24 hours prior to addition of XTT solution. Cells were then incubated for an additional 2 hours before the wavelength was read. X axis refers to the cell number in units of 10^3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ultrasound, even when low power, moderate intensity (20 W, 10 W/cm^2) ultrasound/cytochalasin B-treated cells were compared to moderate power and intensity (60 W, 80 W/cm^2) ultrasound/jasplakinolide-treated cells.

It also appeared that cytochalasin B-treated cells were more sensitive to sonication than vincristine-treated cells or to cells enlarged by serum deprivation and re-addition (Fig. 5A), as all of these groups were sonicated under comparable conditions using 23.5 kHz, 20 W (10 W/cm^2) ultrasound. Similar results for all treated cell groups were attained with 20 kHz, 60 W (80 W/cm^2) ultrasound, with cytoskeletal-disrupting agents potentiating more ultrasonic damage than cytoskeletal-stabilizing agents (Fig. 5B). It is interesting to note that although microfilament-stabilizing jasplakinolide appeared more toxic than microfilament-disrupting cytochalasin B, and required a lower concentration to treat the cells prior to sonication, it conferred the least ultrasonic sensitivity in comparison to the other treatment groups.

Effects of cytoskeletal-disrupting agents on multiple leukemia lines and normal human blood cells

The combination of cytoskeletal-disrupting agents and thirty 1 second pulses of 20 kHz ultrasound (80.6 W; 107.5 W/cm^2) spaced 1 second apart appeared to decrease the viability of multiple leukemia lines (human lines: U937, THP1, K562, and Molt-4; murine line: L1210) more than ultrasound-alone or the administration of

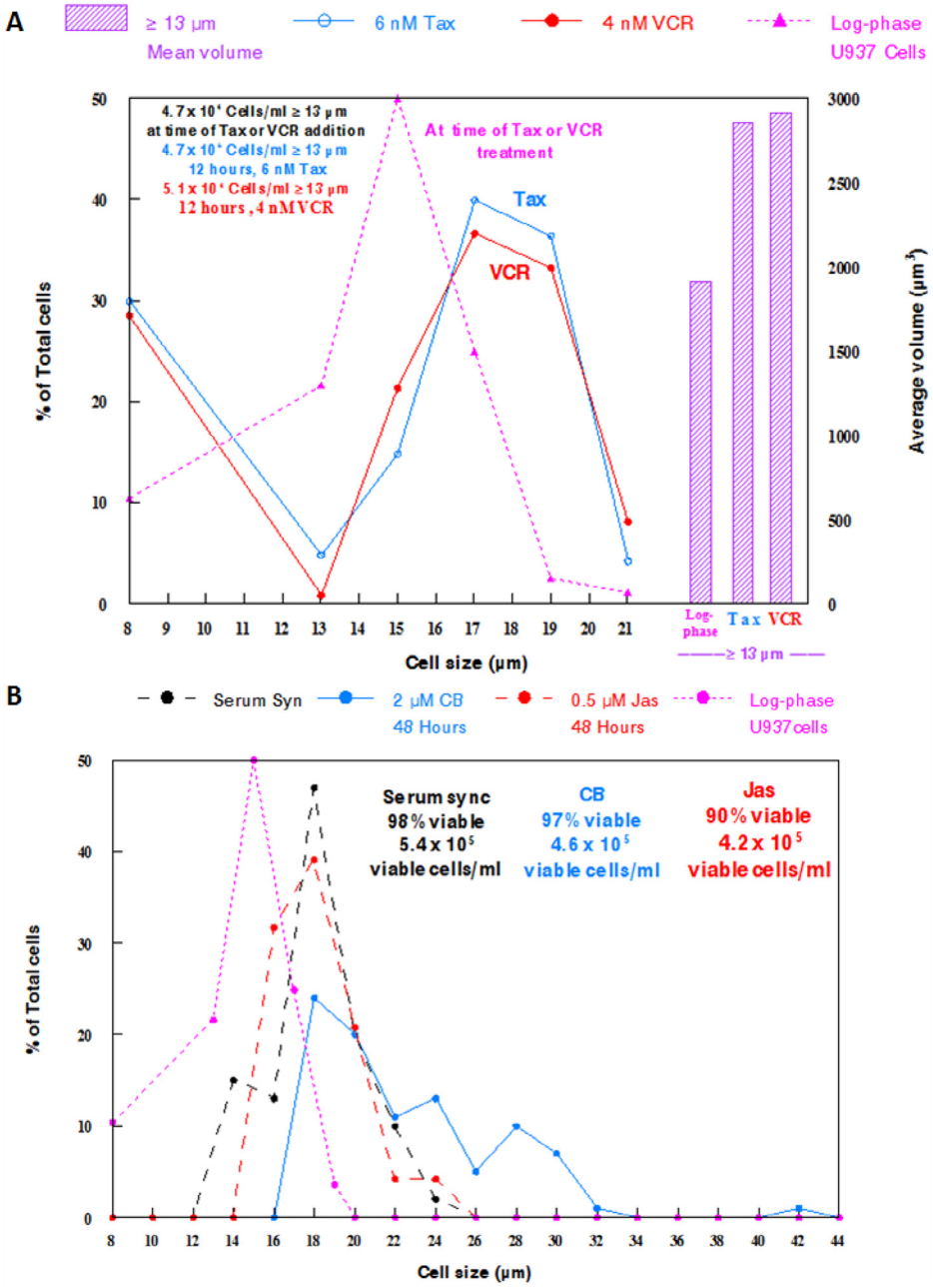

Fig. 2. Size distribution of log-phase U937 cells treated with cytoskeletal directed agents. A) Cells treated with microtubule-directed agents. Cells were either treated with 4 nM vincristine (VCR), or 6 nM paclitaxel (Tax) for 12 hours. The left y axis corresponds to the % total of cells ≥13 µm that attained the cell size given on the x axis, while the right y axis corresponds to the average cell volume of cells ≥13 µm after being treated with the indicated agent. B) Cells treated with microfilament-directed agents or serum deprivation and re-addition. Cells were treated with either 2 µM cytochalasin B (CB) or 0.5 µM jasplakinolide (Jas) for 48 hours.

cytochalasin B or vincristine (Fig. 6A). Further, both U937 and THP1 cells had a much higher reduction in cell viability as assessed by XTT reduction when either cytochalasin B or vincristine was combined with ultrasound, with cytochalasin B-treated cells again demonstrating more ultrasonic sensitivity (Fig. 6B). The effects of cytochalasin B and 23.5 kHz, 20 W (10 W/cm^2) continuous ultrasound on normal human blood cells are particularly intriguing, as hHSCs and human leukocytes are damaged markedly less by the treatment in comparison to U937 cells, even after 2 µM cytochalasin B was added for 48 hours prior to sonication (Fig. 6C). The potential importance of these findings will be elaborated upon in the discussion.

Discussion

As assessed by cell enlarging agents and by varying growth conditions to obtain enlarged cells, SDT preferentially damages U937 cells based on size. Cells ≥19 µm as a consequence of serum deprivation and re-addition, or because of chemotherapeutically-induced enlargement were damaged far more than cells ≥13 µm. Since cytochalasin B- and vincristine-treated cells were more sensitive to ultrasound than cells of the same size produced by cytoskeletal-stabilizing agents (jasplakinolide and paclitaxel), or by exposure to serum-deprivation or plateau-phase subculturing, it is apparent that the method used to obtain enlarged cells has

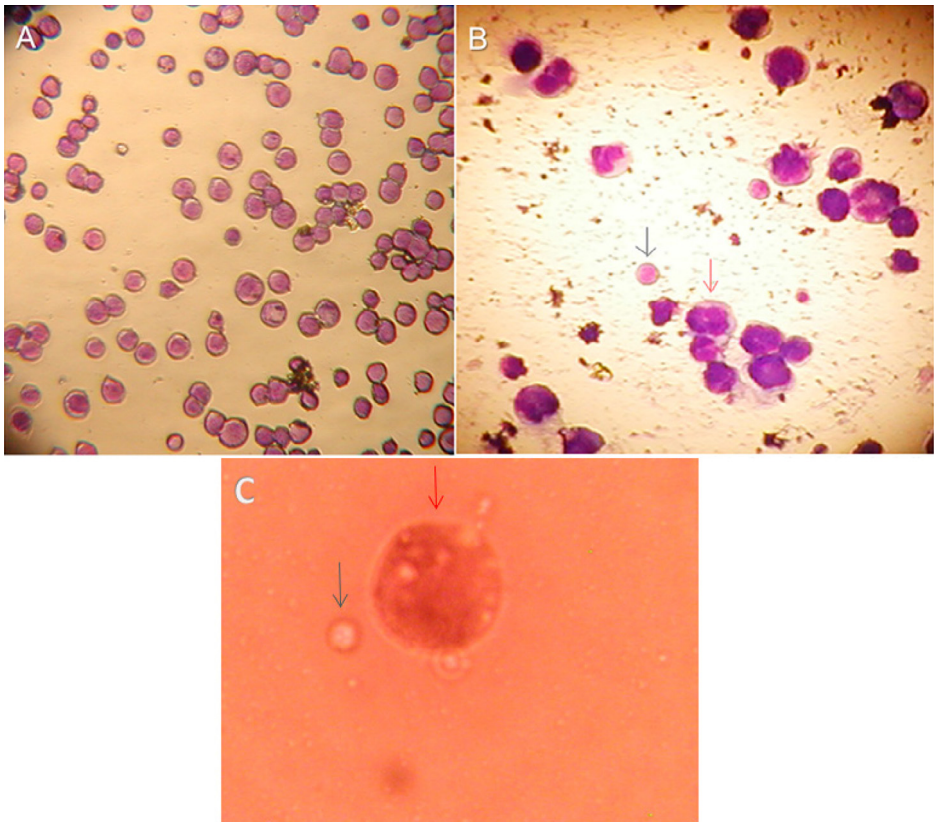

Fig. 3. Effects of cytochalasin B and vincristine on U937 cell size. A) Untreated U937 cells. B) U937 cells treated with 2 μM CB for 48 hours. C) U937 cells treated with 4 nM VCR for 11 hours. Gray arrows indicate a mononucleated cell that was not enlarged by the treatment. Red arrows indicate a cell that was enlarged by the treatment. Nuclei were visualized with Wright–Giesma stain. Photomicrographs for panels A and B were taken under identical conditions of magnification (100×). The photomicrograph for panel C was taken at 400× to conclusively determine whether any multinucleation was present. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a substantial influence on subsequent damage by sonication. It was interesting to note that cytochalasin B-induced microfilament disruption potentiated higher sensitivity than did vincristine-induced microtubule-disruption, suggesting that microfilaments may be a more important target in SDT than microtubule-directed agents. It is reasonable to propose that cytochalasin B or other microfilament-disrupting agents can be combined with microtubule-disrupting agents (vincristine and other vinca alkaloids) to enhance SDT, rather than combining cytochalasin B with agents that rigidify microtubule polymers (paclitaxel and docetaxel/taxotere). Combined cytoskeletal-directed chemotherapy prior to sonication is indeed a sensible prospect, and will be the subject of a future study.

The administration of cytochalasin B or vincristine prior to sonication appears to potentiate the ultrasonic sensitivity of leukemia lines U937, THP1, K562, Molt-4, and L1210 (Fig. 6A). Further, the extent of damage potentiated by these cytoskeletal-disrupting agents and ultrasound is much greater than either ultrasound-alone, or the administration of cytochalasin B or vincristine. These data indicate that cytoskeletal-disrupting agents are potent sonic sensitizing agents in multiple types of leukemia, including acute myeloid leukemia (U937 and THP1), chronic myeloid leukemia (K562), acute lymphoid leukemia (Molt-4), and murine lymphoid leukemia (L1210). In each cell line, cytochalasin B potentiated ultrasonic sensitivity much more than vincristine, indicating that microfilament-disrupting agents are likely more potent sonosensitizers than microtubule-disrupting agents. Further, the ability of both U937 and THP1 leukemia to reduce XTT is substantially decreased following sonication with 20 kHz pulsed ultrasound (Fig. 6B). This is indicative of a marked decrease in mitochondrial activity and cell viability, hallmarks of apoptosis. While XTT assays are unable to fully confirm that apoptosis is occurring, it is likely that this form of programmed cell death is present in sonicated cells. Apoptosis prevents cells from reducing tetrazolium dyes, as mitochondrial activity is degraded in the initial steps of this biological process. Therefore, the data acquired by the present study, as well as multiple studies that have confirmed the presence of apoptosis in human leukemia cells following sonication (including U937 and K562 [20,22–24]), indicate that ultrasound can induce apoptosis in this type of cancer.

Interestingly, U937 cells are preferentially damaged by 23.5 kHz ultrasound over hHSCs and human leukocytes, even after the administration of 2 μM cytochalasin B for 48 hours (Fig. 6C). The extent of preferential damage appears to be significant, as cytochalasin B-treated normal blood cells were damaged markedly less by exposure to 23.5 kHz, 20 W (10 W/cm^2) ultrasound for 4 min than cytochalasin B-treated U937 cells. Therefore, the present study suggests that leukemia cells are preferentially damaged over normal blood cells in the presence of cytochalasin B and ultrasound. This has potential *in vivo* implications, as it may be possible to directly sonicate the blood of leukemia-challenged mammalian models to reduce the load of leukemic blasts in the presence of normal blood components through extracorporeal blood sonication [25]. In addition, it may be possible to use the penetrating power of ultrasound to sonicate the bone marrow of patients to preferentially damage leukemic stem cells in the presence of hHSCs, which are often responsible for propagating the disease [25]. However, these findings should be extended to leukemic stem cells, erythrocytes and megakaryocytes/thrombocytes prior to *in vivo* experimentation.

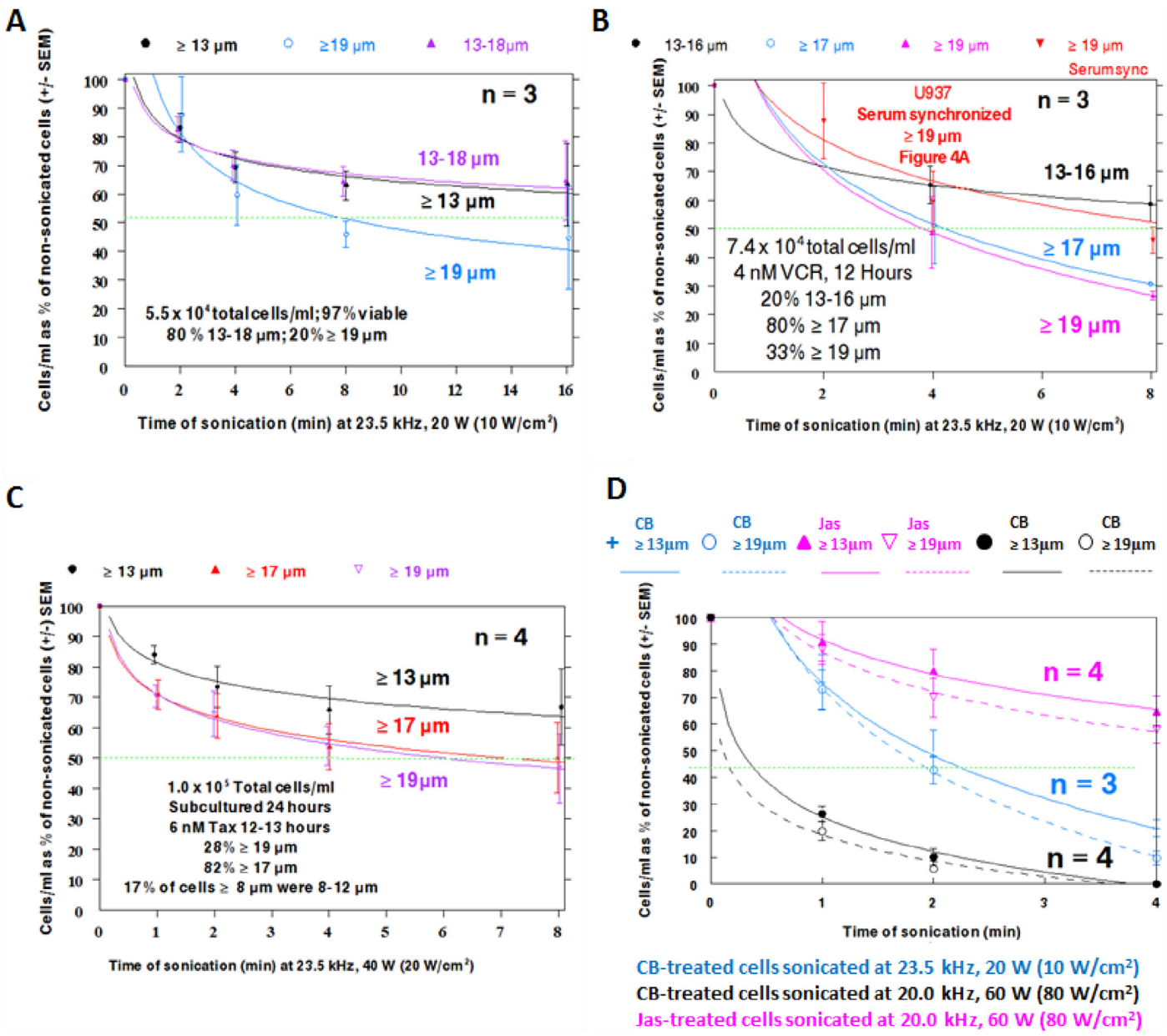

Fig. 4. Effects of sonication on cells treated with cytoskeletal-directed agents or serum deprivation and re-addition. A) Sonication of U937 cells at 6 hours after 20% serum re-addition to cells deprived of serum for 22 hours. Sonications were performed at 20 W (10 W/cm^2), 23.5 kHz. The distribution of cell sizes before sonication is provided on the graph. Bars reflect SEM of 3 independent populations of U937 cells. B) Sonication with 23.5 kHz probe of U937 cells at 20 W (10 W/cm^2) treated with 4 nM vincristine for 12 hours. The distribution of cell sizes after treatment is provided on the graph. The ultrasonic sensitivity of vincristine-treated cells ≥19 μm are compared to the same size range of cells attained from serum deprivation and re-addition. Bars reflect SEM of 3 independent populations of U937 cells. C) Sonication with 23.5 kHz probe of U937 cells at 40 W (20 W/cm^2) treated with 6 nM paclitaxel for 12 hours. Bars reflect SEM of 4 independent populations of U937 cells. D) Comparison of cytochalasin B and jasplakinolide for determining ultrasonic sensitivity of treated U937 cells. CB-treated cells were sonicated at 20 kHz, 60 W (80 W/cm^2) or 23.5 kHz, 20 W (10 W/cm^2). Jas-treated cells were sonicated at 20 kHz, 60 W (80 W/cm^2). Bars reflect SEM of 3 or 4 independent U937 cell populations for CB-treated cells, and 4 independent cell populations for Jas-treated cells.

It is well known from chemotherapeutic approaches used in the clinic that monotherapies employing only a single drug are typically not effective, as primary or secondary levels of drug resistance typically emerge within heterogeneous tumorigenic growths. While the treatment may have valuable initial success, cells resistant to the treatment often arise, and subsequently repopulate tumors with drug resistant cells. Therefore, it is necessary to treat malignancies with a variety of antineoplastic agents concurrently, as it is much more difficult for malignant cells to acquire simultaneous resistance to combinations of chemotherapeutic agents that function by diverse mechanisms [25]. That is why the concomitant use of cytochalasin B and vincristine along with ultrasound appears to be such a sensible prospect. Cytochalasin B-induced microfilament disruption may synergize very well with vincristine-induced microtubule disruption, reducing the ability of leukemia cells to proliferate, and profoundly increasing the sensitivity of these cells to ultrasound through cell enlargement. Indeed, experiments performed by our laboratory assessing the reduction in clonogenicity following concomitant administration of cytochalasin B/vincristine support the synergistic potential of these agents (Fig. 7). Further, we have demonstrated that cytochalasin B/ultrasound substantially decreases clonogenicity in U937 cells [10], and we have

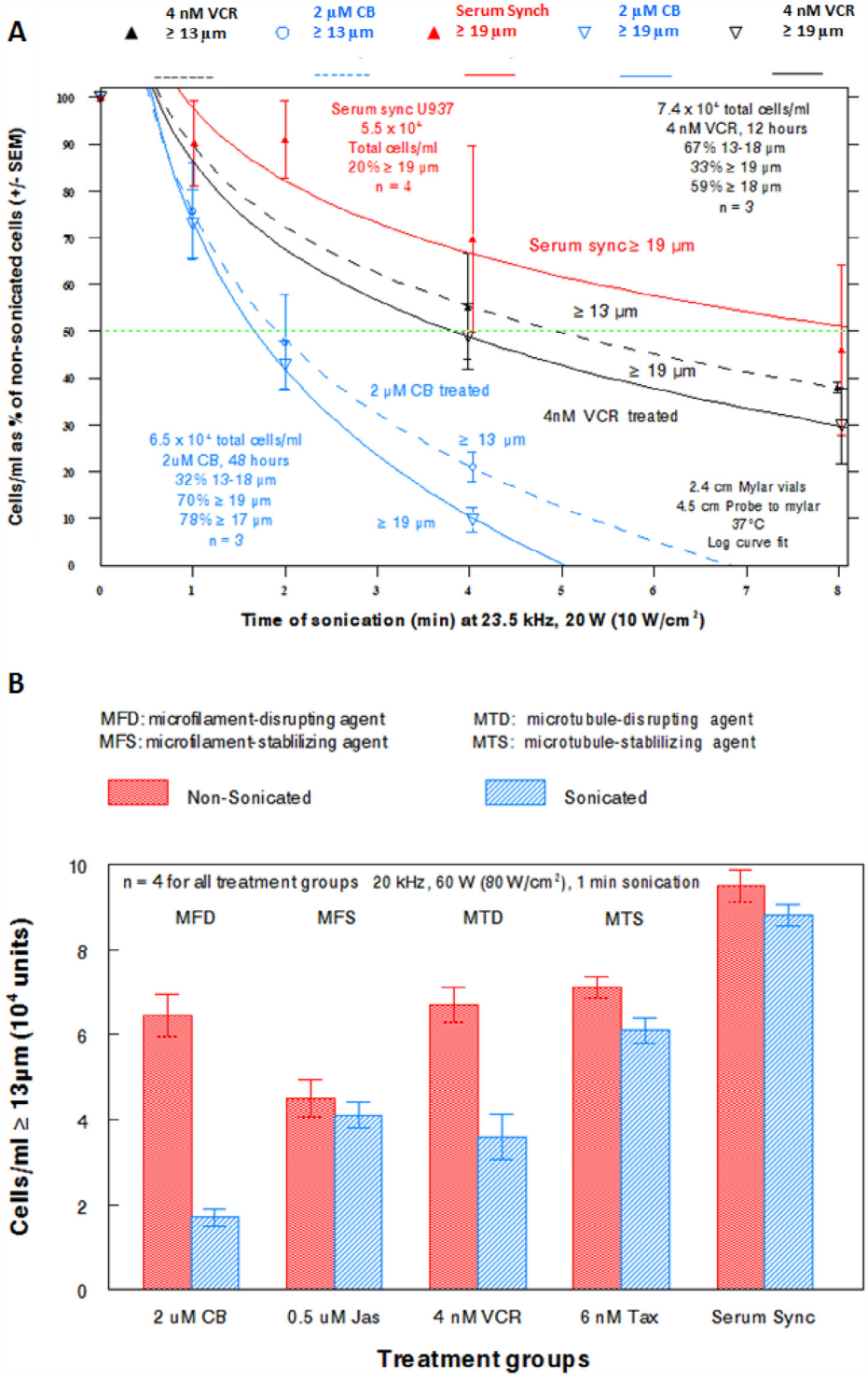

Fig. 5. Comparison of ultrasonic sensitivity potentiated by different cytoskeletal-directed agents under the same sonication protocols. A) Comparison of ultrasonic sensitivity potentiated by cytoskeletal-disrupting agents and serum deprivation and re-addition. All cells were sonicated with 23.5 kHz ultrasound at 20 W (10 W/cm^2). Bars reflect SEM of 4 independent U937 cell populations for cells enlarged by serum deprivation and re-addition, and 3 independent cell populations for CB- and VCR-treated cells. B) Comparison of cell enlarging protocols to determine ultrasonic sensitivity using 20 kHz, 60 W (80 W/cm^2) ultrasound. Abbreviations for the type of cytoskeletal-directed agent are provided in the figure. All cell populations were sonicated for 1 min. Bars reflect SEM of 4 independent U937 cell populations for all treatment groups.

determined that cytochalasin B/vincristine/ultrasound greatly potentiates this clonogenic reduction (Table 1). It is unlikely that the enlarged cellular phenotype will be able to generate biochemically-based resistance to an extrinsic physical agent that damages cell integrity and cell clonogenicity by affecting overall cellular integrity at the macroscopic level as opposed to affecting a defined intracellular signaling pathway. It is not unusual for a sub-population of cancer cells to evade the effects of vincristine and complete nuclear replication. However it is far less probable that the same sub-population could generate resistance to cytochalasin B and circumvent the effects of disrupted microfilaments on cytokinesis. The leukemic cells treated with both classes of

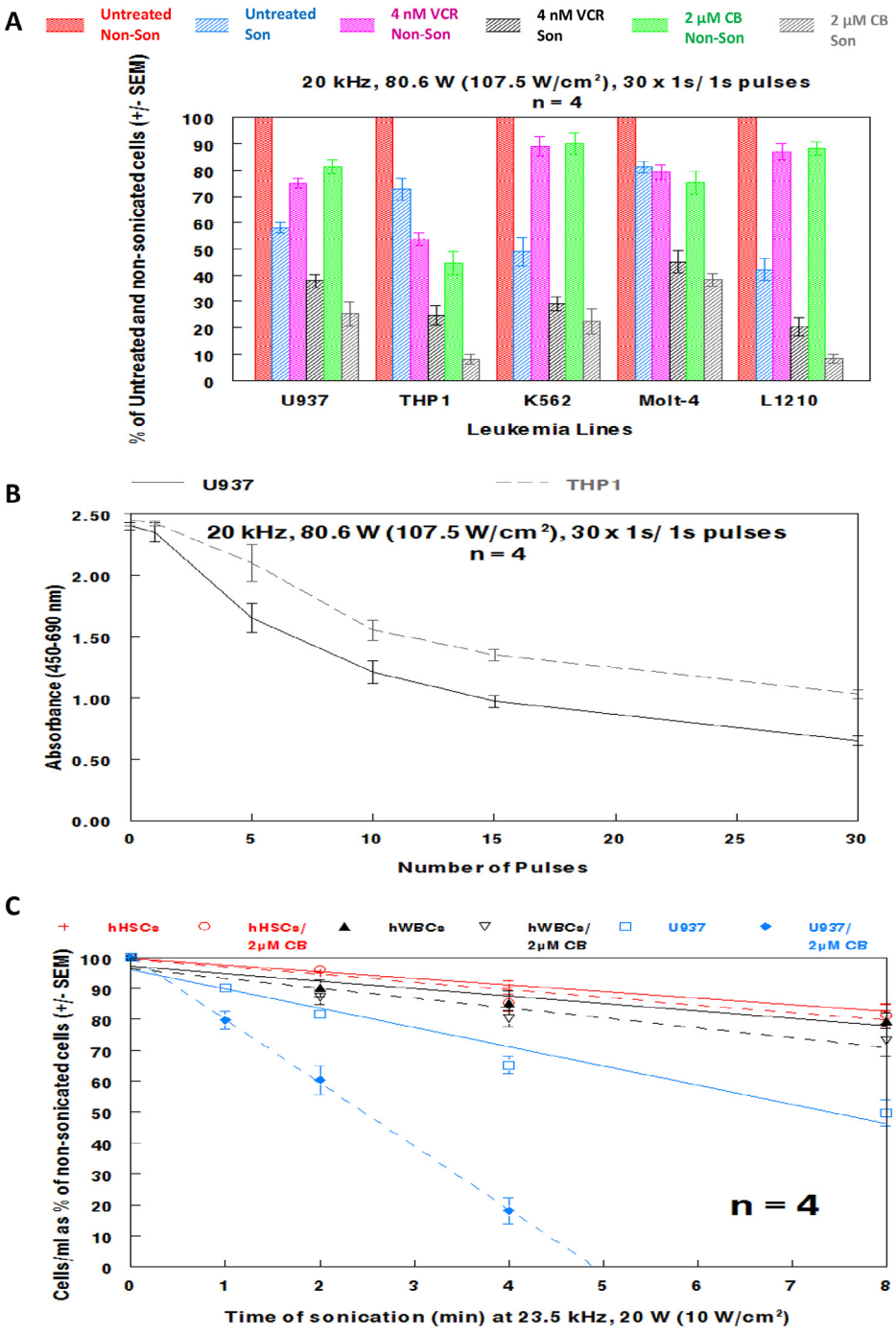

Fig. 6. Effects of cytoskeletal-directed agents and ultrasound on multiple leukemia lines and normal human blood cells. A) Ultrasonic sensitivity of multiple leukemia lines before and after treatment with 2 μM cytochalasin B and 4 nM vincristine. Non-Son (non-sonicated); Son (sonicated). B) Effects of ultrasound-alone and in combination with 2 μM cytochalasin B or 4 nM vincristine on reducing mitochondrial activity and cell viability in U937 and THP1 lines. C) Effects of cytochalasin B and ultrasound on normal human leukocytes and hematopoietic stem cells. Abbreviations used are as follows: hHSCs (human hematopoietic stem cells); hWBCs (human white blood cells, leukocytes).

cytoskeletal-disrupting agents would therefore be forced to become enlarged and ultrasonic sensitive and would be unable to circumvent two cytoskeletal-directed agents that act by completely distinct mechanisms.

Combining ultrasound with cytoskeletal-directed agents may yield improved antineoplastic cell effects. Indeed, the synergistic potential of cytochalasin B and vincristine without ultrasonic treatment has already been well described [26]. When various malignant cell types were exposed to cytochalasin B/vincristine treatments, the resultant DNA fragmentation was greater than the sum of that caused by each agent alone, demonstrating a synergistic relationship. Further, the study indicated that the levels necessary to achieve this potentiation are obtainable *in vivo*, indicative of a potential therapeutic prospect.

While there has been a substantial array of *in vivo* studies that indicate the therapeutic potential of SDT [9,27–30], more studies of this nature are needed before the novel approach can be attempted in the clinical setting. If eventual clinical studies determine

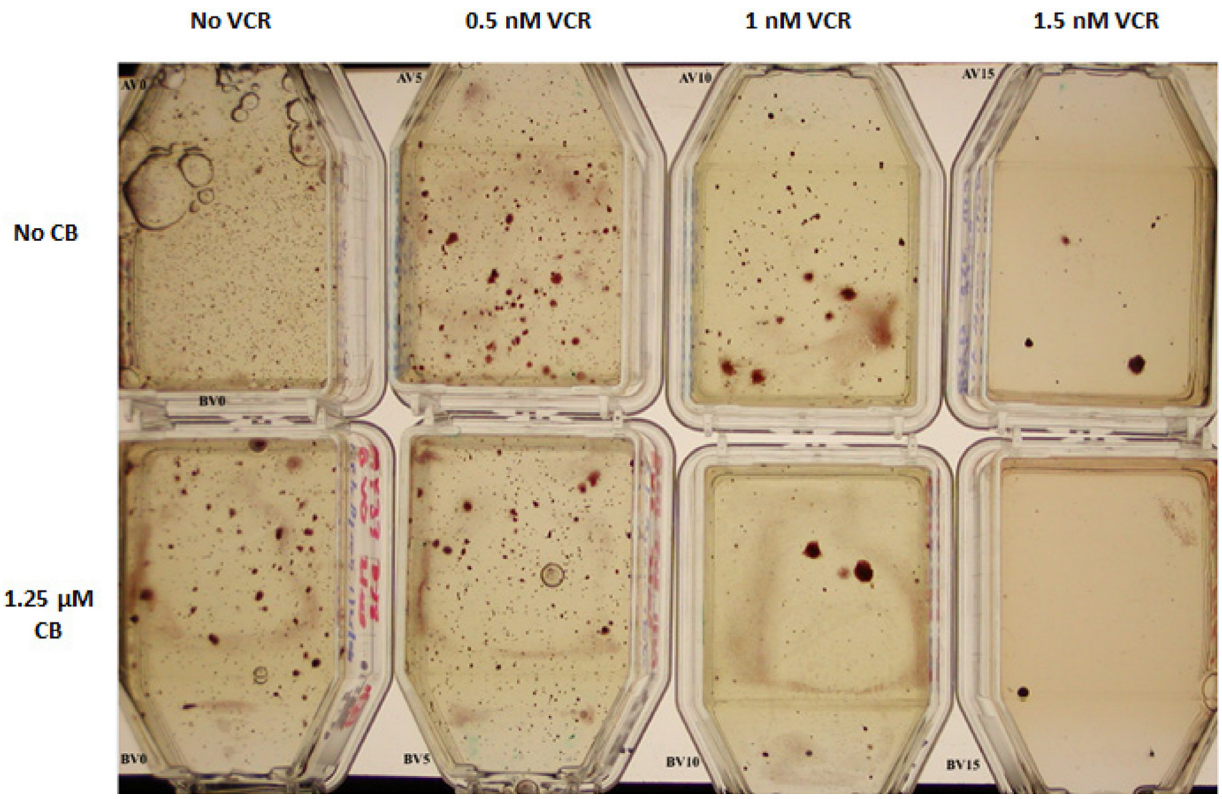

Fig. 7. Effects of cytochalasin B and vincristine as single agents and in combination on the clonogenicity of U937 human monocytic leukemia. Cells were treated with 1.25 μM CB for 4 days, followed by varying concentrations of VCR for an additional 2 days. Cells were then allowed to regrow for 2 days prior to being seeded at ~300 cells in 1% agarose, 20% fetal bovine serum on a 1.5% agarose, 20% fetal bovine serum basement layer. Cells were stained with p-iodonitrotetrazolium violet. Photographs of the flasks were taken 45 days after initial treatment with CB.

that ultrasound combined with cytoskeletal-directed agents alone is not effective, there are a large variety of other cancer chemotherapeutic agents not directed specifically toward the cytoskeleton. These clinically-active chemotherapeutic agents in combination with cytoskeletal-directed agents could potentiate sonic sensitivity by contributing to cell enlargement and other forms of cell disruption, including membrane alteration [10]. Further refinements could be made using other cytoskeletal-directed agents including agents affecting the intermediate filaments, and by using agents affecting cytosolic and membrane integrity and cell size to determine conditions optimal for the preferential destruction of hematological malignancies, and potentially other neoplasms.

Table 1
Reduction in U937 cell cloning efficiency under varying treatment protocols.

U937 cell group	Cloning efficiency (%)	SEM (±%)	% Reduction in cloning efficiency
No treatment			
Control (no treatment)	70	1.1	0
Ultrasound	44	1.2	36
CB treatment			
2 μM CB	14.3	0.9	80
2 μM CB and ultrasound	5.1	0.8	93
VCR treatment			
4 nM VCR	10	0.7	86
4 nM VCR and ultrasound	9	0.4	87
VCR and CB treatment			
4 nM VCR and 2 μM CB	5.5	0.2	92
4 nM VCR and 2 μM CB and ultrasound	1.7	0.2	98

Cloning efficiency of 300 μl (~1000 cells) U937 cells was assessed after an 18 day cloning period. U937 cells assessed for cloning efficiency after sonication were sonicated for 4 min using 23.5 kHz, 20 W (10 W/cm^2) ultrasound. Reduction in cloning efficiency was found by dividing the cloning efficiency by the control cloning efficiency, and subtracting this number from 100%. SEM reflects 3 independent U937 cell populations for all treatment groups.

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Conflict of interest

The authors declare no conflict of interest.

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