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The Promise of Sonodynamic Therapy: Using Ultrasonic Irradiation and Chemotherapeutic Agents as a Treatment Modality

Matthew Trendowski

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Matthew Trendowski
Candidate for Bachelor of Science Degree
and Renée Crown University Honors
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Capstone Project Advisor: _____
Thomas Fondy, Professor

Capstone Project Reader: _____
Stephen Dorus, Associate Professor

Honors Director: _____
Stephen Kuusisto, Director

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Abstract

Sonodynamic therapy (SDT) is a potential cancer treatment modality that has been gaining support due to its effectiveness in both in vitro and in vivo studies. The therapeutic method combines ultrasonic irradiation with drugs known as sonosensitizers that amplify its ability to inflict preferential damage on malignant cells. This is based on the idea that ultrasonic waves have the ability to exhibit profound physical and chemical changes on cellular structure. The mechanisms by which ultrasound disrupts cellular functioning can be further amplified when sonosensitizers are applied. Combining multiple sonosensitizers with ultrasound to create a substantial synergistic effect could be an effective method for destroying tumorigenic growths, while decreasing the likelihood of drug resistance.

Perhaps one of the most intriguing capabilities of ultrasound is its ability to preferentially lyse cells based on size. This known fact invariably gives rise to the idea of grossly enlarging tumor cells to increase their already noticeable size difference with normal cells. Cytochalasin B is a known pharmacological agent that disrupts the actin cytoskeleton and inhibits cytokinesis by interfering with formation of the contractile ring as well as the development of the cleavage furrow. Consequently, the cell does not divide and an immature actin cytoskeleton remains. However, the cell continues to form nuclei and eventually becomes grossly enlarged and multinucleated. Such cells invariably have more DNA targets, increasing the likelihood of apoptosis. Furthermore, the multinucleated cells have a large cell volume, making them more susceptible for direct cell destruction. Preferential damage of malignant cells is actually easily attainable as normal cells exposed to cytochalasin B exit the cell cycle and enter a resting state until sufficient actin levels are restored. Therefore, only malignant cells that have lost the ability to enter the rest phase will become grossly enlarged and multinucleated, providing an ideal target for ultrasonic irradiation.

Work from our lab has indicated that cytochalasin B does indeed only damage leukemia cells, leaving normal blood cells, unaffected. The designated cell line has been promyleocytic leukemia U937 cells as they are a frequent choice for *in vitro* studies. The U937 cells have routinely become grossly enlarged and multinucleated, providing an ideal target based on size. The typical erythrocyte is 6-8µm, while leukocytes fair slightly better with a range of 10-15µm and an average of 12µm. By contrast, work from our lab has shown that cytochalasin B treated leukemia cells easily grow in excess of 20µm with some reaching 40µm in diameter after enough exposure. Such cells have reduced cytoskeletal integrity and are easy targets for ultrasonic irradiation. Furthermore, cytochalasin B treated leukemia cells are substantially multinucleated as cytokinesis is inhibited. This provides plenty of targets for a nucleic acid directed agent such as cisplatin or doxorubicin to attack. To investigate the extent of preferential damage inflicted by cytochalasin B on U937 leukemia/human blood populations, cell mixtures were treated with cytochalasin B and then sonicated under a relatively low intensity ($3W/cm^2$). Results indicated that cytochalasin B preferentially damages U937 cells both before and after sonications. The agent also demonstrates the capability to eliminate rapid proliferation as U937 cells have a marked decrease in clonogenicity. Such findings suggest that cytochalasin B may have profound therapeutic applications when combined with SDT.

Key Words

Sonodynamic Therapy, Ultrasound, Sonosensitizers, Inertial Cavitation, Reactive Oxygen Species, Tumor Vasculature, Preferential Damage

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Executive Summary

The amount of progress cancer therapy has made in recent years is staggering. Clinicians now have the capability to specifically target malignant cells via receptors or protein products that are not found within healthy cells. This ingenuity has resulted in miraculous treatments such as the use of imatinib (Gleevec) in patients afflicted with chronic myeloid leukemia that express the Bcr-Abl gene abnormality, resulting in astounding cure rates. However, despite these compelling discoveries, cancer therapy has only limited efficacy in the clinical setting. One of the most cited shortcomings of chemotherapy in clinical practices is drug resistance acquired by tumors. While initial treatments appear to have remarkably high efficacy, perceptively removing the malignancy from the patient, there are many instances when a few cancer cells remain to repopulate and create a novel tumor; resistant to prior chemotherapeutic approaches. Further, many clinical therapies rely on cancer's well-known characteristic of fast, uncontrolled cell division. By targeting this distinct feature, it is hoped that the treatment regimen will be effective and specific in its destruction. Unfortunately, there are many cells in the body, such as in the gastrointestinal tract and hair follicles, that also divide rapidly and are often drastically affected by treatments. If there was a way to specifically target malignant cells based on other inherent characteristics, higher efficacy rates may be achieved.

As it turns out, cancer cells are often larger than normal ones. In fact, metastatic cells (those that have broken away from the primary tumor to move to other tissue sites) are often much larger than the surrounding blood cells circulating through the bloodstream. Therefore, exploiting this inherent size differential may be a novel characteristic of cancers to target. Further, malignant cells that have reached the bloodstream would be a vital target as it is this metastatic progression that causes so much destruction in the patient. In fact, more than 90% of

patient mortality due to cancer is a direct consequence of metastatic progression. Other than aberrant cell division, there is not a single more unifying characteristic in cancer biology. This ultimately gives rise to the idea that the unique mobility linking virtually all malignant growths can be exploited to improve current chemotherapeutic approaches. That is, use the malignancy's most devastating characteristic to develop novel therapeutic methods that specifically target and damage circulating cancer cells. Such an approach would have monumental importance in mitigating the symptoms of metastatic progression. After all, it is the metastatic phenotype of cancer that subdues most patients.

This form of targeted chemotherapy may be achieved by sonodynamic therapy (SDT), a novel treatment modality that uses ultrasound (frequencies above 20 kHz) with specialized chemotherapeutic agents known as sonosensitizers to preferentially damage malignant cells. It has been shown in many cellular and animal experiments that ultrasound preferentially damages malignant cells based on the size differential between such cells and those that have not become cancerous. This is especially important for tumors that have progressed to the metastatic state as the malignant cells will be in close proximity to normal blood cells. By targeting the malignant cell's inherent size differential with normal cells in circulation, SDT asserts itself as a therapeutic approach that is both effective and specific.

The size differential between malignant and normal cells can be dramatically increased through the use of sonosensitizers that specifically target malignant cells, thereby amplifying the preferential damage of ultrasound. While ultrasonic waves produce remarkable antitumor effects under appropriate settings, such effects are not always widespread and tumor populations often become resistant to ultrasound-only treatments. That is why using chemotherapeutic agents to amplify the effects of ultrasound is such a sensible prospect. Such an approach significantly

enhances the efficacy of ultrasound, while still displaying preferential damage towards malignant cells. Every mechanism by which ultrasound destroys malignant tissue can in fact be amplified when an appropriate sonosensitizer is administered. Such drugs often attack cells through multiple mechanisms as well, creating a potential synergistic effect when sononosensitizers of different classes are used in collaborative efforts. If preferential damage to malignant tissue can be maintained when such drug cocktails are applied, the efficacy of treatments could be truly remarkable. SDT has also been shown to be particularly effective in drug resistant cell lines as both cellular and animal experiments have revealed that SDT has the ability to reverse this potent defense mechanism in a variety of cancer types.

Although SDT has been shown to be effective against a variety of cancer cell lines in cellular and animal studies, it is important to note that this treatment modality may be most effective on specific cancers found in the clinical setting. The cancer type that has shown the greatest response to SDT and is a primary focus of this article is systemic leukemia. In leukemia, leukocytes (white blood cells) revert to a more primitive, embryonic state in which cell division occurs much more frequently. These aberrant cells begin to overcrowd healthy, functional leukocytes, erythrocytes (red blood cells) and megakaryocytes (cells that eventually give rise to blood platelets). The bloodstream can become saturated with these aberrant cells, eventually compromising the immune system, blood clotting and erythrocyte transport. Leukemia cells originate from hematopoietic stem cells that also give rise to erythrocytes and megakaryocytes and overtake the stock of healthy stem cells so that other blood cells are unable to be produced in sufficient quantities. Unlike most cancers, leukemia is inherently metastatic as leukocytes are required to move throughout the bloodstream to elicit an immune response, suggesting that no further mutations are needed for this characteristic. Leukocytes also have the need to leave the

circulatory system in great quantities over a short period of time in order to combat infection and trauma, a process known as diapedesis. While metastatic cells of other cancer variants must acquire mutations that enable them to leave the bloodstream, leukocytes have an innate form of this mechanism. In effect, leukemic cells have natural characteristics due to their cell of origin that allow them to invade other areas of the body, without requiring additional mutations. This is a major reason why leukemias are so commonly found as childhood cancers as less fundamental alterations are required for the development of malignant growths.

However, as indicated in this article, leukemia cells are profoundly sensitive to SDT, especially when a sonosensitizer known as cytochalasin B is administered. Cytochalasin B is a known pharmacological agent that inhibits cytokinesis (cell separation after DNA has been replicated in dividing cells) by preventing the progeny cells from separating. Consequently, the original cell does not divide and an immature cellular structure remains. However, as with virtually all cancers, leukemia cells continue to form nuclei (the structures that contain DNA) due to their increased rate of cell division. As a result, cytochalasin B treated cells eventually become grossly enlarged and multinucleated. As previously mentioned, ultrasound preferentially damages cells based on size, making enlarged leukemia cells ideal targets for SDT. Cytochalasin B has also been shown to increase the metabolic rate of leukemia cells, suggesting that other chemotherapeutic agents that target this activity as well as the increase in nuclei can be used to create a potent synergistic effect. One of the most profound effects of cytochalasin B is its ability to mitigate leukemia's ability to reproduce. A fundamental feature of any cancer is its capability of uncontrolled and often accelerated cell division. It is this characteristic that allows such quantities of aberrant cells to spread throughout the body and cause eventual death if not controlled. Cytochalasin B has demonstrated the ability to dramatically reduce rates of cell

division, eventually halting the process altogether. In effect, cytochalasin B is effectively neutralizing leukemia's ability to produce new cells, mitigating a fundamental component of cancer.

As compelling as the evidence for SDT's efficacy has been, there have yet to be any attempts to translate such results in the clinical setting. In order to use SDT in cancer therapy, effective measures need to be devised in which to administer ultrasound as well as the chemotherapeutic agents to patients. Seeing that SDT has yet to be tested in the clinical setting, there has been no analysis as to how this treatment modality could be practically applied to patients. Although SDT fundamentally relies on an ultrasound system, there are a variety of ways in which the generated ultrasonic irradiation can be delivered. The necessary equipment needed to employ SDT in therapeutic applications is already available and could be readily devised if given the opportunity. Unless clinicians are willing to take a chance on the novel method, the required data necessary to accurately determine whether SDT is a viable approach will not be attained. It will remain as an intriguing, but untested method that has little more than conceptual importance.

The work from this thesis was indeed productive as it resulted in 4 publications as well as presentations at several scientific conferences. Further, the thesis was completed a year in advance as the author graduated a year early. More importantly however, the research has elucidated an exciting novel approach to chemotherapy. Although leukemia was the primary emphasis of the study, SDT has shown considerable efficacy in a substantial variety of cancer types. By using the synergistic effects of ultrasonic irradiation and sonosensitizers, SDT is proving to be a viable treatment modality that has the capability to revolutionize the way in which chemotherapy is administered in the clinical setting. All that remains is the necessary

clinical testing. Only through these necessary trials will enough evidence be compiled to conclude whether SDT is in fact as good as advertised.

Significance

One of the most cited shortcomings of chemotherapy in clinical practices is drug resistance acquired by tumors. SDT has been shown to reverse this potent defense mechanism. Studies have also indicated that the mechanisms by which ultrasound destroys malignant tissue are amplified when appropriate sonosensitizers are administered. Such drugs often attack cells through multiple mechanisms, creating a potential synergistic effect when sononosensitizers of different classes are used in collaborative efforts. Being able to develop treatment regiments in which the synergistic effects of different sonosensitizers are applied can have monumental importance in clinical applications. Such treatments could substantially amplify the capability of ultrasound to preferentially damage malignant cells in order to decrease the rate at which drug resistance is observed.

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Chapter I: Introduction to Sonodynamic Therapy

Introduction

In the enduring battle against cancer, sonodynamic therapy (SDT) is showing promise as a potentially vital alternative to traditional treatment modalities. SDT is a form of ultrasound therapy in which chemotherapeutic agents known as sonosensitizers are administered to increase the efficacy of ultrasound's preferential damage on neoplastic cells [1]. It has been shown in both *in vitro* and *in vivo* experiments that low intensity ultrasound can increase the permeability of the plasma membrane without causing complete cell destruction [2, 3, 4]. The attractive features of SDT emerges from the ability to focus ultrasound energy on malignancy sites buried deep in tissues and to locally activate a preloaded sonosensitizer [5]. Furthermore, SDT has shown to induce cell damage in many cancer types and appears to be a versatile treatment method [6, 7, 8, 9].

Ultrasound is defined as acoustic sound of a frequency above the audible range between 20 kHz and 1GHz and shows a longitudinal wave in fluids [10]. The waves generated by ultrasound are propagations of pressure and density fluctuations and can reverberate through the medium, causing an amplifying effect [1, 3, 10]. Beyond this inherent similarity however, the ways in which ultrasound can be applied varies extensively. Consequently, while all SDT treatment schemes rely on sonosensitizers to increase the efficacy of ultrasound damage, the ways in which ultrasound is administered is not standardized.

While frequencies used in ultrasound vary within the kilohertz to megahertz range, the intensity at which the sound is applied is divided into high and low intensity ultrasound. High intensity focused ultrasound (HIFU) applies high intensity ultrasound energy to locally heat and destroy diseased or damaged tissue through ablation. As an acoustic wave propagates through the tissue, part of it is absorbed and converted to heat. When beams are focused, some of the wave

will penetrate deep in tissues. By focusing at more than one place or by scanning the focus, the tissue can be thermally ablated. HIFU takes advantage of the subsequent penetration to produce an increase in drug uptake ability as well as create a toxic effect of P-glycoprotein (P-gp) to substantiate the effects of lipophilic anticancer drugs [12]. Low intensity pulsed ultrasound (LIPUS) takes a more moderate approach by using waves around 1.5 MHz in frequency with pulses at an intensity of 30mW/cm^2 to generate cell lysis in a non-thermal setting. LIPUS is a non-invasive therapeutic tool that is widely used for clinical applications including physiotherapy, drug delivery, bone fracture healing and thrombolysis [7].

With the seemingly endless possibilities on how to apply ultrasound in SDT, it can be become difficult to determine whether there is a proper way in which the modality should be administered for a maximum effect against neoplastic cells. It is therefore necessary to understand how ultrasound interacts with chemotherapeutic agents in order to find a setting that can maximize tumor cell lysis, while causing minimal damage to surrounding healthy tissue. The ways in which drugs are transported inside of the cell should be examined as well in order to find the optimal method of drug uptake. High efficacy of drug uptake will result in lower doses of sonosensitizer being required to significantly damage malignant growths. Since virtually all chemotherapeutic agents have adverse side effects on patients, such a discovery would have monumental clinical relevance. Finding the mechanisms of ultrasonic irradiation, the synergistic effects of applied sonosensitizers and methods to increase drug transport are vital for the development of SDT as such concepts will turn it from a theoretical approach to a highly reliable cancer treatment.

Chapter II: The Properties of Ultrasound

Ultrasound Settings

There have been countless experiments that have examined the effects of ultrasound on malignant cells. With so many experiments having already been conducted, there is a great wealth of information concerning the settings of applied ultrasound in cancer research. Frequency is a variable often examined by researchers interested in the effects of SDT. While the ultrasound used to sonicate cells varies between kilohertz and megahertz depending on the experimental set up and available equipment, the range of SDT seems to fall between 20 kHz and 5 MHz. The distinct range is due to the nature of sound waves and how they interact with living tissue. Any frequency below 20 kHz is no longer within the ultrasonic range, resulting in longer wavelengths that do not have much of an effect on cell integrity [10]. However, increase the frequency too much and the same effect occurs. The generation of such small wavelengths by frequencies above 5 MHz is also seen as relatively harmless and is in fact the kind of ultrasound used in diagnostic imaging, although their ranges are typically above 10 MHz [12].

While the testable range of ultrasound used in SDT includes both kilohertz and megahertz, changing between the different frequencies is no easy task. In fact, changing the frequency by as little as 5 kHz can prove to be problematic. The issue lies within the fact that there has not yet been a reliable variable frequency ultrasound generator that can be tuned to specific frequencies. Consequently, experimenters who do want to examine the differences of frequency on cell lysis have to often buy separate transducers to generate the varying levels [10]. Any interest above 25 kHz requires the special addition of a piezoelectric crystal transducer that relies on the remarkable characteristic of specific materials such as crystals to generate an electric charge while under mechanical stress. Such a power supply is sufficient in generating the

electricity needed to form the higher ranged frequencies with one major drawback; the specialized transducers have to be tuned to the specific frequency that they are generating [10].

While it is true that the ultrasound frequency range tested for preferential cell lysis is truly immense, there are several noticeable patterns. Due to the fact that no variable frequency generator has been successfully applied to ultrasound, discrete frequencies are used. Although the equipment and generation of ultrasound varies by experiment, researchers often use common frequencies in order to gain a substantial amount of information on the effect frequency has on different variables of SDT. The most common frequency used seems to be 1MHz which can be seen as sort of the baseline from which other frequencies are compared. This frequency has more accumulated data than any other setting and has been used to examine the effects of ultrasound with and without the use of sonosensitizers.

Energy density also needs to be considered when examining frequency at maximum intensity as energy density is the product of intensity and exposure time represented by energy density = It, where I is intensity and t is time [10]. When different frequencies are used with the maximum intensity technically possible, different energy densities are created, making it impossible to compare the frequency effect alone. Since each frequency carries a different energy level when generated by the specified transducer, there needs to be a way to set energy levels equal at different frequencies in order to solely investigate the impact of frequency. While this may seem complicated, the process is actually straight forward. The energy density being received by the sound wave is governed by the power supply the frequency generator is attached to. Altering the voltage of the power supply can increase or decrease the energy density received by the sound wave, allowing frequencies from different transducers to be compared [10].

As important as frequency has been to designing effective ultrasound experiments, it is only one of many variables that researchers have been investigating. The intensity at which ultrasound waves are directed at a target is perhaps the most altered setting in experiments. Sound intensity is defined as the power of the sound per given area represented by $I = P/A$ where I is intensity, P is power and A is the area over which the power is applied. While SI units are in W/M^2, most experiments denote intensity by W/cm^2 which ultimately suggests a more concentrated or intense sound wave. Some experiments even represent the beam used to generate the ultrasonic waves in dB referring to decibels. The range of intensity for the threshold of hearing is truly immense as the softest sound detectable by the average human ear is $1 \times 10^{-12} W/M^2$, while instant perforation of the eardrum results when intensities reach a staggering $1 \times 10^{16} W/M^2$ [13]. The shear range of audible sound intensity is very difficult to express in a linear fashion and is why the logarithmic decibels scale was developed. The sound level in decibels is represented by $\beta = 10 \log I/I_o$ as β represents sound level, I is the intensity being converted and I_o is a reference point for the threshold of hearing ($1 \times 10^{-12} W/M^2$).

Amplitude is the maximum value in which a sound wave can reach and has a direct effect on intensity. In fact, intensity is proportional to the square of the amplitude which ultimately suggests that doubling the amplitude will quadruple the intensity [13]. Since sound has wave properties, amplitude can be increased by directing waves that are in phase with each other towards a target. Known as constructive interference, waves with the same crests (maximums) and troughs (minimums) will interact with each other, causing the amplitudes to be added together. This concept derived from the principle of superposition allows sound intensities to be dramatically increased simply by having multiple outputs produce sound at the same frequency

as the waves will interact through constructive interference to generate larger net amplitudes, dramatically increasing sound intensity.

While not present in every experiment, the use of duty cycles and subsequent pulse dosing is an important component of ultrasound exposure. Duty cycles refer to the percent of time a power supply spends in an active state as a fraction of the total time under consideration [11]. In other words, equipment set to a 50% duty cycle will only spend half of its time on in a given time period. Since duty cycles can be readily altered, cells can be exposed to different cycles in which they are exposed to ultrasonic waves, referred to as pulse dosing [11]. For example, cells could be subjected to ultrasound at a given frequency for the entirety of a minute or they could be given pulses of ultrasound in which waves are received once every 5 seconds for the minute. Such control allows for the implementation of pulse repetition frequency (PRF) in ultrasound and pulses can be spaced within fractions of a second to create different biological effects [11].

Chapter III: Ultrasound as a Cancer Therapy

Mechanisms of Ultrasound

The application of SDT in clinical settings would ultimately depend on the type of sonosensitizer being administered as such drugs have been shown to improve preferential tumor damage induced by ultrasonic waves. This suggests that ultrasound alone already has the capability of reducing the viability of malignant cells. In fact, there have been multiple studies conducted to specifically examine the mechanisms by which ultrasound alters cell structure and viability [3, 9, 10, 13] (Fig. 1). Subsequent results have generated remarkable insight as to how preferential damage is actually produced. Such insight is critical for choosing appropriate drugs

for SDT as sonosensitizers can be selected to amplify the damaging effects of ultrasonic irradiation as well as focus on new targets to reduce cellular resistance.

Microbubbles and Inertial Cavitation

Microbubbles are micron sized (1–10 μm) bubbles that oscillate in response to incident ultrasound [11]. While microbubbles are often systemically injected into patients in order to improve diagnostic imaging, such structures develop naturally when ultrasound is applied [3, 16]. When microbubbles are employed under therapeutic ultrasound exposure levels, their oscillations are capable of increasing the permeability of microvessels which enhances cellular uptake of molecules, nanoparticles and therapeutic agents. The increased permeability is typically due to sonoporation which is the temporary opening of pores in the plasma membrane generated by microbubbles oscillating in a stable motion, known as stable cavitation [11]. Sonoporation has been shown to be an effective method to improve drug uptake and work to promote the delivery of anticancer agents into tumor tissue through microbubble potentiated microvascular permeability enhancement is being investigated in many *in vivo* experiments [7, 18]. This has been motivated by the fact that the effectiveness of many anticancer agents is limited by the inability to reach therapeutic concentrations within tumor tissue [14]. Low intensity ultrasound is ideal for sonoporation as it allows a steady increase of stabilized microbubbles to be established within the cell. However, it should be noted that low intensity ultrasound should not be used to treat cancer cells alone as the sonoporation effect produced under low power can arouse the repair mechanism after cell damage. Without antitumor drugs being administered, tumorigenic growth will be stimulated. In addition, enhanced oxygen supply resulting from the increased permeability of surrounding vessels and cells may also contribute to malignant cell development [4].

Microbubbles are not limited to their ability of increasing drug uptake. Under more vigorous conditions, microbubbles have the capability of causing direct cell damage, eventually resulting in cell lysis. Under higher acoustic pressures, typically greater than 0.60MPa, the expansion and contraction of microbubbles usually become unequal and markedly exaggerated. This activity is known as inertial cavitation and is how microbubbles are able to directly compromise cellular integrity [6]. When microbubbles implode due to the extreme conditions, sharp and forceful mechanical movements of the fluid near vicinity of the implosion site are generated. The presence of fluid flow yields a non-uniform stress on cells known as hydrodynamic shear which is associated with several possible mechanisms by which an ultrasonic field causes cell lysis, including acoustic streaming, bubble pulsations and bubble implosions [3] (Fig. 2). Asymmetrical collapse results in high liquid jets being directed through the center of the microbubble, creating very high shear velocities in the surrounding liquid. The effect of shear flow is to induce a localized tensile stress that can exceed the tensile strength of a cell membrane, ultimately resulting in cell lysis [3]. In general, for a given velocity gradient, the larger the microbubble, the greater the tensile force exerted on that object. Implosions can also be radially symmetrical in which case the part of the cell closest to the microbubble is moved radially inward more than is the part farthest from the microbubble, producing a net tensile force on the cell [3].

The benefits of sonoporation are retained when inertial cavitation develops within a cell. Microbubble destruction by ultrasound exposure generates microstreams or microjets of moving liquid that open transient pores in plasma membranes, effectively producing the same effect of stable cavitation [10]. It should be noted though that this form of sonoporation is less orderly as the fluctuating cell can tear, creating an opening that is no longer selective to small molecules

such as sonosensitizers. The type and level of cavitation activity can actually be identified by frequency outputs as broadband energy corresponds to inertial cavitation and ultraharmonic energy corresponding to stable cavitation [10]. The presence of peaks around 0.5 and 1.5MHz are indicators of substantial microbubble oscillations and the broadband signal across a range of frequencies is associated with inertial cavitation, the hallmark of violent microbubble oscillations [3]. This information is critical for clinical applications as it suggests ultrasound can be monitored and effectively controlled to produce the desired cavitation form.

Inertial cavitation has also been shown to preferentially damage cells based on size. This was elucidated by a study that created size differentials in erythrocytes through HIV infection [3]. Cells exposed to HIV either retained their normal proportions (normocytic) or became grossly enlarged in size (macrocytic). Subsequent results indicated that cells with a smaller volume required a greater amount of shear force to induce lysis. This suggests that larger cells are more sensitive to ultrasonic disturbance and can be preferentially lysed by controlling intensity. The preferential destruction of larger cells was shown to be unrelated to HIV infection as the overall sensitivity of normocytic erythrocytes was no different than normal, healthy cells. This insight is invaluable for the treatment of mobile cancer cells such as leukemias or metastatic fragments as malignant cells are typically larger than erythrocytes and leukocytes found in the bloodstream. If appropriately administered, sonication of large areas could target malignant cells not associated with a large tumor mass.

Inertial cavitation also exerts effects on surrounding vascular tissue used by tumors to satisfy their increased metabolic rates (Fig. 3). Since microbubbles are used as contrast agents for ultrasonic imaging to observe organs as well as the vessels themselves, it seems logical that such structures could exert the effects of inertial cavitation on the vasculature connected to the tumor

[6]. At sufficiently high intensities, ultrasound has been proven to induce significant vascular damage, shutting down blood flow to the tumor [10, 16]. Other research has found that ultrasound disrupts the already uneven distribution of oxygen and nutrients delivered to the tumor through the connecting vasculature as the vessels develop and harbor hypoxic regions. As a result, many developing vessels fail to mature, depriving the tumor of nutrients and inducing apoptosis [6]. Seeing that angiogenesis, the process by which the existing vascular network expands to form new blood vessels is inherently required for the growth of solid tumors, specifically targeting these structures through applied ultrasound could significantly inhibit neoplastic development.

The generation of inertial cavitation is essential for ultrasonic irradiation to lyse malignant cells. Finding ways to promote the phenomenon in cells would be of extreme benefit to SDT as it would allow clinicians to induce the damaging effects of inertial cavitation through relatively precise means. It turns out that pulse dosing may hold the key to effective inertial cavitation treatments. High PRF treatments have been shown to generate significant cell lysis of erythrocytes *in vitro* [10]. The study further suggested that as pulse duration increases, the number of potential cavitation nuclei generated increases. The amount of inertial cavitation activity and level of hemolysis produced were found to be dependent on the peak negative acoustic pressure level as well as the pulse length and PRF. When constant acoustic energy was delivered, ultrasound exposures with longer pulse length or high PRF generated more microbubbles. This was due to the fact that as pulse duration increased, the number of potential cavitation nuclei generated by ultrasound increased. The longer pulses generated more and larger bubbles that could serve as nuclei than did short pulses, allowing more vigorous inertial cavitation activity which in turn generated more microbubbles that increased in size due to the

aggregation of nuclei. The explosive increase of generated microbubbles known as the cascade effect was most frequently observed when long pulse lengths or high PRF conditions were applied. Such concepts can be used to facilitate and then amplify the effects of inertial cavitation, providing yet another opportunity to selectively lyse malignant cells.

Effects on the Cytoskeleton

Aberrant cytoskeletal structure is perhaps one of the most defining characteristics of neoplastic cells. While SDT can use agents to specifically target such structures, ultrasound alone induces a variety of effects on the cytoskeleton. The most noticeable of these alterations is the fluidization and resolidification of the cytoskeletal network. When the cytoskeleton becomes stressed by outside forces such as those generated by ultrasonic irradiation, filaments that maintain the integrity of the structure become stretched [17]. There is an acute response to such stretches as the cytoskeleton can stiffen, increase traction forces and reinforce the stressed filaments or soften and fluidize to reduce strain on the filaments. When the filaments become fluid in nature, the cytoskeleton will often reorganize and then resolidify when the stress has been removed. The fluidization response is therefore accompanied by a dramatic, but reversible acceleration in the rate of cytoskeletal remodeling. Such structural remodeling is typically mediated by events at the levels of signaling or energy metabolism, but also can be mediated mechanically by direct application of physical forces such as shear or tensile stress [17]. The extent of fluidization depends on the amount of stress applied to cytoskeletal filaments and offers a promising opportunity to undermine the altered structures found in malignant cells.

The reorganization of filaments would ultimately suggest rapid depolymerization of actin subunits as they are a major component of the cytoskeleton. This has been shown to be the case when cells are exposed to ultrasound. When subjected to a higher acoustic intensity, the actin

network is progressively disrupted and disassembles within 3 minutes following exposure [17]. Short exposure to low intensity pulsed ultrasound (LIPUS) can drive this reorganization through fluidization followed by slow recovery in which filaments resolidify to reform the network. While microtubules were not examined in the study, it is likely that they are undertaking a similar process when considerable stress is applied as they have an analogous polymerization and depolymerization mechanism that can result in dramatic reorganization of subunits in a very short period of time. The instability of fluidization opens up the opportunity for cytoskeletal directed sonosensitizers as these drugs could exert a dramatic effect on filaments reorganizing within a malignant cell. There are numerous cytoskeleton agents available that prevent polymerization as well as depolymerization of actin filaments and microtubules. For example, paclitaxel (taxol) stabilizes microtubules in order to inhibit depolymerization, interfering with the normal breakdown of tubulin subunits during cell division. Conversely, colchicine inhibits microtubule polymerization by binding to tubulin subunits. If there was a method by which paclitaxel and colchicine could be selectively taken in by malignant cells during ultrasound, the effects of these agents combined with increased cytoskeletal fluidity might destroy the cell's ability to form a microtubule network. This would effectively neutralize the inherent uncontrolled proliferation cancers use for rapid development, allowing sustained treatments to prevent further tumor growth.

Ultrasound Induced Apoptosis

Apoptosis is a form of programmed cell death that normal cells undergo when they are no longer required or have been severely mutated, resulting in abnormal characteristics that would be harmful if passed onto subsequent generations. Cells in the process of apoptosis are generally characterized by organized cytoskeletal shrinkage, chromatin condensation, inner nucleosomal

DNA fragmentation and caspase activation [6, 15, 19, 21]. The self-destruct mechanism is often induced by actions of mitochondria which have the capability to activate caspases by releasing cytochrome-c and other caspase activators. This is often spurred on by the stimulation of various cell death triggers such as increased cellular Ca^{2+} or oxidant concentrations, activation of Bax proteins and increased ceramide production which is known to have significant apoptotic effects in malignant cells [19, 20, 22]. Further, the dissipation of electrochemical gradients found within mitochondria has been shown to induce disruption of cristae organization and inhibition of mitochondrial fusion leading to mitochondrial fragmentation [19, 23]. This provides an opportunity to significantly inhibit tumor growth as malignant cells are known to have increased levels of metabolism. Destroying the mitochondria found within these cells would undermine their ability to satisfy increased energy requirements, ultimately resulting in apoptosis.

Although neoplastic cells typically develop mechanisms to evade apoptosis, ultrasonic irradiation has the capability of inducing this natural cellular response in the presence of aberrant changes. Ultrasound has been shown to influence the genes relating to apoptosis. This results from the fact that there are two main apoptotic pathways; the extrinsic (receptor mediated) and the intrinsic (mitochondria mediated). While the extrinsic pathway would require sonosensitizers to interact with receptors of interest, the intrinsic pathway of apoptosis can be triggered by both internal and external stimuli, including ultrasound [6]. The most representative regulators of the mitochondria mediated pathway are P53, an inducer of apoptosis and Bcl-2, a molecule that suppresses apoptotic activation. Studies involving the effects of ultrasound on neoplastic cell viability have found that both P53 and Bcl-2 are impacted by applied treatments, subsequently inducing increased rates of apoptosis [6]. Cell signaling pathways also play an important role in the mitochondria mediated apoptosis of cancer cells. The mitochondria-caspase signaling

pathway has been shown to be activated in ultrasound induced apoptosis as treatments promote the expression of pro-apoptotic proteins such as Bax which increases the mitochondria's outer membrane permeability, resulting in the activation of caspases [6].

There has been extensive research examining the use of ultrasound to treat various forms of leukemia as the modality has been shown to be very effective against unattached malignant cells. Consequently, the mechanisms by which ultrasonic irradiation induces apoptosis in leukemia cells have been well studied and several patterns have emerged that could bring forth the application of novel sonosensitizers to specifically enhance such cellular responses. In leukemia, poorly differentiated leukocytes begin to overcrowd normal healthy functional leukocytes, erythrocytes and thrombocytes. The vasculature can become saturated with these aberrant cells, eventually compromising the immune system, blood clotting and erythrocyte transport [2]. Leukemia cells originate from hematopoietic stem cells that also give rise to erythrocytes and thrombocytes and overtake the stock of healthy undifferentiated cells so that other blood cells are unable to be produced in sufficient quantities [2]. Unlike most cancers, leukemia is inherently metastatic as leukocytes are required to move throughout the vascular system and no mutation is required for anchorage independent growth. This is a major reason why leukemias are so commonly found as childhood cancers as less fundamental alterations are required for the development of malignant growths [24].

Ultrasonic treatment induces the characteristic features of apoptosis in leukemia cells such as mitochondrial transmembrane potential disturbances, loss of phosphatidylserine asymmetry, morphological changes and eventually DNA fragmentation [19, 32, 33]. Soon after a treatment is administered, an important decrease of intracellular glutathione level is observed, suggesting that oxidative stress plays a hand in ultrasound induced apoptosis. Loss of glutathione

typically results in oxidative damage and has been suggested to constitute early signaling events in apoptotic cell death. Under the exact same conditions, healthy leukocytes as well as erythrocytes are much less sensitive to ultrasound than leukemia cells. This difference in behavior between healthy and malignant cells is most likely due to modifications of fundamental cell mechanisms such as p53 regulation, signaling pathways and resistance to oxidative stress which alter components of apoptosis [19].

While individual leukemia cell lines have inherently unique characteristics due to variable mutations, different lineages have been shown to have similar, if not the same mechanism of apoptosis derived from ultrasonic treatment. Such is the case when both U937 and K562 cells are sonicated *in vitro*. U937 cells have Ca^{2+}/Mg^{2+}-dependent endonucleases and increased intracellular calcium ion concentration plays a major role in apoptosis [19]. When Ca^{2+} concentrations are examined immediately after sonication, there is a transient and heterogeneous increase in Ca^{2+}, apparently due to an influx from the extracellular environment (Fig. 4). Cells treated with calcium ion chelators such as BAPTA-AM have almost no sign of DNA fragmentation and loss of mitochondria membrane potential is partially inhibited [19]. Such results indicate that ultrasound causes a transient increase in Ca^{2+} that is directly correlated with DNA fragmentation and partially affects mitochondrial function. As with most leukemia variants, U937 cells also display a loss of glutathione concentration when exposed to ultrasound. This notion is supported by the observation that cells treated with N-acetylcysteine (NAC) have DNA fragmentation and caspase-3 activation inhibited after ultrasonic irradiation. NAC is known to act as a glutathione precursor as it is readily deacetylated in cells to yield cysteine which is the rate limiting amino acid in glutathione synthesis [19]. Cells treated with NAC

therefore recuperate the losses of glutathione brought on by ultrasound and are significantly less susceptible to apoptosis.

The mitochondria induced apoptosis pathway is one of the fundamental mechanisms by which U937 cells are eradicated through ultrasound. After a successful ultrasound treatment, Bax often undergoes a conformational shift and becomes integrated into the mitochondrial membrane. The subsequent activation of caspases includes caspase-3 which is responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [17, 28] (Fig. 5). Losing such a vital DNA repair mechanism often signals apoptosis in malignant cells as other proofreading enzymes have been lost to prior mutations, giving the cell no way to correct the resulting deformations. Experiments have indicated that cleaved PARP fragments are often found within sonicated U937 cells, supporting caspase-3 activation and a subsequent apoptotic response [17, 19]. As expected, caspase-3 and PARP genes' mRNA levels are also significantly increased, further supporting apoptosis through the mitochondria pathway [17] (Fig. 6).

Ultrasound also appears to have considerable control over apoptotic gene regulation in U937 cells. One study in particular exposed U937 cells to the frequency of 1 MHz with 100 Hz pulse repetition frequency ultrasound [6]. Analysis of gene expression within these sonicated cells suggested that ultrasound could induce apoptosis by down regulating 193 genes and up regulating 201 genes. Most down regulated genes were associated with cellular growth and proliferation, gene expression, or cellular development, while up regulated genes were associated with cellular movement, cell morphology and cell death. Such a discovery sheds light on the truly remarkable impact ultrasonic waves have on malignant cells and provides a tremendous amount of potential targets for sonosensitizer development.

While sonicated K562 cells follow the same mitochondria dependent pathway as U937 cells, one study uncovered even more information regarding inhibition of leukemias [19]. Along with having cleaved PARP fragments, K562 cells were annexin V positive, suggesting the expression of phosphatidylserine on the cell surface. Caspases other than caspase-3 were also activated, resulting in the fragmentation of actin filaments as well as gelosin, an actin binding protein that regulates actin filament assembly and disassembly. Since ultrasound is known to promote cytoskeleton fluidization that results in the rearrangement of such filaments, the combination of both events should have a severe impact on K562 cell colonies. Although unrelated to apoptosis, the study also discovered that ultrasound significantly decreased the ability of K562 cells to multiply and form a colony which is the foundation of cell viability. In contrast, normal hematopoietic stem cells had cloning efficiency completely unaffected by ultrasound even after successive treatments, further substantiating the idea of preferential damage. The combined effect of apoptosis and reduction in viability indicates even more mechanisms by which ultrasound can damage malignant cells, further substantiating its validity as an effective treatment modality.

Reactive Oxygen Species

Malignant cells are notoriously resilient entities that are capable of adapting to multiple forms of cancer therapy, resulting in a tumor resistant to almost all forms of known treatment. This is not surprising due to the shear heterogeneity of cells that populate a tumor. While a treatment may be extremely effective against the majority of cells within the tumor, there can often be a few exceptions that survive. These cells are then free to repopulate the tumor, now filled with resistant cells incapable of being affected by the previous treatment that appeared to work so well before. Treatment modalities therefore need to attack cells using multiple

mechanisms simultaneously in order to increase the likelihood that any trace of the malignancy is successfully eradicated.

The inertial cavitation produced within cells during ultrasonic irradiation is not limited to its capability of undermining cellular integrity through physical stress. Inertial cavities that grow to near resonance size often expand to a maximum before violently collapsing. The extensive amount of energy released by the imploding cavities produces temperature and pressure in excess of 5000K and 800atm [5]. These extreme conditions may induce a series of chemical reactions within and surrounding the collapsed microbubble, including a concentration of energy sufficient to generate light, known as sonoluminescence [5]. When appropriate endogenous molecules as well as some forms of sonosensitizers are exposed to the sonoluminescent light, the compounds are activated from their ground state into an excited state. As the activated compound returns to the ground state, the energy released can generate reactive oxygen species (ROS) which mediate cellular toxicity directly [7, 29, 30]. When enough ROS have been generated, the cell will activate a cascade of events that ultimately results in apoptosis (Fig. 7). Along with singlet oxygen, various other free radicals can be generated through sonoluminescence. Inertial cavitation is such a violent process, it can result in pyrolysis of water vapor inside the microbubble, generating the very reactive hydroxyl radical as well as a hydrogen atom [5]. The induced cavitation also produces hydroxyl radicals through Fenton's reaction. The net result is a simultaneous oxidation and reduction of hydrogen peroxide that creates 2 different oxygen radical species with water as a byproduct.

The reaction sequence is given below:

1) $Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + HO\bullet + H_2O$

2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$

Normally, a Fenton reaction might be limited in biological tissues by the low availability of free iron. Therefore, it has been suggested that exposure to ultrasound generates superoxide radical ions that augment the release of iron from ferritin, providing a pool of active Fe^{2+} to catalyze the Fenton reaction [5].

Electronic excitation during ultrasound through sonoluminescent light has indeed been indicated to activate sonosensitizers that generate the highly reactive singlet molecular oxygen [5, 6]. However, such reactions have also been indicated to occur in leukemia cells without the addition of a sonosensitizer. Several reports have suggested that inertial cavitation induces single-strand breaks in DNA by the action of residual hydrogen peroxide [15]. Ultrasound is also known to generate active oxygenated species that result in a significant reduction of intracellular thiol levels. This is significant as endogenous thiols are integral to the buffering of intracellular ROS levels. Loss of such compounds will result in a dramatic elevation of ROS, bringing forth apoptotic signaling [31].

There was even a study that specifically examined the effects of intracellular ROS on multiple leukemia cell lines (K562, HL-60, KG1a, and Nalm-6) after being treated with ultrasound [15] (Fig. 8). In the procedure that was described, ultrasound at low energy was used to induce apoptosis specifically in leukemic cells without the use of any sonosensitizer. Data obtained in the presence of histidine, a quencher of oxygen, suggest the importance of singlet oxygen in the induction of apoptosis under the low energy conditions as malignant cells survived much more frequently under these conditions. Mannitol, an inhibitor of hydroxyl radicals also protected against ultrasound induced apoptosis, implying the generation of such radicals. These observations inherently suggest a sonochemical mechanism. In fact, the combination of histidine and mannitol resulted in more than 60% inhibition of apoptosis, providing convincing evidence

of the importance free radicals have in ultrasound induced apoptosis for leukemia cells. The study was conducted at low energy to prove that the ROS generated were indeed coming from within the cells and not due to solvent interactions with ultrasonic waves. At the chosen frequency and intensity (1.8MHz, 0.22W/cm^2), ultrasound does not directly generate free radicals from the sonolysis of the solvent. Therefore, increased ROS levels are being generated within the cells from endogenous photoabsorbing molecules such as porphyrins and flavoproteins. Implications of endogenous porphyrins in photodynamic DNA damage have shown to be a viable mechanism and helps explain how intracellular compounds generate such a cytotoxic effect.

With high levels of ROS already being generated by leukemia cells after sonication, applying sonosensitizers through SDT which amplify such levels could produce complete destruction of even the most resistant cells. Indeed, ultrasonic irradiation has shown the capability of making drug resistant cancer cells more sensitive to anticancer drugs, providing a noninvasive physical approach for chemo-drug resistance reversal [6, 25]. Through the use of RT-PCR on HepG2/ADM cells (hepatocellular carcinoma cells), it has been found that ultrasound could significantly down regulate the expression of P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) at the mRNA level, while producing excess ROS levels. The study not only confirmed that ultrasonic irradiation could reverse the chemotherapeutic resistance of cancer cells, but also found its mechanism [6]. Such results provide hope that once deadly drug resistant tumors will eventually become successfully treated after SDT is properly developed.

Chapter IV: Unique Advantages of Ultrasound

Virotherapy

The selective uptake of sonosensitizers by malignant cells is a fundamental concept of SDT that must be accomplished for the treatment modality to be successful. Since some of the sonosensitizer might be taken in by normal cells as well, it is also very important to increase the efficiency of drug uptake so that lower doses can be administered. This will decrease any adverse effects that applied treatments could have on patients, resulting in healthier hosts that are more capable of fighting off the malignancy. While sonoporation induced naturally by ultrasound could be accentuated with various drugs, another option has been investigated that shows signs of promise.

Although viruses are more known for their ability to induce tumors such as the notorious human papillomavirus (HPV), there has been extensive research on the idea of using adenoviruses to increase membrane permeability. Clathrin mediated endocytosis is a critical component of adenovirus transduction as direct injection of the virus into the cytoplasm has actually been shown to result in less transduction than cellular incubation with the virus in the extracellular environment [19]. When ultrasound is applied in conjunction with adenovirus infection, transduction rates increase significantly as many more viral capsids are found in the cytoplasm after treatment [12, 19]. Closer examination of the cells using transmission electron microscopy (TEM) revealed that ultrasound treatments stimulated formation of both clathrin coated pits as well as other pits not associated with the protein. This indicates that sonication stimulates clathrin mediated endocytosis as well as other endocytic pathways [19]. The enhancement of clathrin mediated endocytosis has been further substantiated by an experiment in which HeLa cells (cervical cancer) were treated with chlorpromazine (CPZ), a known inhibitor

of clathrin pits in the plasma membrane. Treatment with CPZ significantly reduced the levels of adenovirus transduction in cells even after ultrasonic irradiation was applied [19]. Cells that are treated with ultrasound have a substantially higher amount of clathrin coated pits that are increasingly perpetuated when adenovirus infection is present. Sonosensitizers that are able to enter cells through such pits would be able to take advantage of the increase in openings, resulting in a tremendously high drug efficiency rate.

Besides increasing membrane permeability, viruses have also been investigated for their ability to preferentially infect and destroy tumor growths. Oncolytic viruses which have the capability of selectively self-amplifying within cancer cells based on extracellular receptors have seen clinical use, but have been restricted by limited delivery from the bloodstream into the tumor. The chaotic nature of tumor associated vessels due to facilitated angiogenesis results in a highly heterogeneous network, leaving many regions poorly perfused. Such heterogeneity makes the delivery of oncolytic viruses a non-uniform process that is often hard to predict [13]. This suggests an improved infection mechanism needs to be developed before oncolytic viruses are considered a viable treatment method. It just so happens that microbubbles under duress from inertial cavitation provide such a pathway. When a study used high pressure (1.2MPa) ultrasound with microbubbles to instigate inertial cavitation, delivery of the oncolytic virus into tumorigenic mice dramatically increased [13]. Every single ultrasound treated tumor expressed higher oncolytic virus levels than the non-ultrasound control growths. By slightly altering the way in which the virus was introduced to malignant tissue, the infection efficacy was significantly improved, providing a potential solution to the flaws of traditional viral transmission.

Variable Effects of Ultrasound

With any new potential cancer therapy, one of the most fundamental questions is whether the treatment will exert the same cytotoxic effects on healthy cells. Ultrasound has been shown to preferentially damage malignant cells as indicated by the reduction in leukemia cell count, while hematopoietic stem cells remained unaffected [15]. However, most treatment modalities are designed for more than a single form of cancer and SDT needs to be versatile enough for use on other malignancies. Various studies have already indicated the positive effects ultrasound can have on normal cells. It has been demonstrated that ultrasound promotes osteogenesis, protein synthesis and calcium uptake. Further, the induction of DNA synthesis has also been shown as ultrasound promotes DNA synthesis in human osteoblast, gingival fibroblast and periosteal cells [10]. Such qualities suggest potential therapeutic benefits in patients who have had important cell types depleted due to chemotherapy.

The extent of preferential damage on cancer cells was exemplified in a study that investigated the variable effects ultrasonic irradiation had on different cell types [10]. The study used a human cardiac microvascular endothelial cell line (hcMEC) and a canine kidney epithelial cell line (MDCK) for normal cell representatives, while a neuroblastoma (Neuro2A) and an adenocarcinoma cell line (HT29) were chosen to investigate effects on malignant cells [10]. The results indicated that ultrasound has a profound effect on cell proliferation rate that is dependent on the cell line. Sonication at a medium energy density ($25 Ws/cm^2$) resulted in a significant increase in the proliferation rate of hcMEC and MDCK. However, the exact same treatment dramatically decreased proliferation in both Neuro2A and HT29, suggesting that ultrasound has the capability to preferentially damage malignant cells based on proliferation reduction. Seeing that one of the fundamental tenets of cancer is uncontrolled cell proliferation, such a discovery is

truly remarkable. However, it should be noted that the beneficial effects of ultrasound are reversed when extremely high energy densities are applied. The application of 600Ws/cm^2 was actually shown to have a positive proliferative effect on Neuro2A as well as HT29 [10]. Such results are troubling as they completely contradict the goal of preferential damage and suggest that optimal conditions are not simply the highest ultrasound intensities.

Perhaps one of the most profound discoveries of the study was the capability to partially induce differentiation in Neuro2A cells. When ultrasound was applied, the expression of neurofilament was increased in neuroblastoma cells, evidence of beginning differentiation. This remarkable shift towards differentiation was found at each energy density that was examined in the study. Such findings indicate that the differentiation of Neuro2A can be enhanced by ultrasound, but only in terms of neurofilament expression. Further differentiation steps like neurite outgrowth were not affected under the specific protocol, but might occur when more suitable protocols such as incorporating pulse dosing are used [10]. Differentiated Neuro2A cells would lose their capability of uncontrolled cell proliferation, effectively neutralizing tumorigenic growth.

Chapter V: Combining Ultrasound with Chemotherapeutic Agents

Sonosensitizers

While the beneficial effects of ultrasound are truly immense, they are only half of the story in SDT. Based on the known mechanisms of preferential damage induced by ultrasound, sonosensitizers have either been developed or selected from the diverse array of chemotherapeutic agents that already exist. Although using a combination of various sonosensitizers may yield the most effective treatments, studies have typically only focused on a

particular class of drug. The results of such studies have given significant credibility to the idea of using sonosensitizers to maximize the preferential damage of ultrasonic irradiation.

Known Chemotherapeutic Agents

Although often initially successful, many known chemotherapeutic agents run into the issue of drug resistance when a few unaffected cells end up repopulating a tumor. Increasing the efficacy of these proven cytotoxic drugs would be a significant discovery in its own right and could significantly increase the rate at which successful treatments are attained. Ultrasound already has the immediate benefit of sonoporation which would allow smaller doses of the same drug to be administered for the same net effect. The smaller doses would result in fewer side effects for patients who are forced to endure such grueling regiments. However, it has also been shown that sonication actually increases the overall effectiveness of the drug.

Doxorubicin (DOX), also referred to as Adriamycin is an anthracycline antibiotic that works by inserting itself between base pairs, thereby intercalating DNA. The drug is commonly used in the treatment of a wide range of cancers, including hematological malignancies, carcinomas and soft tissue sarcomas. As with most chemotherapeutic agents, it faces the same issues with drug resistant tumors. However, a study that incorporated DOX as a sonosensitizer yielded substantial results [2] (Fig. 9). Ultrasonic irradiation combined with DOX significantly increased its efficacy on the human leukemia multidrug resistant cell line K562/A02, indicating that sonication of the given parameters (20 kHz, 0.25W/cm^2, 60s intervals) can significantly increase DOX concentration within malignant cells to fortify the destructive effect. Such effects were derived from a cell line shown to be resistant to both ultrasound and DOX-alone control treatments, further substantiating the amplifying effect sonosensitizers have in SDT. There has been a similar effect when ultrasound/DOX treatments are applied to U937 cells, suggesting the agent

can be effective against multiple leukemia cell lines when used in tandem with ultrasonic irradiation [34]. The study also indicated that DOX was damaging the cells through increases in ROS content, indicating an additional mechanism by which the agent can damage malignant cells (Fig. 10).

Other studies designed around the potential synergistic effects of chemotherapeutic drugs combined with ultrasound include the use of alkylating agents. Often a clinical favorite for neoplastic growth inhibition, alkylating agents attach an alkyl group to guanine nucleotides in DNA, subsequently resulting in apoptosis. However, such agents are also toxic to normal cells, resulting in substantial damage to cells that divide frequently and methods to increase the efficiency of drug uptake is critical to decrease side effects in patients. It is known that hyperthermia is especially effective at enhancing the effects of alkylating agents [35, 36]. The temperature increases needed to induce hyperthermia can be locally concentrated by using sustained ultrasound of moderate intensities, allowing for significant increases in drug efficacy. Such treatments would also provide the additional benefit of increased epithelial cell growth (as shown with MDCK cells) to help patients replenish labile cells found in the gastrointestinal tract that are lost after treatment with alkylating agents [10]. Studies so far have shown promise for both cisplatin and diaziquone which show increased cytotoxicity to multiple cancer types in the presence of ultrasonic irradiation [5]. Such results offer hope that a refined SDT approach could be used to enhance the efficacy of known chemotherapeutic agents, decreasing the frequency of drug resistance found in clinical practice.

Reactive Oxygen Species Agents

The ability of inertial cavitation to generate ROS provides a wonderful opportunity to amplify apoptosis in malignant cells. Ultrasound generated sonoluminescence causes electronic

excitation of many compounds such as porphyrins through energy transfer, initiating a photochemical process. The compound is then converted into the formation of cytotoxic free radicals such as singlet oxygen. These ROS accumulate within the cytoplasm and organelles, damaging lipids, proteins and DNA. This deterioration of cellular organization is coupled with the additional factors of mitochondrial dysfunction, ion balance deregulation and loss of membrane integrity, culminating in apoptosis of affected cells [37, 38]. In contrast to most anticancer drugs, porphyrins and other ROS agents are nontoxic in the absence of ultrasound as many are naturally found within cells [5]. Preferential uptake of the sonosensitizer by the tumor, followed by ultrasonic irradiation will therefore provide an effective treatment with minimal side effects.

One sonosensitizer in particular shows promise for treating patients afflicted with leukemia. HMME (Hematoporphyrin monomethyl ether) is a porphyrin derived sonosensitizer that has been developed in efforts to substantiate SDT as a viable treatment modality for clinical applications [27, 39]. As with all porphyrins, HMME is a heterocyclic macrocycle (compound containing a ring of nine or more atoms that can coordinate to a metal center) composed of 4 modified pyrrole subunits interconnected at their α carbon atoms via methine bridges. Due to their unique structure, porphyrins are aromatic as they obey Hückel's rule and are highly conjugated systems, indicating that such compounds are extremely stable. Multiple studies have shown that HMME has a higher selective uptake by tumor tissue as well as a more pronounced cytotoxic effect when combined with ultrasonic irradiation than other porphyrin related agents [27]. Such selectivity and stability is important for *in vivo* applications as sonosensitizers will be exposed to a highly variable molecular environment. HMME is also known to trigger rapid dissipation of electrochemical gradients when exposed to sonoluminescent light, indicating the

interruption of oxidative phosphorylation. Loss of electrochemical gradients is vital for the destruction of mitochondria as it invokes cristae fragmentation, removing any sort of integrity from the inner membrane. Cancer cells unable to carry out respiration will subsequently activate apoptosis as it becomes impossible to support their increased metabolic rates.

HMME has been used for multiple cancer cell lines and has shown commendable efficacy, particularly in a study that involved U937 cells [27]. Immediately after administration, intracellular HMME concentrations rapidly increased within the U937 cells, reflecting its high affinity for malignant tissue. The treated cells were incubated for 4 hours to assess the cytotoxicity of HMME-mediated SDT. As expected, there was not any cell toxicity in the HMME-alone group and ultrasound-alone only caused slight cell damage to the U937 cell population. However, the synergistic effect of ultrasound (1.1 MHz, 1W/cm^2, 60s intervals) with HMME showed significant cell destruction, vindicating the necessity of sonosensitizers in ultrasound mediated therapy (Fig. 11). Flow cytometry with DCFH-DA staining confirmed that HMME-ultrasound treated cells had markedly increased ROS levels compared with the control, HMME and ultrasound-alone groups. Further analysis of damaged cell populations revealed that oxidative stress was present and that cells had indeed undergone apoptosis. These results not only confirm the linkage between ROS and apoptosis within U937 cells, but ultimately suggest a novel approach to treat leukemia patients. This damage can be further enhanced with the use of DOX in collaboration with ultrasound/HMME as a study with QBC939 cells (leukemia) has indicated [40]. The study indicated that the ultrasound/DOX /HMME group had a higher reduction in cell viability than either the ultrasound/DOX or ultrasound/HMME groups, suggesting the need to investigate the cumulative effect of multiple sonosensitizers (DOX was shown to increase ROS content in [20]).

Analysis of other ROS agents has produced similar results. The sonodynamically induced effects of a chlorin derivative, ATX-S10 (4-formyloximethylidene-3-hydroxy-2-vinyldeuterio-porphynyl (IX)-6,7-diaspartic acid) has a substantially longer sonoluminescent lifetime than other porphyrin agents, providing more opportunity to generate singlet oxygen [5]. The co-administration of ATX-S10 with ultrasonic exposure (2 MHz) stopped the growth of implanted colon-26 cells in mice when ultrasound alone showed only a slight antitumor effect. Cellular uptake of ATX-S10 localized within the mitochondria and resulted in significant dysfunction following activation with ultrasound. The sonosensitizer triggered rapid dissipation of electrochemical gradients, indicating impairment of mitochondrial respiration and ultimately suggests interruption of oxidative phosphorylation [5]. Loss of electrochemical gradients is vital for the destruction of mitochondria as it invokes cristae fragmentation, removing any sort of integrity from the inner membrane. Cancer cells unable to carry out respiration will subsequently activate apoptosis as it becomes impossible to support their increased metabolic rates.

The possibilities for ROS agents are truly endless as sonosensitizers have been developed to generate extremely high ROS content within cells. Indeed, porphyrin agents such as ATX-S10, have substantial sonoluminescent lifetimes, providing more opportunity to generate singlet oxygen [5]. Hydroxyl radicals are another ROS of interest as they have been shown to damage virtually all types of macromolecules including carbohydrates, nucleic acids (causing mutations), lipids (peroxidation) and amino acids. In fact, lipid peroxidation by itself gives way to a whole other class of ROS agents as hydroxyl radicals absorb electrons from lipids to obtain a stable octet, causing oxidative degradation that ultimately compromises the plasma membrane [41, 42]. Hydroxyl radicals have been shown to be produced *in vitro* as Ehrlich ascitic tumor (EAT) cells had markedly increased levels after exposure to ultrasonic irradiation (2.17 MHz, 3 W/cm^2,30

and 60s) [41]. This demonstrates yet another ROS by which ultrasonic irradiation can damage malignant cells. Dissolution of membrane integrity (hydroxyl radicals) combined with the destruction of mitochondria (singlet oxygen) would give malignant cells populating a tumor very little opportunity of acquiring drug resistance and those that are capable of surviving can be eradicated by using a collection of sonosensitizers that cause damage through other mechanisms.

Cytoskeleton Agents

By default, malignant cells have a perturbed cytoskeleton due to the effects of dysplasia and subsequent anaplasia. Dysplasia refers to the increase of immature cells within a given area of tissue that reflects a corresponding decrease in the number and location of mature cells. This transformation consequently produces the following pathological abnormalities: anisocytosis(cells of unequal size), poikilocytosis (abnormally shaped cells) hyperchromatism (excessive pigmentation) and presence of mitotic figures (an unusual number of cells which are currently dividing) [43]. Each of these aberrant characteristics inherently suggests a cytoskeletal alteration that must have been due to the fundamental change and reorganization of appropriate filaments. This reversion to an undifferentiated cell form is furthered by anaplasia which is characterized by pleomorphism (variability in the size, shape and staining of cells as well as nuclei) [43]. Such alterations produce a whole host of abnormalities to the cell, officially changing it to a malignant state. Nuclei become characteristically hyperchromatic and enlarged. Giant cells that are considerably larger than their neighbors may be formed and possess either one enormous nucleus or several nuclei known as syncytia. The chromatin eventually clumps together, producing nucleoli of incredible size. While these alterations are only partially due to abnormal filament structure, mitosis which is entirely dependent on the cytoskeleton is distinctly atypical. Not only does the rate of mitosis significantly increase, anarchic spindles with multiple

polarities are often seen [43]. Anaplastic cells also usually fail to develop recognizable patterns of orientation to one another as they lose normal polarity, a direct consequence of filament aberrancy.

With so many alterations present in malignant cells, the cytoskeleton provides an ideal opportunity for preferential damage. With so many alterations present in malignant cells, the cytoskeleton provides an ideal opportunity for preferential damage. Ultrasonic waves already show a tremendous capability of inducing cytoskeletal fluidization that ultimately results in filament reorganization [17]. Drugs that enhance this effect could cause malignant cells to cycle through states of aggravated fluidization, culminating in a complete loss of cytoskeletal integrity. The study that investigated the effects of ultrasound-alone treatments on filament rearrangement also examined its effects combined with known cytoskeleton agents, histamine and cytochalasin D. As expected, histamine treated cells became stiffer with a higher density of actin–myosin bonds. This is due to the fact that histamine is released naturally from the immune system during detection of known pathogens, causing an inflammatory response that induces muscle (actin and myosin) stiffness [17]. In such cells, the same acoustic pressure (170, 290kPa) applied to untreated cells supplied less energy per single bond resulting in a smaller increase in remodeling rates. Conversely, cells treated with cytochalasin D, a known inhibitor of actin polymerization, had a lower density of actin–myosin bonds. These cells attained higher energies per bond, resulting in a larger increase in the rate of structural rearrangement. Ultrasound induced alterations in cytoskeletal structure are clearly enhanced when cytochalasin D is applied, providing a novel avenue to generate preferential cell damage.

Docetaxel (taxotere) is a taxane cytoskeleton agent that has shown a synergistic effect with ultrasonic irradiation. As with its relative paclitaxel (taxol), the principal mechanism of

action is the disruption of microtubule function. Microtubules are essential for mitosis and taxanes stabilize GDP-bound tubulin polymers, thereby inhibiting cell division. Docetaxel was paired with ultrasound in an *in vivo* study that hoped to inhibit the growth of PC3 (human prostate cancer) tumors in athymic mice [12]. Subsequent results demonstrated a pronounced enhancement of docetaxel antitumor activity through its combination with ultrasound treatments (1 MHz, 50ms bursts (0.00024 duty cycle), 1.65MPa). The combined effects were significantly higher than both the docetaxel and ultrasound-alone groups. Analysis of treated mice revealed the docetaxel-ultrasound combination not only produced significant damage through the typical cytoskeletal mechanism, but induced antivascular effects as well. This discovery could dramatically improve the delivery of docetaxel in a clinical setting as it is recognized that a significant issue limiting the antitumor activity of the drug is post extravasation transport within tumor tissue. By destroying the vascular network associated with the malignancy, the drug will no longer be transported away from the tumor, therefore dramatically increasing efficacy.

Perhaps one of the most intriguing capabilities of ultrasound is its ability to preferentially lyse cells based on size. This known fact invariably gives rise to the idea of grossly enlarging tumor cells to increase their already noticeable size difference with normal cells. Cytochalasin B is a known pharmacological agent that disrupts the actin cytoskeleton and inhibits cytokinesis by interfering with formation of the contractile ring as well as the development of the cleavage furrow [44]. Consequently, the cell does not divide and an immature actin cytoskeleton remains. However, the cell continues to form nuclei and eventually becomes grossly enlarged and multinucleated. Such cells invariably have more DNA targets, increasing the likelihood of apoptosis. Furthermore, the multinucleated cells have a large cell volume, making them more susceptible for direct cell destruction. Preferential damage of malignant cells is actually easily

attainable as normal cells exposed to cytochalasin B exit the cell cycle and enter a resting state until sufficient actin levels are restored. Therefore, only malignant cells that have lost the ability to enter the rest phase will become grossly enlarged and multinucleated, providing an ideal target for ultrasonic irradiation.

The concept of preferential damage induced by cytochalasin B was put to the test in a study that involved sonication of U937 cells [unpublished data]. Sonic sensitivity for U937 cells treated with cytochalasin B was shown to be significant when using enough wattage. The cells often grew to 20µm or greater and were unable to tolerate ultrasound treatments in which normal erythrocytes remained stable. For cells that were 24µm or greater (26% of the total cells), 50% cell destruction was reached at a modest 36 watts in only a single minute of sonication. Contrastingly, all U937 control cells did not show sonic sensitivity for the same given amount of wattage when 1 minute of continuous ultrasound was applied. Such findings further elucidate the significance of sonosensitizers in SDT as ultrasound-alone groups are simply unable to generate the same extent of malignant cell destruction.

Without question, cytoskeleton agents have a clear synergistic effect with ultrasound. However, there is an additional benefit of sonication that may be even more provocative than its effect with known cytoskeleton agents. Both cycloplatin and methotrexate are known anticancer agents that destroy malignant cells through DNA modification. Cycloplatin achieves this by alkylating guanine nucleotides, thereby inhibiting DNA replication, while methotrexate causes competitive inhibition of dihydrofolate reductase, an enzyme that participates in tetrahydrofolate synthesis [45]. Tetrahydrofolate is required for the production of thymidine as well as all purine bases, essentially making it necessary for 3 out of the 4 nucleotides present in DNA.

Clearly, cycloplatin and methotrexate are nucleic acid agents that have targets far away from the cytoskeleton. However, a study that examined the effects of these drugs combined with ultrasonic irradiation indicated an astonishing synergistic effect on the cytoskeleton of HeLa cells (cervical cancer) [45]. Ultrasonic treatment (1.8 MHz, 0.22W/cm^2) with both drugs resulted in the following common features: thinning of actin and microtubule bands (especially at the cell periphery), fragmentation of microtubules with the formation of tubulin granule-like structures and partial loss of stress fibers. The combined effect of ultrasonic irradiation and sonosensitizers intensified all changes and produced a distinct decrease in cell volume accompanied by aggregation of microtubules into thick bundles as well as accumulation of remaining stress fibers in the peripheral regions. Their subsequent faulty repolymerization resulted in development of the observed pathological features. As with all sonosensitizers, the results were much more observable in the drug-ultrasound treated groups and both cycloplatin and methotrexate demonstrated a certain similarity in their effect. The findings of this study suggest that ultrasound can produce novel effects of known anticancer agents, thereby creating an entire new mechanism by which these drugs attack malignant cells. When drugs have multiple methods of attack, the likelihood of drug resistance should be significantly decreased, thereby increasing the overall efficacy of chemotherapeutic treatments.

Vascular Disrupting Agents

With exception to leukemias and other hematological malignancies, primary tumors are inherently dependent on nutrients they receive from available vascular networks. Such tumors are relatively confined to the areas in which they develop and their increased metabolic activity absolutely necessitates a direct supply of blood from the vasculature. Since tumors are initially without this supply, they must create their own through the process of angiogenesis. Without

such a supply, tumors are fundamentally limited to a relatively small size and the likelihood of metastasis is significantly decreased. Vascular Disrupting Agents (VDAs) have therefore been widely acknowledged by the medical community as a potential source of limiting malignant growth [12]. There is also the potential to induce necrosis as significant amounts of the tumor will experience hypoxia due to the decrease in available oxygen and such cells will be unable to survive.

There has been considerable attention paid to 2 types of VDAs in particular as pronounced enhancements of taxanes have been achieved when combined with other tubulin binding agents as well as flavonoid derivatives [12]. Tubulin binding agents used for endothelial cells such as Combretastatin A-4-Phosphate have the remarkable capability of selectively destabilizing the cytoskeleton of proliferating endothelial cells, resulting in cell rounding. This is followed by a cascade of ensuing events such as the exposure of basement membranes, transiently enhanced permeability and erythrocyte extravasation which culminate in a pronounced shutdown of blood flow. Vadimezan is a flavinoid derivative that has a tubulin independent mechanism of action involving direct and indirect antivascular effects. While the biochemical pathways of vadimezan action are not fully understood, endothelial cell apoptosis is known to be induced within 15-30min. This is a consequence of changes in endothelial cell morphology, the exposure of microvascular basement membranes and platelet accumulation. Indirect antivascular activity also occurs, associated with the influx of neutrophils and the upregulation of a range of cytokines, including tumor necrosis factor.

As a monotherapy, VDAs have exhibited only limited effectiveness in achieving sustained antitumor effects. This has been attributed in part to a vascular rebound effect as the outer layers of the malignant growths are less affected by treatments due to their proximity with

the extracellular environment, acting as a site for revascularization and subsequent regrowth [12]. However, when these agents are combined with taxanes (paclitaxel or docetaxel), there is a substantial additive effect towards inhibiting tumor growth. The observed synergy is due to the fact that VDAs and taxanes preferentially damage different areas of the tumor. VDAs have been shown to damage the fragile tumor vasculature, thereby inducing necrosis in the tumor centers where blood supply is already limited. By contrast, taxanes preferentially affect highly proliferating, well perfused tumor rims [12]. Since both the inside and outside layers of malignant tissue are under attack, the tumor is often left significantly debilitated.

Ultrasonic irradiation has been suggested to increase the efficacy for this already potent combination for a simple reason; it enhances the activity of VDAs as well as taxanes. The synergistic effect of sonication and taxanes has already been well established as docetaxel has been shown to increase its cytotoxicity when exposed to ultrasonic waves [12]. VDAs should also display a synergistic effect with ultrasound as both attack tumor vasculature. Collapsing microbubbles remain trapped and subsequently dismantle the endothelial lining, causing thrombopoiesis in the vessels to prevent blood leakage. Elevated thrombocyte levels block the blood supply of the malignant tumor, inducing either apoptosis or necrosis. Other studies have found that ultrasound can facilitate anti-angiogenic gene delivery that has been shown to inhibit prostate tumor vasculature growth *in vitro* and *in vivo* [6]. Required nutrients such glucose and oxygen become unequally delivered through the tumor vasculature and vessels subsequently develop hypoxic regions. The endothelial cells are put under severe oxidative stress and vessels fail to mature, inducing the apoptosis of malignant cells.

With such a profound effect on tumor vasculature, it seems only natural to combine ultrasonic irradiation with potent VDAs to cripple a tumor's ability to sustain rapid cell

proliferation. While this aspect of SDT has gone largely unnoticed, there has been a study that looked at the synergistic effects of microbubbles applied to metronomic chemotherapy, a treatment modality that specifically focuses on preferential endothelial cell destruction in tumor vasculature [46]. The definition was coined due to the repetitive low dosage of VDAs administered (akin to a metronome) in order to target the endothelium or tumor stroma, while retaining low toxicity levels. The study was conducted with MDA-MB-231 cells (breast cancer) implanted in athymic mice. The VDA of choice was metronomic cyclophosphamide (MCTX), a known alkylating agent and was coupled with Definity, a commercial microbubble agent. Ultrasonic irradiation (1 MHz, 0.00024 duty cycle, 1.6MPa) combined with the VDA and microbubble agent to produce impressive results (Fig. 12). The combined Definity-MCTX treatment group showed significant growth inhibition and survival prolongation relative to the ultrasound-only and MCTX-only treatment groups. Such results are promising and hopefully will stimulate further studies to investigate the potential synergistic effect of ultrasound mediated microbubble destruction and VDAs in order to eradicate the vascular network supporting malignant growth.

Echo Contrast Agents

The capability of microbubbles to enhance SDT is truly remarkable as they can be used alone or in tandem with sonosensitizers (VDAs) to wreak havoc on malignant tissue as well as its supporting vasculature. Such damage is typically brought on by inertial cavitation, a violent oscillation pattern that culminates in the collapse of microbubbles, bringing forth damage to cytoskeletal structures as well as generating significant amounts of heat and pressure to generate ROS. While microbubbles are produced naturally by cells during ultrasonic irradiation, the amount can be significantly increased through the use of echo contrast agents. Also known as

ultrasound contrast agents, these drugs were initially designed to improve the quality of diagnostic ultrasound, a procedure that uses frequencies way above SDT requirements. Such agents are injected intravenously into systemic circulation. The microbubbles remain in systemic circulation for a certain period of time and ultrasonic waves are directed towards the area where diagnostic imaging is required. Microbubbles exposed to the high frequencies oscillate and reflect a unique echo that stands in stark contrast to the surrounding tissue due to the difference between microbubble and tissue echoes, allowing blood to be distinguished from surrounding tissue [3]. This procedure uses less intense ultrasonic irradiation as stable cavitation is required for the microbubbles to oscillate without collapsing.

At first glance, echo contrast agents may appear to be relatively harmless drugs that would only benefit cancer treatments through its ability to enhance imaging of located tumors. As mentioned however, diagnostic imaging is done under relatively light conditions in which only stable cavitation is required. Increase the intensity and decrease the frequency of applied ultrasound and a whole assortment of interesting observations are made. In a previously mentioned study designed to find whether sonication could preferentially lyse larger, macrocytic erythrocytes, Albunex, a well-known echo contrast agent was applied to examine its effect on microbubble levels [3]. Gas based ultrasound contrast agents such as Albunex have been shown to increase erythrocyte sonolysis, presumably by enhancing inertial cavitation activity [9]. Subsequent results concluded that macrocytic erythrocytes were damaged by the increased proportion of microbubbles at intensities that left normal erythrocytes intact. When whole human blood is infused with Albunex, microbubbles come into close contact with nearby erythrocytes. In order to expand under the influence of applied ultrasound, each bubble must push against surrounding erythrocytes or whatever fluid-filled space is available between the cells. These

conditions perpetuate asymmetric oscillations known to induce collapsing due to the availability of inrushing fluid not being equal everywhere on the microbubble [3]. The larger cells are, the more likely they are to experience the force of multiple unequal oscillations, putting the cytoskeleton under considerable stress. Subsequent microbubble collapses prove to be too much for enlarged cells, culminating in their destruction.

One echo contrast agent of particular interest for enhancing preferential leukemia cell damage is Levovist, a drug that not only increases microbubble levels, but has also been proven to significantly improve Ca^{2+} influx into U937 cells when ultrasound is applied [15]. Since the induction of apoptosis in leukemia cells is increased when high intracellular Ca^{2+} content is present, the echo contrast agent has the capability of attacking malignant growths through multiple mechanisms. Levovist has the additional benefit of providing nuclei for microbubbles to accumulate during unstable oscillations which in effect increases the blast radius of collapsing microbubbles, thereby enhancing destruction due to inertial cavitation [15].

Due to its unique characteristics, Levovist has been examined in multiple studies to determine whether the drug could be a viable chemotherapeutic agent when combined with ultrasonic irradiation. The ensuing results have been quite promising, especially when Levovist was applied to multiple leukemia cell lines (Jurkat, Molt-4 and U937) in an in vitro study [47]. Levovist was applied to cell lines before being exposed to ultrasonic irradiation (1 MHz, $0.3W/cm^2$, 10% duty factor pulsed at 100Hz). As expected, the results indicated that loss of viability and apoptosis was induced in each cell line with apoptosis being the highest in Molt-4 cells. Further analysis revealed that cells exposed to the Levovist-ultrasound treatment had low mitochondrial membrane potential, high superoxide production, increased intracellular calcium concentration, and phosphorylation of histone H2AX after sonication. Such observations were

seen most frequently and to the highest extent when both Levovist and ultrasound were applied. Each factor is a known contributor of apoptosis and further elucidates the versatility Levovist has in inducing malignant cell destruction.

Significance of Sonosensitizers

The sheer variety of sonosensitizers available to enhance ultrasonic irradiation is truly immense. Such agents can induce preferential damage on malignant cells in mechanisms ranging from increasing ROS content to disrupting the vascular networks of malignant tissue. The sonosensitizers discussed in this report have been grouped and categorized to summarize their modes of attack when combined with ultrasound in order to elucidate the sheer variety of potential treatments (Table 1). While sonosensitizers are grouped by their differences in primary mechanism, many of these drugs have additional methods of attack that are similar or even the same as other types. Therefore, it is likely that a cocktail of sonosensitizers would have a substantial synergistic effect, playing off the strengths of each other by overlapping in methods of attack. Tolerable doses would create a potent anticancer regiment when combined with ultrasonic irradiation as this energy form has been shown to damage cells by virtually identical mechanisms. Such collaborative efforts are necessary for SDT to stand out as a viable clinical approach as it will come across an innumerable variety of neoplastic growths, each packed with different methods of overcoming applied treatments.

Chapter VI: Implementing Sonodynamic Therapy in the Clinical Setting

Comparison to Other Treatment Modalities

The benefits of SDT have been expressed in explicit detail in order to convey its relevance to current techniques used in the clinical setting. However, SDT needs to offer distinct advantages over available treatment options if money and effort are going to be invested into its

development. SDT already offers a clear advantage of providing a synergistic effect with chemotherapeutic agents currently being administered to patients, suggesting that lower dosages can be used to reach the same net result. Such a benefit would be well received by patients as lower dosages can likely reduce aberrant side effects experienced during treatment regimens. However, other physical and chemical treatment methods are already being used in tandem with chemotherapy. Patients often endure rounds of x-ray irradiation or undergo invasive surgery to remove the primary tumor in conjunction with receiving chemotherapy. Further, a similar approach known as photodynamic therapy (PDT) which uses light to stimulate activating agents is already being used in clinical settings to treat various types of skin cancers [49].

While there are indeed multiple adjuvant and neoadjuvant combinations currently used with chemotherapy, SDT offers distinct advantages over such available methods. One of the most common criticisms of x-ray derived treatments is the extent of visible side effects. Patients are often left with reduced hair growth and superficial burns that are difficult to resolve and sometimes never improve [1, 43]. Further, patients often complain of nausea, body aches and a sense of overall fatigue when exposed to successive treatments of radiation therapy. The method also has the less publicized side effect of having the potential to induce other cancers in patients as the use of ionizing radiation can mutate previously healthy cells, providing an opportunity for the acquisition of neoplastic characteristics [43]. SDT avoids these problems altogether as ultrasonic irradiation has not caused any visible side effects in available *in vivo* studies. Ultrasonic irradiation also does not produce the energy required to alter molecular structure through ionization as is the case with radiation therapy, preventing the development of subsequent mutations.

Invasive surgery is often one of the first few steps in cancer treatment as it has the capability to remove an entire tumor within a matter of hours. This modality is extremely beneficial for patients with low grade tumors that have not yet metastasized as the operation effectively removes any trace of cancer cells when done properly. However, when not properly done, there is the chance of artificially spreading the disease as such instances have been reported [24]. Some patients are also unable to undergo surgery as their performance status is too low for such a taxing procedure. Perhaps the most fundamental limitation of surgery is its inability to effectively treat metastatic disease. Removing a primary tumor will not be very beneficial when there are already other tumors in development. Adjuvant treatment with chemotherapy will therefore have reduced efficacy [43]. Although lacking the instantaneous results of surgery, SDT has the capability of providing sustained treatments that gradually eradicate malignant growths. Ultrasonic irradiation can penetrate deep into internal tissue and effect metastatic emboli that have reached the circulatory system through extravasation [1, 24]. In fact, SDT is showing the most promise with leukemia and other hematological malignancies that have inherent metastatic capabilities due to their derived cell origins. While surgery can never be replaced as an initial means of destroying primary tumors, SDT provides a useful alternative when operation is deemed an unviable approach.

With the obvious similarities between PDT and SDT, it can appear difficult to ascertain why one treatment would be more effective over the other. After all, both activate a preloaded sensitizing drug which often attack by similar mechanisms. In fact, some photosensitizing agents have been shown to be effective sonosensitizers when applied *in vivo* [5]. The main difference therefore lies within the energy source used to activate the drugs. While PDT has indeed been shown to be effective against particular squamous carcinomas, the effective range of the

treatment does not extend far past the skin barrier. Consequently, PDT has limited utility in cancer therapy. SDT uses ultrasonic irradiation that can easily penetrate deep tissue layers where some malignancies reside. Further, the synergistic effect of SDT and therapeutic agents is not replicated in PDT as light does not inflict damage through as many mechanisms as ultrasonic irradiation [50]. While PDT can effectively active ROS agents and other species dependent on a light activating source, cytoskeletal alterations and perturbed tumor vasculature networks simply do not occur. Therefore, PDT is also limited in the variety of sensitizing agents that are available. SDT attacks malignant cells through multiple mechanisms, providing the opportunity to utilize a vast array of therapeutic agents. This could significantly reduce the frequency of drug resistant tumors found within patients as cells would have to be considerably resilient to overcome the various mechanisms that SDT can implement.

The only major con of SDT so far is that it has not yet been appropriated to clinical testing. Therefore, the impressive results obtained in *in vitro* and *in vivo* studies might be grossly attenuated when actually applied in a clinical setting. The efficacy of SDT on real patients is simply unknown. Unless clinicians are willing to take a chance on the novel method, the required data necessary to accurately determine whether SDT is a viable approach will not be attained. It will remain as an intriguing, but untested method that has little more than conceptual importance. That is why the results mentioned in this review are of particular importance. Numerous studies have demonstrated the potential benefits SDT could offer to cancer therapy; now all that needs to be done is transition the treatment modality into the clinical setting. This is the only true way of knowing whether SDT is indeed as good as advertised. If initial trails are successful, further refinements could be made to determine conditions optimal for malignant cell destruction. As

with any novel treatment, the only way to determine actual efficacy is to give the therapeutic approach real world experience.

Likely Targeted Cancers

Although SDT has been shown to be effective against a variety of cancer cell lines in both *in vitro* and *in vivo* studies, it is important to note that this treatment modality may be most effective on specific cancers found in the clinical setting. The cancer type that has shown the greatest response to SDT and is a primary focus of this article is systemic leukemia. This hematological malignancy is unique to cancer biology as it is inherently metastatic. The leukocytes that dedifferentiate into leukemia are required to move throughout the vascular system, indicating that no mutation is required for anchorage independent growth [24]. This helps explain why leukemias are so commonly found as childhood cancers as less fundamental alterations are required for the development of malignant growths. However, leukemia is also unique in that it typically does not form primary tumor sites, but rather saturates the vasculature with aberrant cells, eventually compromising the immune system, blood clotting and erythrocyte transport [51, 52, 53, 54]. As such, leukemia cells are often freely floating alongside healthy blood cells. Being in such close proximity to cells that are vital for normal physiological functioning, it seems appropriate that SDT should have the capability to preferentially damage the malignant cells, while leaving healthy cells intact. Studies have confirmed that SDT has such a remarkable capability and as will be discussed later, the size differential between leukemia and normal blood cells can be dramatically increased using appropriate chemotherapeutic agents.

However, there are other cancers in which it is unclear whether SDT will be effective. Brain cancers are difficult to treat in the clinical setting as the monumental importance and sensitivity of this organ necessitates the use of precise, targeted therapies such as gamma knife

radiation that are capable of selectively damaging a specific area. However, such treatments are often incapable of completely eradicating the malignant cells. In particular, glioblastoma multiforme is characterized by strong local invasiveness and rapid growth, destroying vital brain functions in only a matter of months. With the limitations of current chemotherapeutic and radiation approaches, SDT could be proposed as an alternative treatment as it has been shown to reverse drug resistance in a variety of tumor types [1, 2, 6, 32]. As with any untested therapeutic approach, there may some difficulty with implementing SDT in brain cancer chemotherapy as an *in vivo* study using rats has demonstrated that SDT could be potentially harmful to the vital blood-brain barrier (BBB) [55]. Specifically, electron microscopy revealed that sustained ultrasonic irradiation (1.04 MHz, 100W/cm^2, 3min) resulted in swelling and denaturing of astroglial cells. The protoplasm of endothelial cells and mitochondria were also observed in the center and border of regions of where ultrasonic irradiation was administered. These observations were coupled with numerous pinocytotic vesicles in the cytoplasm of the endothelial cells; disruption of the cytoplasmic membrane of endothelial cells and astroglias were found in these regions. Such findings suggest that ultrasonic irradiation increases blood vessel permeability as a result of severe damage to the BBB as well as disruption of the cytoplasmic membrane of endothelial cells.

As troubling as these results may appear, human ultrasound therapy studies have indicated that the brain can indeed be safely sonicated. In particular, recent advances have enabled delivery of HIFU through the intact human cranium with magnetic resonance imaging (MRI) guidance. Such technology has been shown to improve essential tremor occurrences in patients treated with MRI-guided focused ultrasound thalamotomy (selected ablation of thalamus regions) [56, 57]. Further, various studies have shown that low intensity ultrasound of much

shorter lengths of time can significantly increase the permeability of the BBB, without harming any cellular structures [58, 59]. Such a discovery is potentially monumental for clinical applications of ultrasound as the BBB often impedes chemotherapeutic agents. This is particularly problematic for the treatment of malignant gliomas which are characterized by their diffuse infiltration into normal brain tissue where neoplastic cells are protected by an endogenous BBB.

Despite its potential issues with brain cancer therapy, SDT is still a viable treatment modality for a substantial diversity of malignancies. Indeed, a considerable variety of carcinomas, hematological malignancies and sarcomas have been indicated to be preferentially destroyed by SDT using various sonosensitizers [1, 2, 6, 7, 60]. While the rest of the article will focus primarily on leukemia therapy as it has shown the most promise and viability, it is important to note that SDT has been shown to be effective against a tremendous diversity of cell lines in both *in vitro* and *in vivo* studies. Success in leukemia chemotherapy will hopefully inspire clinicians to implement SDT for other malignancies if the treatment modality indeed lives up to its promise.

Administering Sonodynamic Therapy to Patients

Seeing that SDT has yet to be tested in the clinical setting, there has been no analysis as to how this treatment modality could be practically applied to patients. Although SDT fundamentally relies on an ultrasound system, there are a variety of ways in which the generated ultrasonic irradiation can be delivered. The 3 procedures that the author believes to be the most salient for leukemia therapy are heating the ankles and wrists as ultrasound is applied to these areas (Heat and Treat), using an ultrasonic probe to scan the body for malignant growths (Target and Destroy) and extracorporeal blood sonication (EBS) through dialysis. Each method offers a

unique set of benefits and concerns that will need to be weighed before it is chosen for clinical trials. Finding proper frequency ranges and sound intensities for ultrasound will also be of clinical importance, but considerable work in this area has been done *in vivo* and such data should be readily extrapolated for human therapy (Table 2). Therefore, the ways in which ultrasonic irradiation can be applied to patients in clinical trials remains the most pressing issue.

Heat and Treat

The vascular system is truly a remarkable piece of biological architecture as it provides a reliable solution to the oxygenation of tissues far away from the thoracic cavity where the heart and lungs are located. Blood cells are unique among the great diversity of cell types used in physiological functioning as they are required to move throughout the entire body in a timely manner. In fact, the approximate 5.6 liters of blood within the body circulates at a remarkable 3 times per minute [66]. As such, most blood will pass through the wrists and ankles in a short amount of time. Therefore, applying ultrasonic irradiation to the ankles and wrists could be a reliable method for making sure the entirety of leukemia cells within a patient are effectively sonicated.

Heat comes into play as it has been shown to increase the efficacy of specific chemotherapeutic treatments that could be used in SDT. Mild hyperthermia (39°C-43°C) is an adjuvant therapy that has yielded substantial benefits in the treatment of a variety of tumor types. Hyperthermia increases tumor blood flow and vascular permeability, promoting drug delivery to the targeted site (essential for effective SDT treatments). The slight increase in temperature enhances the uptake and efficacy of numerous chemotherapeutic agents, including cisplatin, resulting in increased cytotoxicity (Fig. 13) [35, 36, 67]. In addition to these biological responses, hyperthermia has been shown to be an effective drug-release trigger for temperature-

sensitive nanoparticles, resulting in an improved and more targeted drug delivery system [68, 69]. The degree of thermal enhancement of hyperthermia is inherently dependent on the ability to localize and maintain therapeutic temperature elevations. Due to the often heterogeneous and dynamic properties of tissues (most notably blood perfusion and the presence of thermally significant blood vessels), therapeutic temperature elevations are difficult to spatially and temporally control [70]. Ultrasound can in fact provide a dual role as heat source for the treatment as it has been shown to have a higher degree of spatial and dynamic control of heating compared to other commonly used heating modalities. These advantages include a favorable range of energy penetration characteristics in soft tissue and the ability to shape the energy deposition patterns [70].

The set up for Heat and Treat would be relatively straightforward. The patient could be placed comfortably in a chair, while ultrasonic devices are attached near the wrists and ankles. There could be 2 ultrasound machines used (one for hyperthermia and the other for treatment) or alternatively, the patient's hands and feet could be immersed in hot water. However, some form of ultrasound is inherent in this procedure as it is the sound energy (ultrasonic irradiation) that inflicts preferential damage on malignant cells through diverse mechanisms of action. While sonosensitizers are used to amplify the effects of these fundamental mechanisms, they will not have as great as an effect by themselves, even with thermal ablation from other heating sources [1, 6]. The sonosensitizers used in the treatment would be administered intravenously before sonication, allowing the chemotherapeutic agents to accumulate in the bloodstream. Once an effective dosage has been applied, the patient would be connected to the ultrasonic devices, necessitating a waiting period between application of sonosensitizers and ultrasound. The applied ultrasound could be run continuously or in short bursts during the treatment. The length of each

individual SDT treatment remains unclear and would have to be determined by clinicians after initial trials. However, the simplicity and relatively low potential risks to the patient during Heat and Treat provides compelling reasons for using ultrasonic irradiation in the clinic. Although incidental normal blood cell (erythrocyte, leukocyte, megakaryocyte) destruction may be a potentially hazardous issue, it can be monitored by attending clinicians to ensure preferential damage is indeed occurring.

Target and Destroy

Although Heat and Treat is a potential avenue for treating leukemias and other hematological malignancies, sonicating the ankles and wrists will yield little benefit for other cancers that are often concentrated at a primary tumor site. Further, some patients have leukemia cells remain trapped in the bone morrow, therefore proving inaccessible to the Heat and Treat method. This is commonly seen in patients suffering from aleukemia in which the bone marrow contains cancerous leukocytes that disrupt the normal production of blood cells, but remain in the marrow instead of entering the vasculature. Without the luxury of using the vascular system to transport blasts to readily accessible areas such as the ankles and wrists, another sonication approach needs to be devised. One that is capable of scanning the body for concentrated pockets of malignant cells and then blasting such sites with high-intensity ultrasound.

In fact, such technology already exists and could be readily applied to the clinic with a few minor adjustments. Ultrasonic probes are devices capable of delivering high frequency or intensity ultrasound to localized areas and are commonly used in the medical world for diagnostic imaging or even breaking up calcified kidney stones (as in extracorporeal shock wave lithotripsy). Medical ultrasonography uses a substantial variety of ultrasonic probes and many operational systems are available for testing with SDT. In fact, such probes are currently being

used in the clinic for extracorporeal shock wave lithotripsy (ESWL). Breaking up calcified stones found in the gall bladder or kidney with ultrasound requires considerable intensity. The lithotripter used in such procedures breaks up stones with tolerable collateral damage by using an externally-applied, focused, high intensity acoustic pulse; the exact kind needed for SDT [71, 72]. ESWL can actually be seen as a proof of concept of SDT as it breaks up calcified deposits through inertial cavitation, just as malignant cells are in SDT.

Due to the advances in medical imaging, it is now possible to readily locate primary tumor sites, providing the basis for Target and Destroy SDT procedures. By injecting sonosensitizers subcutaneously at tumor aggregates prior to treatment, ultrasonic probes can be locally applied to the affected site, thereby allowing a highly specified and hopefully effective chemotherapeutic approach. Such a therapeutic method has apparent clinical implications outside hematological malignancies as a great diversity of cancers could be treated using Target and Destroy. However, the true diversity of cancers that can be treated through this form of SDT will only be determined through substantial clinical trials.

Extracorporeal Blood Sonication

The potential for Heat and Treat and Search and Destroy is enough to warrant preliminary clinical trials for SDT. Of course, both treatment methods inherently rely on ultrasound waves traveling through the skin barrier as well as complex internal structures. As such, ultrasonic irradiation loses some of its intensity as it travels through the human body. Instead of increasing the wattage to obtain the same amount of intensity, if there was a way to remove malignant cells from the body so they could be treated in an extracorporeal environment, there would be no sound inhibitors protecting such cells from the preferential damage of SDT.

Although such an approach is unfeasible for most malignancies, leukemia is unique in that it does not form a primary tumor site. Rather, it flows through the blood, alongside normal cells as it slowly overcomes the natural defenses of the immune system. However, its most beneficial asset can be exploited to become a profound fatal flaw. Since most leukemias are localized in the blood, it would be rather straightforward to draw the malignant cells out their hiding place through dialysis. While dialysis is typically used on patients to act as an artificial replacement for lost kidney function due to renal failure, it could just as easily be used to treat leukemia in an extracorporeal setting. Sonosensitizers would be injected intravenously as in Heat and Treat with roughly the same amount of time passing before injection and sonication. The patient would then undergo a typical hemodialysis procedure in which blood is pumped outside of the body, thereby removing the natural sound barriers of human anatomy (Fig. 14). There would be nothing standing in the way between the malignant cells and the ultrasonic waves that are able to inflict such profound preferential damage. In effect, this SDT procedure allows an *in vivo* setting to become almost *in vitro*. Since the *in vitro* studies of SDT with leukemia have yielded impressive results, this may be the most effective way to administer ultrasonic irradiation to such patients. However, it goes without saying that the sound intensities used for Heat and Treat and Search and Destroy would likely be inappropriate for extracorporeal blood sonication (EBS). There is very little standing in the way between the blood and the high intensity ultrasound being administered. While normal erythrocytes and leukocytes are more resistant to SDT, they are not invulnerable. Sufficient sound intensities will cause just as much damage to these cells as the malignant cells that are within close proximity [3]. Therefore, the sound intensity used in EBS would likely have to be considerably reduced. Nevertheless, it still provides the most direct route for sonicating the dedifferentiated blasts that cause so much

devastation in patients. This method could potentially be used in tandem with Target and Destroy so that malignant cells caught within the bone marrow are significantly reduced. It may even be possible to use all 3 treatments in a comprehensive scanning and removal of leukemia cells found within the patient.

Chapter VII: Cytochalasin B as the Prototypical Sonosensitizer

Experimental Evidence of Cytochalasin B

To demonstrate the utility of SDT in clinical applications, one of the approaches of SDT will be exhibited in great detail. It is hoped that this comprehensive experimental evidence will convince readers of SDT's promise as a viable treatment modality for leukemia patients. The experiments involved preferentially lysing U937 promyeleocytic leukemia cells in the presence of normal blood cells. Only cytochalasin B was used as a sonosensitizer in order to indicate how effective this pharmacological agent is with ultrasound. Later studies will investigate the potential synergistic effects of using nucleic acid and mitochondrial agents with cytochalasin B in an attempt to develop a highly effective therapeutic approach.

Materials and Methods

U937 Cell and Normal Blood Cell Preparation

U937 human promyelocytic leukemia cells were placed at 5.2×10^4 viable cells/ml in 20% Fetal Bovine Serum (FBS) in Isocove's medium with the following: 2% by volume of 10,000 units/ml penicillin, 10mg/ml streptomycin, 0.5% gentamicin sulfate and 2mM glutamine. Human blood cells acquired from SUNY Upstate Medical University (Syracuse, NY) mixed with human hematopoietic stem cells (hHSCs) (10% concentration of hHSCs) from the same patient were cultured under equivalent conditions. To ensure that cytochalasin B would alter U937 cells in the predicted manner, U937 cells were first treated individually with a 1.5μM concentration for 48

hours, corresponding to two cell cycles. Cells were seeded at 1×10^5 cells/ml before being examined. Cells were subsequently Wright-Giemsa and DAPI (4',6-diamidino-2-phenylindole) stained to examine nuclear structure. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA; Wright-Giesma is a histological stain that is used primarily to stain peripheral blood smears and bone marrow aspirates. It is commonly used to stain chromosomes to facilitate diagnosis of syndromes and diseases due to its ability to readily visualize cell nuclei [73].

It has been well cited that leukemia cells have exceedingly high mitochondrial activity due to increased metabolic rates [1, 6, 7]. Therefore, testing whether cytochalasin B further amplifies mitochondrial activity of malignant cells has tremendous utility as this would open the door for mitochondrial-based agents. Mitochondrial activity of U937 cells was assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a colorimetric assay that relies on NADPH-dependent cellular oxidoreductase enzymes found within the organelle [74]. Under appropriate conditions, the enzymes reduce MTT to an insoluble product that has a purple color. The amount of mitochondrial activity is then readily assessed based on the deepness of purple that each sample provides.

Once it was confirmed that cytochalasin B had the suspected effect on U937 cells (i.e. cells were grossly enlarged and multinucleated) more U937 cells were prepared under the same conditions to ensure healthy U937 cells would be introduced to the normal blood cell populations. Before each experiment, U937 cells were mixed at a 20% concentration with normal blood cells and incubated for 24 hours prior to treatment, ensuring stabilization of the heterogeneous cell population. After U937 cells were mixed with healthy blood cells, 1.5µM cytochalasin B was administered for 48 hours.

Sonication of Cell Populations

The U937–normal blood cell mixtures were put into 2.4cm diameter vials with Mylar bottoms for sonication. To be sure cytochalasin B was truly impacting the extent of preferential damage, controls of normal blood cells alone, U937 cells alone and U937–normal blood cell mixtures (no cytochalasin B) were prepared. The cells were seeded in 1.0ml of 20% FBS medium with 1% Gibco® Fungizone (Life Technologies, Grand Island, NY, USA). Each vial contained 1000µl of cells. Cells were sonicated using a Fisher Scientific® Sonic Dismembrator (23.5kHz, 6.0cm diameter cup) system (Fisher Scientific International Inc., Hampton, NH, USA) along with a Bellco® Orbital Shaker (Fisher Scientific International Inc., Hampton, NH, USA). Mylar vials were placed in 7.0cm deionized, distilled and degassed water and located 6.0cm from the sonic horn before sonication. Cells were sonicated at a constant 3W/cm² for 1-4min. Trypan Blue staining was used to identify non-viable cells after sonications were performed: 50µl of cell suspension and 50µl of 0.4% Trypan Blue stain in isotonic saline were mixed and transferred to a hemocytometer counter chamber after sonication experiments. A Z2 Beckman-Coulter® Particle Count and Size Analyzer (Beckman Coulter Inc., Brea, CA, USA) along with a Bio-Rad® TC10 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used for size determination, as well as for identifying the number of enlarged, multinucleated cells post-sonication.

Longitudinal Effects of Cytochalasin B

In order to examine the longitudinal effects of cytochalasin B on U937 cells, clonogenicity was assessed for both untreated and treated cells. A healthy U937 cell population was divided into two groups with one receiving 1.5µM cytochalasin B, and the other acting as a control. One and a half Corning® 384 well immunoassay plates (Sigma-Aldrich Corp., St. Louis,

MO, USA) were used for each cell population. Each well was loaded with 0, 1, or 2 cells that ranged from 13-19µm in diameter for untreated cells and 14-24µm for treated cells (48 hours after cytochalasin B administration). After loading, the cells were incubated for 12 days in 5% CO_2 at 37°C. Individual wells were then assessed for the presence and number of clones.

Results

Effects of Cytochalasin B on U937 Cells

Administering cytochalasin B to U937 cells resulted in profound alterations to cytological structure and physiology. Wright-Giesma staining revealed that malignant cells had become remarkably multinucleated 48 hours after 1.5µM cytochalasin B was introduced to the cell population (Fig. 15). When compared to normal U937 cells, it became readily apparent that cytochalasin B had a profound effect on cytoskeletal structure as is expected from a cytokinesis inhibitor. Aberrant cytoskeletons are often a hallmark of perturbed cellular integrity, suggesting that such cells would be highly sensitive to physical disruption (ultrasonic irradiation). The extent of multinucleation was further confirmed by DAPI staining as aggregates of DNA were readily detected (Fig. 16). Results from flow cytometry and both cell counters revealed a profound shift in U937 cell size, indicated by the apparent increase in peak height. Both normal and cytochalasin B-treated U937 cells were then compared with normal blood cells for a further analysis of size differential. Subsequent results revealed a profound difference in average cell size (Fig. 17). The extent of physiological disturbance induced by cytochalasin B was further demonstrated by MTT assays of normal and cytochalasin B-treated U937 cells (Fig. 18). Cytochalasin B-treated cells had an approximate fourfold increase of absorbance at 590nm, indicating enhanced number or activity of mitochondria. The increased mitochondrial activity

coincides with the fourfold higher DNA content on average which was previously indicated by Wright-Giemsa and DAPI staining.

Effects of Sonication on Cytochalasin B-Treated U937 cells

While the effects of cytochalasin B on U937 cells were readily apparent, the malignant cells were still viable at 48 hours post-treatment. Therefore, a physical catalyst is needed to promote cell death (either by apoptosis or necrosis) of the U937 cells. Results from sonication at $3W/cm^2$ suggest that ultrasonic irradiation is a viable catalyst. The extent of preferential damage was readily detected post-sonication with Trypan Blue staining (Fig. 19). While normal blood cells (including hHSCs) did have slight sensitivity to sonication at $3W/cm^2$, it was minor compared to the profound sensitivity U937 cells exhibited. This sensitivity was dramatically amplified when cytochalasin B was administered, as evidenced by the significant difference in cell viability at 4 minutes.

Longitudinal Impact of Cytochalasin B

The effects of cytochalasin B on U937 cell clonogenicity were readily apparent. After the 12-day incubation period, cytochalasin B-treated cells had a cloning efficiency of approximately 12% and exhibited the same hallmarks of increased size and multinucleation, with some being in excess of 40µm. By contrast, the control, untreated U937 cells had a cloning efficiency of 71% (Fig. 20). With such a low cloning efficiency, it is apparent that most cytochalasin B-treated U937 cells lose the ability to readily proliferate. It is important to note that the very large cytochalasin B-treated U937 cells (24µm) had lost their capability to proliferate after the 12-day incubation period. This suggests that further along the multinucleation process (indicated by their increased size and nuclei) U937 cells continue to lose their proliferative capability. , This could

indicate that the most rapidly proliferating U937 cells ultimately lose their clonogenicity at the fastest rate.

Discussion

Cytochalasin B appears to be a versatile chemotherapeutic agent that amplifies the damage ultrasonic irradiation preferentially inflicts on malignant cells. Exposed U937 cells consistently became grossly enlarged and multinucleated after being administered a relatively small dosage of 1.5μM cytochalasin B. By contrast, normal blood cells exhibited no change in cell morphology and remained stable in size throughout the 48-hour incubation period. When exposed U937 cells were assessed for mitochondrial activity using MTT assay, the cells exhibited a fourfold increase in activity in comparison to U937 cells of typical histology. Such a dramatic increase in metabolic rate inherently suggests using mitochondrial agents in tandem with cytochalasin B during ultrasound treatments. Indeed, Reactive Oxygen Species (ROS) agents often target the mitochondrial induced apoptotic pathway of leukemia cells, providing a viable method for developing synergistic treatments. This approach could be further supported by nucleic acid agents as cytochalasin B-treated U937 cells are considerably multinucleated. It is very likely that only a single nucleus will have to undergo apoptosis in order for the malignant cell to be destroyed; having so many nuclei present greatly increases the likelihood of this event.

By itself, a cytochalasin B, mitochondrial- and nucleic acid-directed drug cocktail appears to be a viable method for generating preferential damage to malignant cells in patients with leukemia. However, this combinatorial therapy appears to have the most promise when it is used to amplify the effects of ultrasonic irradiation. Cytochalasin B-treated U937 cells are remarkably sensitive to relatively low sound intensities ($3W/cm^2$). Although U937 cells are much more sensitive to ultrasound than normal blood cells, the damage caused by ultrasound

pales in comparison to the preferential damage inflicted on cytochalasin B-treated cells. This is why sonosensitizers are so important in SDT. Using chemotherapeutic agents make susceptible cancer cells much more sensitive to ultrasound, indicating that less intense sound intensities will be needed to generate substantial preferential damage. Ultrasound-only therapies would necessitate much higher intensities in order to inflict the same amount of damage to the malignant cell population. As observed with blood cells, normal cells are not immune to the effects of ultrasound. Increasing the sound intensity needed to lyse malignant cells would dramatically reduce the specificity of damage. Therefore, sonosensitizers hold the key to the efficacy of SDT, which is why more research should be invested in determining what drug combinations produce the greatest synergistic effects.

One of the most profound results of this study is the considerable loss of clonogenicity of cytochalasin B-treated U937 cells. A fundamental feature of any cancer is that it is capable of uncontrolled and often accelerated cell proliferation. This phenotypic effect is what allows such quantities of aberrant, dedifferentiated cells to spread throughout the body and cause eventual death if not controlled. Cytochalasin B has the capability of mitigating this phenotype as demonstrated by the dramatic reduction in U937 cell clonogenicity. It is likely that the first few SDT treatments will not destroy every leukemia cell found in a patient as some cells may persist in the bone marrow or spleen. However, if cytochalasin B can effectively remove the cell's ability to proliferate, it will be effectively neutralized. It is also very unlikely that an enlarged, multinucleated cell would be able to survive for extended periods of time due to its increased metabolic needs. Taking all of the evidence together, it appears that ultrasound administered with cytochalasin B is an effective method for generating preferential damage of leukemia cells in the presence of human blood cells.

While treatments with cytochalasin B-alone could yield substantial results for patients with leukemia when combined with US, the fact that affected cells become profoundly multinucleated, as well as grossly enlarged, provides the opportunity for synergistic effects with a nucleic acid agent. Although ultrasound has been shown to increase the efficacy of multiple nucleic acid agents, one agent of particular note is DOX as it has been shown to attack malignant cells through a novel mechanism when applied in SDT, enabling the chemotherapeutic agent to damage DOX-resistant cell lines. The human leukemia multidrug-resistant cell line K562/A02 has been shown to be damaged by ROS when DOX is applied with ultrasound, a mechanism that is not typically seen for a DNA intercalating agent [2]. Such effects were derived from a cell line shown to be resistant to both ultrasound and DOX-alone control treatments, further substantiating the amplifying effect sonosensitizers have in SDT. There is a similar effect when ultrasound/DOX treatments are applied to U937 cells, suggesting the agent can be effective against multiple leukemia cell lines when used in tandem with ultrasonic irradiation [1, 34].

Cytochalasin B has also been shown to increase the metabolic activity of U937 cells, indicating that it could have a profound synergistic effect with mitochondrial agents. HMME (Hematoporphyrin Monomethyl Ether) has been used for multiple cancer cell lines and has shown commendable efficacy, particularly in a study that involved U937 cells [27]. Immediately after administration, intracellular HMME concentrations rapidly increased within the U937 cells, reflecting its high affinity for malignant tissue. The synergistic effect of ultrasound with HMME showed significant cell destruction, indicating the necessity of sonosensitizers in ultrasound-mediated therapy. Flow cytometry with DCFH-DA (2'-7'-Dichlorodihydrofluorescein diacetate) staining confirmed that HMME-ultrasound treated cells had markedly increased ROS levels compared with the control, HMME and ultrasound-alone groups. Further analysis of damaged

cell populations revealed that oxidative stress was present and that cells had indeed undergone apoptosis. These results not only confirm the linkage between ROS and apoptosis within U937 cells, but ultimately suggest a novel approach to treating patients with leukemia. This damage can be further enhanced with the use of DOX in collaboration with ultrasound/HMME, as a study with QBC939 cells (leukemia) has indicated [40]. The study demonstrated that the ultrasound/DOX /HMME group had a higher reduction in cell viability than both the ultrasound/DOX and ultrasound/HMME groups, suggesting the need to investigate the cumulative effect of multiple sonosensitizers. Such results reflect a synergistic effect as DOX has the ability to increase ROS content in malignant cells. Since HMME and DOX can increase production of singlet oxygen when activated by ultrasound, the drugs act in tandem to create an environment that malignant cells find particularly cytotoxic due to their decreased levels of endogenous thiol buffers.

Chapter VIII: The Lasting Utility of Sonodynamic Therapy

Conclusion

Ultrasonic irradiation appears to be a viable approach to preferentially damaging malignant cells. This stems from inherent cellular responses such as the generation of microbubbles that under enough intensity will collapse, producing the phenomenon known as inertial cavitation. The energy released from the collapse produces shear forces that damage the cytoskeleton as well as generate extreme temperatures and pressures that allow sonoluminescene to be observed; a key step in dramatically increasing ROS content within the cell. In enough concentration, ROS are known to have a severe cytotoxic effect as singlet oxygen disrupts normal mitochondrial function and hydroxyl radicals misappropriate electron distribution in the plasma membrane, causing lipid peroxidation. In addition, ultrasound is known to have a

profound effect on disrupting tumor vasculature as the thin endothelial linings provide ideal targets for microbubble destruction. Endothelial cells within these regions are put under severe oxidative stress due to the development of hypoxic regions and vessels fail to mature, thereby inducing the apoptosis of malignant cells. Even malignant cells that do not rely on extensive vascular networks such as metastatic fragments or leukemias can be effectively destroyed through sonication as ultrasonic waves have the capability of being directed towards any part of the body, producing a form of search and destroy treatment.

Clearly, ultrasonic waves produce remarkable antitumor effects under appropriate settings. However, such effects are not always widespread and tumor populations often become resistant to ultrasound-alone treatments. That is why SDT is such a sensible prospect as it significantly enhances the efficacy of ultrasonic irradiation, while still displaying preferential damage towards malignant cells. Every mechanism by which ultrasound destroys malignant tissue can in fact be amplified when an appropriate sonosensitizer is administered. Such drugs often attack cells through multiple mechanisms as well, creating a potential synergistic effect when sononosensitizers of different classes are used in collaborative efforts. Therefore, studies should also concentrate on examining the synergistic effects between sonosensitizers in an effort to create potent drug cocktails that very few malignant cells could survive. If preferential damage to malignant tissue can be maintained when such drug cocktails are applied, the efficacy of treatments could be truly remarkable.

One of the most cited shortcomings of chemotherapy in clinical practices is drug resistance acquired by the tumor. Already, multidrug resistant HepG2/ADM cells have been shown to be severely damaged by ultrasonic irradiation. Such capabilities are continued in SDT as the efficacy of DOX towards multidrug resistant K562/A02 cells is significantly increased,

indicating that the treatment modality can help overcome one of cancer's most potent defense mechanisms. There may even be the possibility of using relatively low doses that still produce a substantial effect on malignant growths as ultrasound has the additional benefit of sonoporation. Such activity provides openings by which small molecules (sonosensitizers) can use to gain entry into the intracellular environment. It has even been shown that particular classes of viruses further increase the entry points that sonosensitizers have available. With so many points of entry, relatively small doses of drug treatments can still have considerable efficacy in generating preferential tumor damage.

Seeing that SDT has yet to be attempted in the clinical setting, appropriate methods in which to administer ultrasound have not been devised and must be determined if the treatment modality is to have a future in oncology. The methods expressed here all have their own unique benefits as well as potential side effects. Further, Heat and Treat as well as EBS have only specific clinical implications as they would most likely only be used to treat leukemias. This leaves Search and Destroy as the only proposed method to treat other cancers found in patients. Of course, if SDT finds success with leukemia patients, further research could be set forth to discover novel ways in which ultrasound can be administered.

It should be noted that there are significant similarities between metastatic cancer cells and leukemia [24]. As such, it is likely that SDT could be applied in the clinical setting to preferentially damage circulating metastases. Successful treatment could have a profound influence on patient survival as complications from metastatic progression result in more than 90% of cancer mortality [75]. As shown by ESWL for calcified stone removal, ultrasound has the propensity to fragment large chemical aggregates. Since carcinomas often circulate through the blood as metastatic emboli to avoid the unsuitable environment of the circulatory system, it

seems likely that ultrasound could be used for breaking up such aggregates, thereby exposing the cells to the unsuitable environment. Without the protective embolism, it is likely that most metastatic cells in circulation would die, significantly reducing the likelihood of disease migration. While this would not account for micrometastases that have already reached the intended secondary site, patients can always be monitored after treatments have concluded to safeguard against such surprises.

Throughout this report, there has been considerable attention paid to the effects of SDT on leukemia cells. While there is a substantial amount of data available on such cells and SDT, there is another reason why particular importance was paid to leukemia. This type of hematological malignancy is responsible for more incidences and deaths related to childhood cancer in the United States than any other type of neoplastic growth. Leukemias account for 33% of all cancers for individuals between the ages of 0-14; much higher than any other type of malignancy [75]. Further analysis reveals that 30.4% of all deaths attributed to childhood cancer are in fact some form of leukemia. In fact, leukemia is the leading cause of disease-related death associated with children (0-14), taking more of America's youth than any other ailment. The fact that leukemia is responsible for the deaths of more children than any other type of malignancy alone warrants the need to develop more effective treatments.

The startling findings actually make sense when the nature of leukemia is examined. Leukocytes are needed to travel throughout the body as they serve as the core of the host's natural immune system. Such motility is inherently maintained when leukocytes begin to develop neoplastic features. In effect, leukemia cells are free to travel throughout the body without ever having the need to acquire subsequent mutations as with carcinomas and other types of malignancies. This inherent metastasis is pivotal in understanding why leukemias are so

common in young children. While other cancers take years to develop mutations required for malignant growth, leukemia already has several key aberrancies acquired from its derived cells when neoplastic characteristics begin to develop. This is coupled with the fact that leukemias are often found in children with immune disorders as defective leukocytes are more prone to acquire mutations [24]. The weakened immune system is also less likely to amount a response when aberrant cells are detected, increasing the likelihood of neoplastic development. Therefore, leukemia typically takes less time to develop than other malignancies, explaining why it is so prevalent in childhood cancer.

That is not to say all of the efforts put into developing SDT should be directed towards leukemia as there are other malignancies that take the lives of many more individuals each year. It just should be noted that SDT has found particular promise with leukemia and that countless lives full of potential could be saved if effective treatments are developed. Being able to develop treatment regiments in which the synergistic effects of different sonosensitizers are applied can have monumental importance in clinical applications. Such treatments could substantially amplify the capability of ultrasound to preferentially damage malignant cells in order to decrease the rate at which drug resistance is observed. Unfortunately, such drug cocktails could induce aberrant side effects when given to patients. Many potential sonosensitizers are drugs already used in the clinic and are known to damage normal labile cells, along with malignant tissue when higher doses are required. Therefore, it is necessary to investigate approaches to increase drug uptake by cancer cells so that lower doses can be administered to yield the same net effect. Sonoporation seems to be a viable approach as considerable increases in membrane permeability has been indicated when the phenomenon is observed. At any rate, the idea of combining ultrasound with drugs that amplify the ways in which it preferentially damages malignant cells is

gaining more legitimacy as successful studies have vindicated the potential of SDT. By using the synergistic effects of ultrasonic irradiation and sonosensitizers, SDT is proving to be a viable treatment modality that has the capability to revolutionize the way in which chemotherapy is administered in the clinical setting.

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Appendices

Tables

Table 1: Sonosensitizers tested in sonodynamic therapy.

Sonosensitizer	Class	Primary Mechanism	Synergistic Effect with Ultrasound
Doxorubicin (Adriamycin)	Anthracycline	intercalates DNA, preventing DNA replication and protein synthesis	increased efficacy with multidrug resistant K562/A02 cells at 20 kHz, 0.25W/cm^2, 60s intervals [2]; similar effects observed with U937 cells [34]; can be used in tandem with HMME on U937 cells for a greater effect [40]
Cytochalasin B	Cytoskeleton Agent	disrupts actin cytoskeleton, prevents cytokinesis by interfering with formation of the contractile ring as well as the cleavage furrow, cells do not divide and become grossly enlarged and multinucleated	sonic sensitivity was increased in U937 cells when cytochalasin B was administered at 1.5µM, cells often grew to 20µm or greater and were unable to tolerate ultrasound treatments in which normal blood remained stable, increased mitochondrial activity and reduced clonogenicity were also observed [44]
Methotrexate	Cytoskeleton Agent, Antimetabolite Agent	causes competitive inhibition of dihydrofolate reductase, an enzyme that participates in tetrahydrofolate synthesis, prevents production of thymidine as well as all purine bases; causes the same effects to the cytoskeleton as cycloplatin	aberrant features with the cytoskeleton of HeLa cells were observed using 1.8 MHz, 0.22W/cm^2 [45]
Cycloplatin	Cytoskeleton Agent, DNA Alkylating Agent	alkylates guanine nucleotides, inhibiting DNA replication and protein synthesis; interferes with the cytoskeleton by thinning out actin and microtubule bands, fragmenting microtubules with the formation of tubulin granule-like structures and partial loss of stress fibers	aberrant features with the cytoskeleton of HeLa cells were observed using 1.8 MHz, 0.22W/cm^2 [45]
Docetaxel (taxotere)	Cytoskeleton Agent, Taxane	stabilizes GDP-bound tubulin polymers, thereby inhibiting mitosis	inhibited growth of PC3 tumors in athymic mice using 1 MHz, 50ms bursts (0.00024 duty cycle), 1.65MPa [12]
Cisplatin	DNA Alkylating Agent	alkylates guanine nucleotides, preventing DNA synthesis	increased cytotoxicity to multiple cancer types; ultrasound replenishes labile cells lost due to treatments [5]
Diaziquone	DNA Alkylating Agent	alkylates guanine nucleotides, preventing DNA synthesis	increased cytotoxicity to multiple cancer types; ultrasound has same effects on depleted labile cells [5]
Albunex	Echo Contrast Agent	increases microbubbles in systemic circulation to enhance effects of inertial cavitation	macrocytic (grossly enlarged) erythrocytes were damaged by the increased proportion of microbubbles at intensities that left normal erythrocytes intact using 1.15 MHz, 3MPa, indicates SDT preferentially damages based on size [3, 11]
Levovist	Echo Contrast Agent	increases microbubbles in systemic circulation to enhance effects of inertial cavitation, cells exposed to drug treatment have low mitochondrial membrane potential, high superoxide production,	multiple leukemia cell lines (Jurkat, Molt-4, U937) were significantly damaged using 1 MHz, 0.3W/cm^2, 10% duty factor pulsed at 100Hz [47]

		increased intracellular calcium concentration, and phosphorylation of histone H2AX after sonication	
Cetuximab	Monoclonal Antibody	anti-epidermal growth factor receptor (EGFR) antibody, induces apoptosis as Phospho-EGFR expression is downregulated, whileCaspase-3 activation is upregulated	more cell killing features were evident in the COMB (combined cetuximab and ultrasound) group in HSC-3 and HSC-4 head and neck cell carcinomas compared with the other groups; phospho-EGFR expression was much more downregulated in the COMB group compared with that in the other groups; caspase-3 activation was much more upregulated in the COMB group than that in the other groups; experiments used 1.0 MHz at 0.5W/cm^2 [48]
ATX-S10	ROS Agent, Porphyrin	has a substantially longer sonoluminescent lifetime than other porphyrin agents, providing more opportunity to generate singlet oxygen; follows the same mechanism as other porphyrins	inhibited growth of colon-26 cells injected into athymic mice [5]
Hematoporphyrin Monomethyl Ether (HMME)	ROS Agent, Porphyrin	generates singlet oxygen that disrupts mitochondrial membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases	significant destruction of U937 cells with 1 MHz, 1W/cm^2, 60s intervals, increases intracellular singlet oxygen content [27], shows synergistic effect with DOX as both produce ROS [34]
Metronomic Cyclophosphamide (MCTX)	Vascular Disrupting Agent, Alkylating Agent	alkylates guanine nucleotides, inhibiting DNA replication and protein synthesis; converted in the liver to an active form for chemotherapeutic effects	combined with Definity to inhibit growth of MDA-MB-231 cells in athymic mice using 1MHz, 0.00024 duty cycle, 1.6 MPa [46]

Chemotherapeutic agents with diverse mechanisms of action have been shown to improve the efficacy of SDT. Monoclonal antibodies have also been tested in SDT, with similar improvements observed.

Table 2: Success of sonodynamic therapy in *in vivo* studies.

In Vivo Parameters	*In Vivo* Efficacy	Class of Sonosensitizer	Primary Mechanism of Sonosensitizer
Doxorubicin-loaded microbubbles (DOX-MBs) were administered intravenously in Lewis rats while one of the two tumors (pancreatic carcinomas) was exposed to ultrasound (1.3 MHz; mechanical index 1.6). DOX tissue concentration was measured in tumors and control organs after the experiment [18].	All rats survived the DOX-MB administration without any sign of embolisation/occlusion of the pulmonary vasculature. Ultrasound targeted destruction of DOX-MBs resulted in a 12-fold higher tissue concentration of DOX and a significantly lower tumor growth in the target tumor compared to the contralateral control tumor.	**DOX:** Anthracycline, ROS Agent	intercalates DNA, preventing DNA replication and protein synthesis, has been shown to reverse drug resistance in drug resistant K562/A02 leukemia cells [2], as well as produce ROS [[40]
Metronomic cyclophosphamide (MCTX) was employed administered through drinking water to athymic mice that harbored MDA-MB-231 breast cancer tumors. Ultrasound stimulated microbubble treatments were conducted at 1 MHz employing short bursts (0.00024 duty cycle) at 1.6 MPa in combination with the commercial microbubble agent Definity [46].	The USMB induced an acute reduction of blood flow as confirmed with US contrast imaging and DiOC7 perfusion staining. Longitudinal experiments demonstrated that significant growth inhibition occurred in MCTX-only and USMB-only treatment groups relative to control tumors. The combined USMB and MCTX treatment group showed significant growth inhibition and survival prolongation relative to the USMB-only and MCTX-only treatment groups.	**MCTX:** Vascular Disrupting Agent, Alkylating Agent, **Definity:** Echo Contrast Agent (increases microbubble concentration)	MCTX alkylates guanine nucleotides, inhibiting DNA replication and protein synthesis; converted in the liver to an active form for chemotherapeutic effects, increased microbubbles from Definity amplifies inertial cavitation
A novel porphyrin-derived sonosensitizers designated DEG (7,12-bis(1-(2-(2-hydroxyethoxy)ethoxy)ethyl)-3,8,13,17-tetramethylporphyrin-2,18 dipropionatomanganese) was injected into SCID mice xenograft models with MKN-74 gastric cancer cells, followed by ultrasound (1.0MHz, 1.0W/cm^2 output intensity, and 10% duty cycle for1–2min) [37].	SDT with DEG three times a week for 2 weeks potently inhibited tumor growth compared to ultrasound-only or no treatment. It was shown that ROS are generated and mediate sonotoxicity of ultrasound with DEG on MKN-74 cells.	**DEG:** ROS Agent, Porphyrin	generates ROS after excitation from sonoluminescent light that disrupts mitochondrial membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases
Epirubicin hydrochloride (EPI) inhibition on tumor growth by ultrasound was tested using five-week-old male nude mice injected subcutaneously with HL-60 human promyelocytic leukemia cells. 1-MHz ultrasound and 3W/cm^2 output power density were applied through aquasonic coupling gel for 30s to the tumor region of a mouse [61].	Ultrasound applied locally to the tumor resulted in a substantially increased drug uptake in tumor cells. The inhibition on tumor growth depended on the position of drug injection and phospholipid-based microbubble (PMB) application. Artificial sonoporation nuclei significantly enhanced transient pore formation on cell membranes which facilitates outside drugs entry into the cells.	**EPI:** ROS Agent, anthracycline	generates ROS after excitation from sonoluminescent light that disrupts mitochondrial membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases
The taxane docetaxel (Taxotere) was used for evaluating SDT as it has previously been shown to have potent antitumor effects when combined with small molecule vascular disrupting agents. Experiments were conducted on PC3 human prostate cancer cell tumors implanted in athymic mice. USMB treatments were performed at a frequency of 1 MHz employing sequences of 50 ms bursts (0.00024 duty cycle) at 1.65 MPa. USMB treatments were administered on a weekly basis for 4 weeks with docetaxel (DTX) being given intravenously at a dose level of 5 mg/kg [12].	The USMB treatments, either alone or in combination with DTX, induced an acute reduction in tumor perfusion, accompanied by significantly enhanced necrosis and apoptosis after 24 hours. Longitudinal experiments showed a modest prolongation in survival but no significant growth inhibition occurred in DTX–only and USMB-only treatment groups relative to control tumors. The combined USMB-DTX treatment group produced tumor shrinkage in weeks 4–6, and significant growth inhibition and survival prolongation relative to the control, USMB-only and DTX-only treatment groups.	**DTX:** Cytoskeleton Agent, Taxane	stabilizes GDP-bound tubulin polymers, thereby inhibiting mitosis
The sonodynamically induced antitumor effect of porfimer sodium (PF) was evaluated on a chemically induced mammary tumor in Sprague-Dawley rats.	The synergistic effect between PF administration and ultrasonic exposure on the tumor growth inhibition was significant. The ultrasonic intensity	**PF:** ROS Agent, Hematoporphyrin Derivative	generates ROS after excitation from sonoluminescent light that disrupts mitochondrial

The timing of 24 hours after the administration of PF was chosen for the ultrasonic exposure, based on pharmacokinetic analysis of the PF concentrations in the tumor, plasma, skin and muscle. The rats were exposed to ultrasound (3W/cm^2) for 15 min [62].	showed a relatively sharp threshold for the synergistic antitumor effect, which is typical of an ultrasonic effect mediated by acoustic cavitation. Therefore, a marked synergistic effect between PF administration and ultrasonic exposure on the tumor growth inhibition was observed at a PF dose of 2.5 mg/kg and at a free-field ultrasonic intensity of 3W/cm^2.		membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases
5-aminolevulinic acid (ALA), a precursor to the ROS agent Protoporphyrin IX (PpIX) was investigated for its anti-angiogenic potency *in vivo*. SAS human oral cancer cell suspensions were subcutaneously injected into the flanks of BALB/c mice. ALA was intraperitoneally injected into mice in the ALA and ultrasound + ALA groups at a dose of 250 mg/kg body weight. After 4 hours of administration of ALA, the mice were placed on a plexiglass plate with the tumor immersed in degassed water. Tumors were irradiated by ultrasound (1.1MHz, 2W/cm^2, 50% duty cycle) for 5 min [20].	Ultrasound treatment significantly decreased microvessel density (MVD) compared with control, and the reduction of MVD was more prominent in the ultrasound + ALA group. Accordingly, the expression level of VEGF, a critical proangiogenic factor, was reduced in tumors treated with ultrasound irradiation. Ultrasound plus ALA induced more significant decrease in VEGF expression than ultrasound alone. It also inhibited the secretion of VEGF in SAS cells more significantly in the presence of ALA.	**ALA:** ROS Agent, Precursor to Hematoporphyrin Derivative	generates ROS after excitation from sonoluminescent light that disrupts mitochondrial membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases
Reversal of DOX resistance was investigated in a study of low-intensity ultrasound. Athymic nude mice were inoculated with HepG2 multidrug resistant hepatocellular carcinoma cells. Ultrasound with pulsed irradiation (0.5W/cm^2) was administered for 10 min to both ultrasound/DOX and ultrasound only groups [63].	Ultrasonic treatment resulted in an average 62% reduction in tumor volume a month later. The relative levels of MDR1 and MRP were dramatically reduced in ultrasound/DOX groups, suggesting a reversal of drug resistance.	**DOX:** Anthracycline, ROS Agent	intercalates DNA, preventing DNA replication and protein synthesis, ROS agent
The study was conducted on CT26 colon carcinoma tumors in BALB/c mice. In the respective groups, protoporphyrin IX (PpIX) or the gold nanoparticle-protoporphyrin IX conjugate was injected into the tumors. Ultrasound irradiation (1.1MHz, 2W/cm^2, 3min) was performed on the tumors 24 hours after injection [64].	A significant difference in the average relative volumes of the tumors 13 days after treatment was found between the ultrasound + gold nanoparticle–protoporphyrin IX group and the other groups. The longest doubling and 5-folding times were observed in the ultrasound + gold nanoparticle–protoporphyrin IX and ultrasound + protoporphyrin IX groups.	PpIX: ROS Agent Hematoporphyrin Derivative	generates ROS after excitation from sonoluminescent light that disrupts mitochondrial membrane potential, loss of electrochemical gradient, causes cristae to fragment, induces apoptotic cascade to trigger caspase proteases
C57BL/6J female mice were inoculated subcutaneously with Hepa1-6 hepatocellular carcinoma cells. Herpes simplex virus thymidine kinase under the control of kinase domain-containing receptor (KDR, angiogenic growth factor's corresponding receptor) promoter was used for targeted gene therapy. Plasmid DNA with or without microbubble contrast agent of SonoVue was intravenously. injected. Ultrasound (1 MHz, 2W/cm^2, 5 min) was delivered to hepatic carcinomas in mice. The KDR-tk gene transfer was followed by ganciclovir injection for 10 days and then the diameters of tumors were measured every 4 days for 28 days [65].	Compared with the group treated by ultrasound alone, KDR-tk gene treatment treated by ultrasound combined with SonoVue restrained tumor growth and increased survival time of tumor-bearing mice; microvessel density in group mediated by ultrasound and SonoVue was significantly lower than that in group ultrasound alone. An apoptosis index increased in the group treated by ultrasound and SonoVue compared with the group treated by ultrasound alone, whereas there was no significant difference between group mediated by SonoVue alone and group phosphate-buffered saline alone.	SonoVue: Echo Contrast Agent	increases microbubbles in systemic circulation to enhance effects of inertial cavitation, substantially increases the efficacy of viral gene transfer

It is important to note that the studies were conducted using diverse cell lines and sonosensitizers, suggesting SDT has clinical potential in a variety of cancers. SDT also has the potential to improve viral gene transfer, providing an additional mechanism for the therapeutic approach [65].

Figures

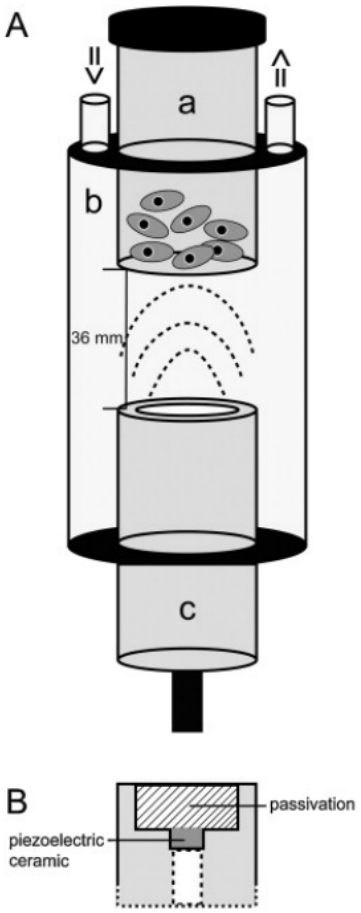


Fig. 1: Typical set up of an *in vitro* ultrasound experiment. The cells are located in a suspended container that sits either above or below the ultrasonic transducer. Cells of various concentrations can be sonicated in the presence or absence of sonosensitizers. Ultrasound settings are usually specified by the experimental set up. Image courtesy of [10].

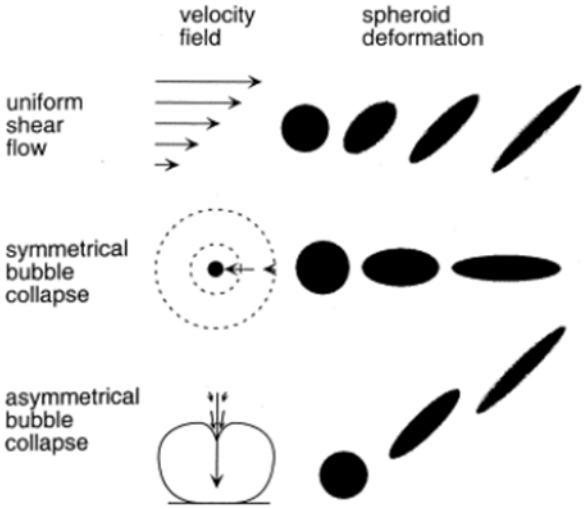


Fig. 2: Representation of inertial cavitation. Microbubbles are unevenly stretched by ultrasonic waves, causing an unequal distribution of force. Subsequent stress results in microbubble implosion, creating considerable amounts of energy. Image courtesy of [3].

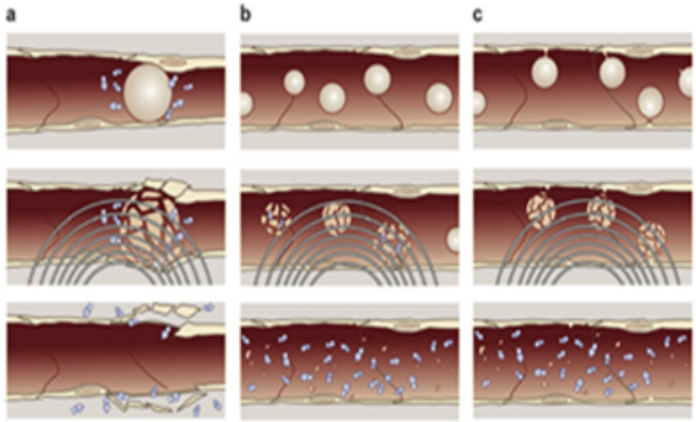


Fig. 3: Vasculature Disruption. Many tumors rely on angiogenesis to sustain increased metabolic activity. Microbubbles enter the tumor vasculature. At sufficiently high amplitudes, ultrasound induces significant vascular damage, shutting down blood flow. The vessels develop and harbor hypoxic regions, causing oxidative stress; lack of nutrients and increased acidity induce apoptosis. Image courtesy of [50].

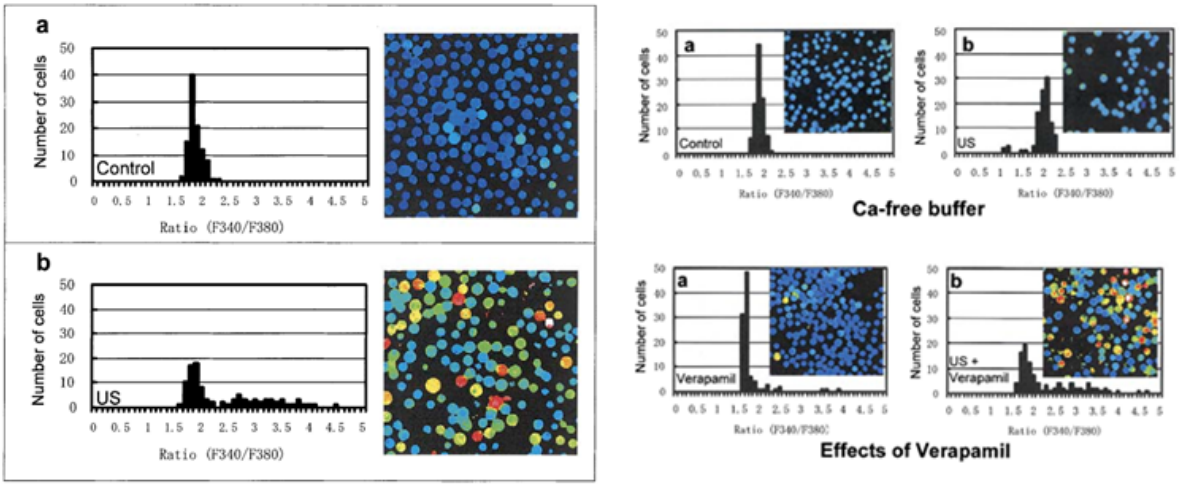
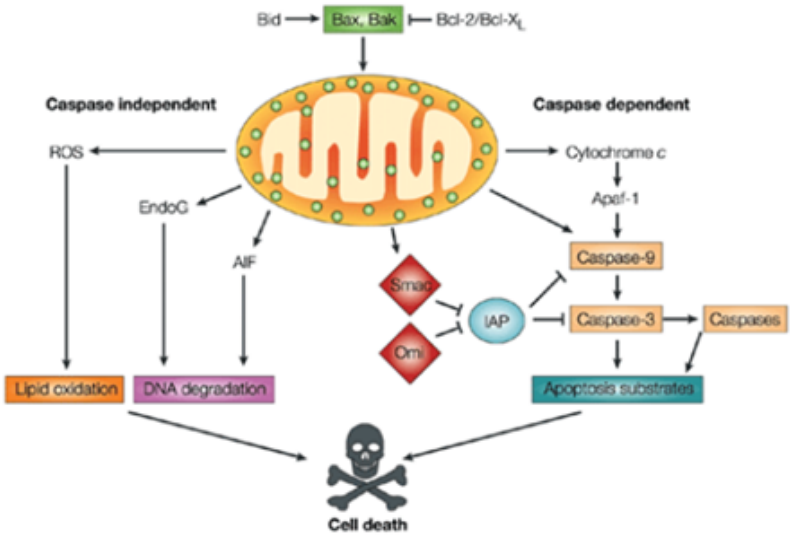


Fig. 4: Effect of Ultrasound on Intracellular Ca^{2+} Concentration. Graphs on the left: Fura-2-loaded U937 cells were sonicated; then had Ca^{2+} concentrations examined. The histogram of 100 randomly selected cells immediately after sonication showed that the number of cells with higher Ca^{2+} increased in the sonicated sample. Graphs on the top right: To explore whether the increase in Ca^{2+} induced by sonication was due to inflow from outside of cells or release from intracellular store sites, the cells were sonicated in HEPES buffer containing 1 mM ethyleneglycoltetraacetic acid (EGTA). When cells were sonicated under the condition without Ca^{2+}, no significant increase was observed. Graphs on the bottom right: Verapamil, a known voltage-dependent Ca^{2+} channel blocker was utilized. When the cells were sonicated in the presence of verapamil at concentrations of 1, 10 and 100M, a similar increase in Ca^{2+} was observed. This influx occurred regardless of the presence or absence of verapamil. The pseudocolor image and histogram of Ca^{2+} are shown. These results indicate that sonication induces the rapid increase in Ca^{2+} inflow from outside of the cells which appears to be independent of the voltage dependent Ca^{2+} channel. Graphs courtesy of [19].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Caspase independent
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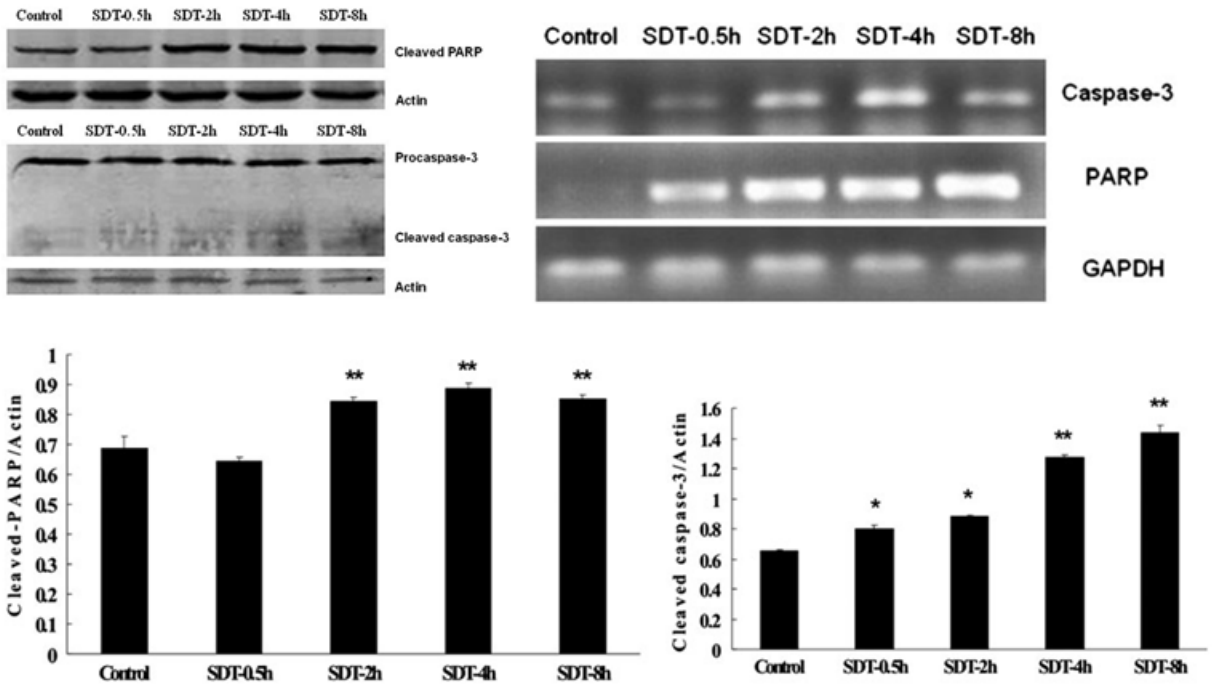
DNA degradation
Apoptosis substrates
Cell death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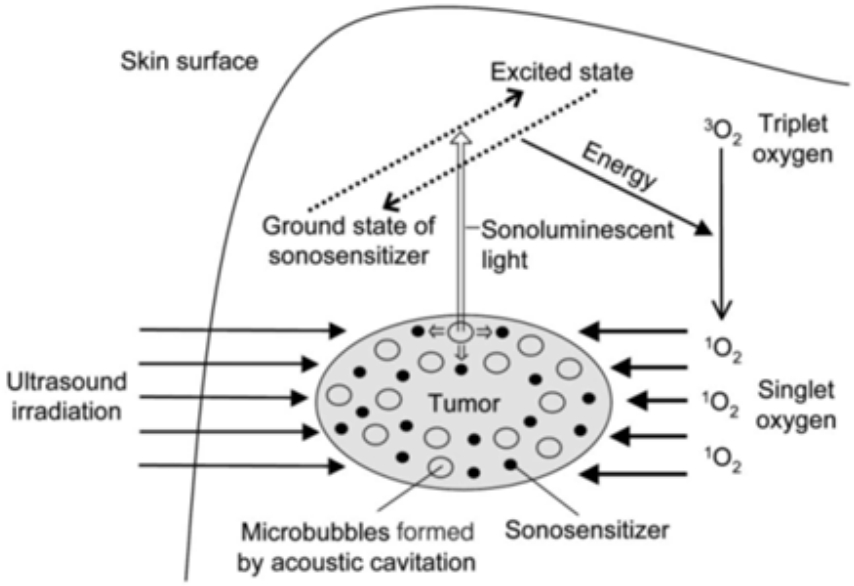

Fig. 7: Mechanism of ultrasound induced sonoluminescence. The energy provided by the collapse of microbubbles allows sonoluminescent light to be produced within the cell. The light subsequently activates endogenous compounds within the cell that release ROS when returning to the ground state. Image courtesy of [7].

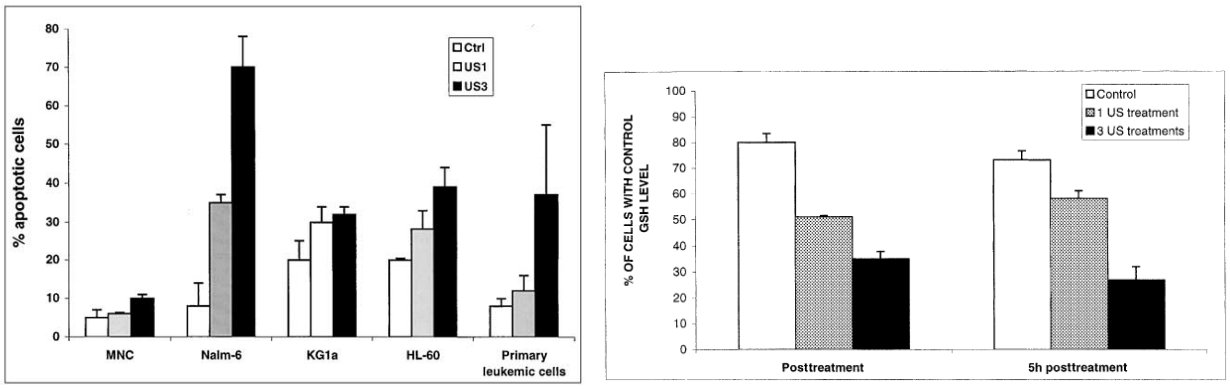

Fig. 8: Ultrasound alone damages multiple leukemia cell lines. Graph to the left: Apoptosis was evaluated by annexin/PI assay 5 hours posttreatment directly in the case of cell lines but after a CD45 gating strategy from primary blast cells. Results are mean SEM of 5 independent experiments. Important to note: normal mononuclear cells (MNC) show significantly less damage than leukemia cell lines, suggesting preferential damage can be attained for malignant cells. Graph to the right: Intracellular GSH content was evaluated by the method described in [15]. Dead cells that had lost the capacity to exclude propidium iodide were gated out from glutathione analysis. Data are expressed in percentage of cells displaying glutathione level comparable to untreated cells. Values are mean SEM of the data from three independent experiments. GSH is an important ROS buffer, providing a possible explanation of the increased ROS content in malignant cells. Graphs courtesy of [15].

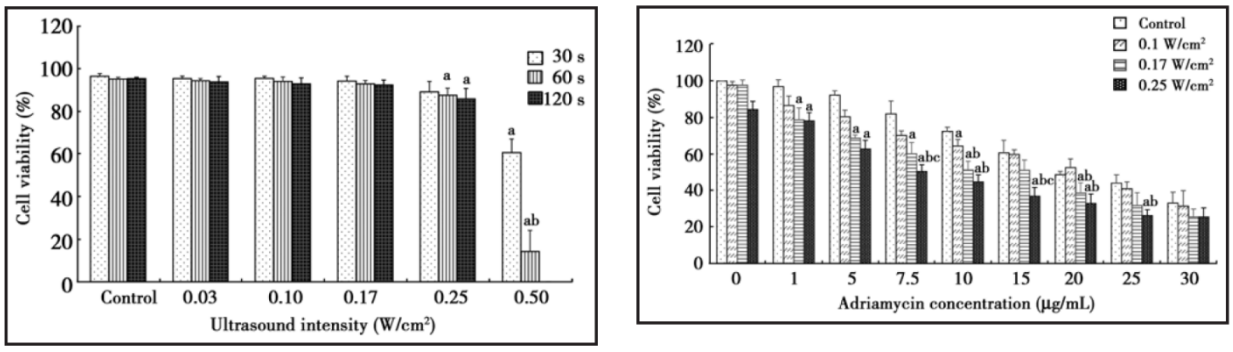

Fig. 9: The synergistic effect of ultrasound/DOX (adriamycin) on K562/A02 cells. Graph on the Left: Effect of ultrasound at various intensities and duration on the cell viability of K562/A02 cells. Results are presented as mean ± SD of three independent experiments. (A) $p < 0.05$ vs. control group; (B) $p < 0.05$ vs. 30 s group. Graph on the right: Effects of ultrasound and DOX on the viability of K562/A02 cells. Results are presented as mean ± SD of four independent experiments. A) $p < 0.05$ vs. control group; (B) $p < 0.05$ vs. 0.1 W/cm^2 group; (C) $p < 0.05$ vs. 0.17 W/cm^2 group. There is a considerable drop in cell viability when ultrasound/DOX treatments are applied. The cell line was shown to be completely resistant to DOX-alone before ultrasound treatments as indicated in [20]. Graphs courtesy of [2].

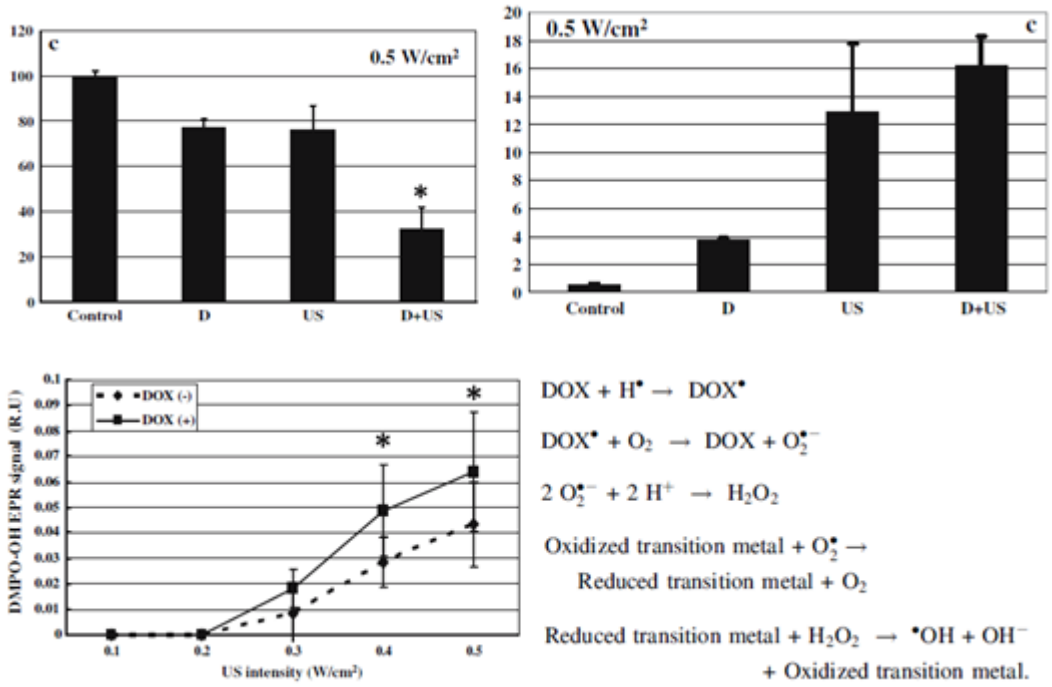

Fig. 10: Doxorubicin (DOX) significantly damages U937 cells through a ROS mechanism. Top left: Enhancement of DOX-inducing cell killing by US. In the DOX + US treated group, the cells were exposed to 5 µM DOX for 30 min and then sonicated at intensities of 0.2, 0.3, 0.5 W/cm^2 for 60s (0.5 W/cm^2 shown here). Cell survival was evaluated by Trypan blue dye exclusion test 6 h after sonication. The data indicate the mean ± SD calculated from more than four different experiments. Asterisk assessed as synergy by two way factorial ANOVA. Top right: Enhancement of DOX-inducing apoptosis by US. The cells were collected after a 6 h culture and subjected to flow cytometry after staining with FITC labeled Annexin V and propidium iodide (0.5 W/cm^2 again shown here). Data indicate mean ± SD calculated from more than four different experiments. Bottom left: Effect of DOX on producing free radicals by US. An aqueous solution with or without 5 µM DOX was sonicated for 1 min at intensities from 0.1 to 0.5 W/cm2. The OH• formation was detected on EPR using 10 mM DMPO as a spin-trapping agent. Data indicate mean ± SD calculated from more than six different experiments.*P < 0.05 (Student's t-test). Bottom right: The proposed mechanism of DOX free radical generation. Graphs and image courtesy of [34].

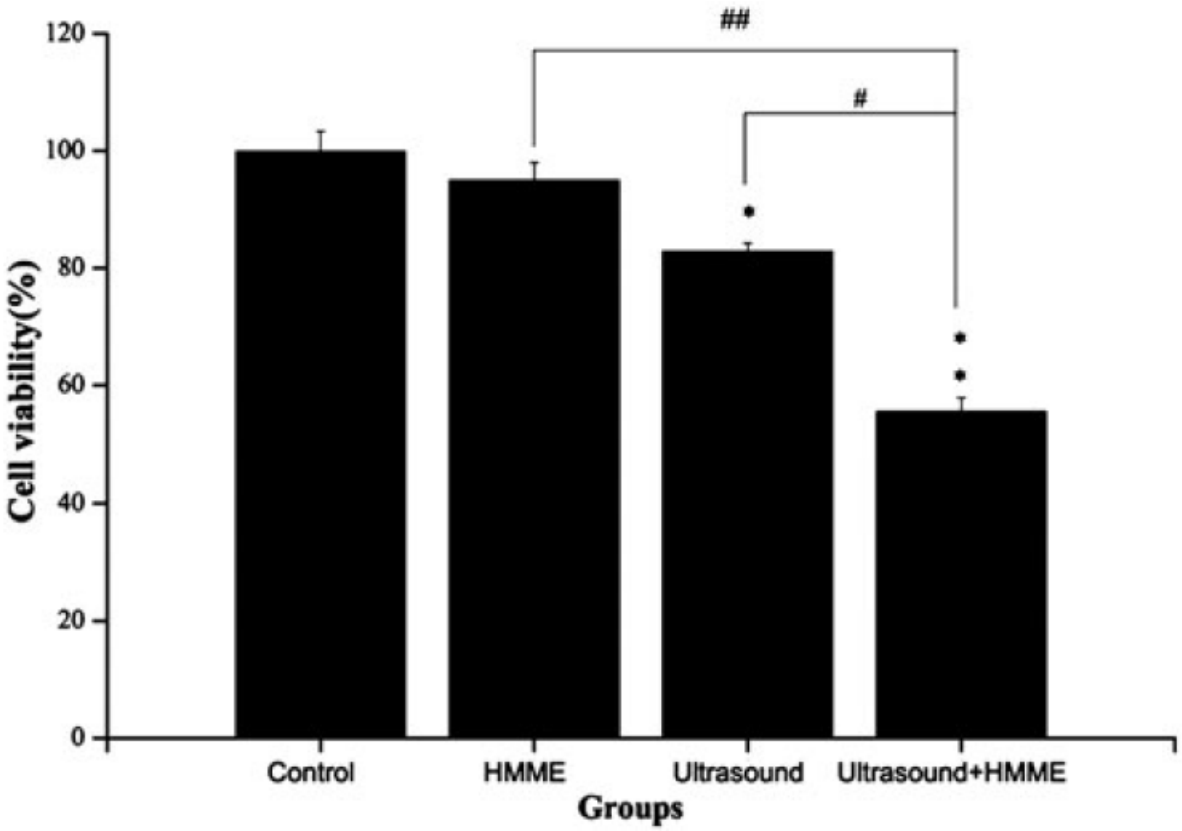

Fig. 11: The synergistic effects of ultrasound/HMME on U937 cells. The viability of U937 cells at 4 hours after Ultrasound/HMME was assessed by MTT assay. The control was cells without any treatment. Cells were treated with 10µg/ml HMME in alone and with ultrasound experiments. Cells were irradiated with 1W/cm^2 ultrasound alone. Ultrasound + HMME, cells were irradiated with 1W/cm^2 ultrasound. *$p < 0.05$, **$p < 0.01$ versus untreated controls. ##$p < 0.01$, versus HMME. #$p < 0.05$, versus ultrasound. The treatment halved cell viability in comparison to the control group. Graph courtesy of [27].

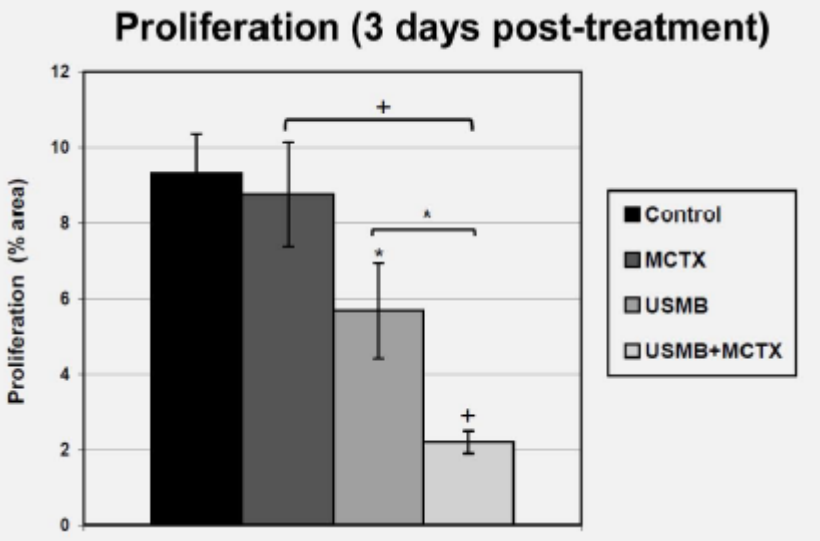

Fig. 12: Cell proliferation is dramatically decreased with ultrasound/MCTX. The 3-day MDA-MB-231 cell proliferation results indicate that the combined USMB(Ultrasound Microbubble)/MCTX treatment group has significantly lower cell proliferation levels than the control and individual treatment groups. *, **, and + indicate p values of less than 0.05, 0.01, and 0.001 for differences between means of groups; symbols immediately above bar indicate significance with respect to controls. Image courtesy of [46].

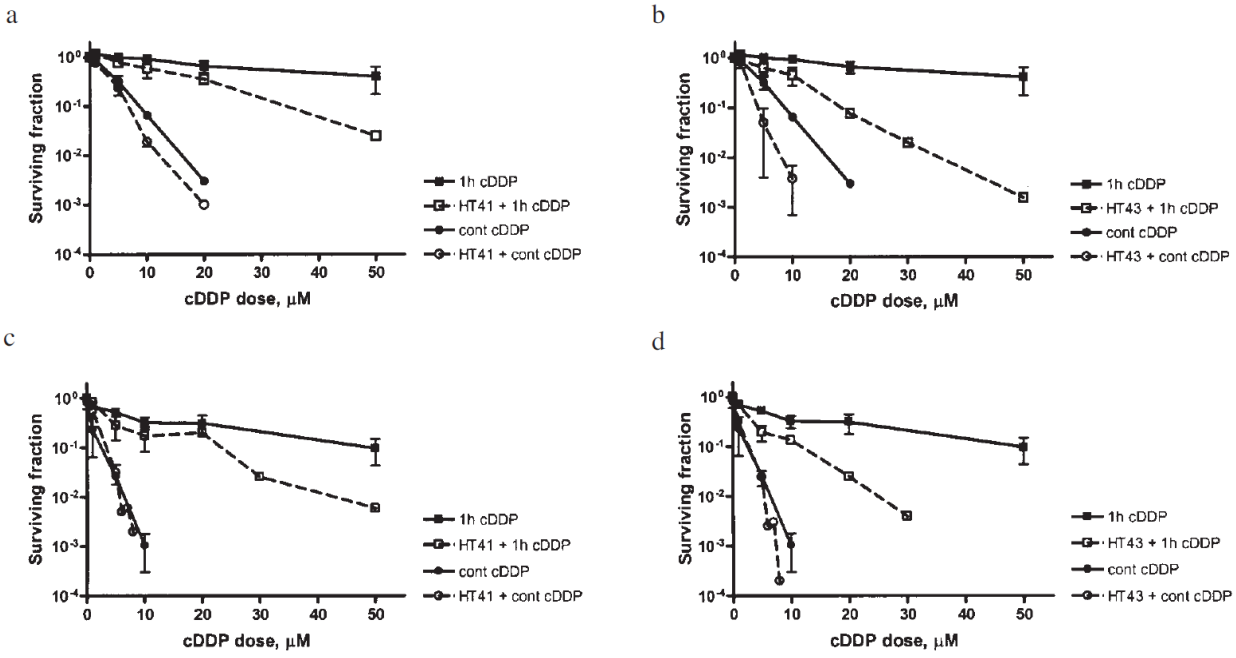

Fig. 13: Mild Hyperthermia Increases the Efficacy of Cisplatin. (a) Survival after 41˚C hyperthermia and cisplatin in SW-1573 cells. (b) Survival after 43˚C hyperthermia and cisplatin in SW-1573 cells. (c) Survival after 41˚C hyperthermia and cisplatin in SiHa cells. (d) Survival after 43˚C hyperthermia and cisplatin in SiHa cells. Mean ± SEM are shown for at least three separate experiments. Each cell line has the lowest survival rate when mild hyperthermia is applied with cisplatin Graphs courtesy of [67].

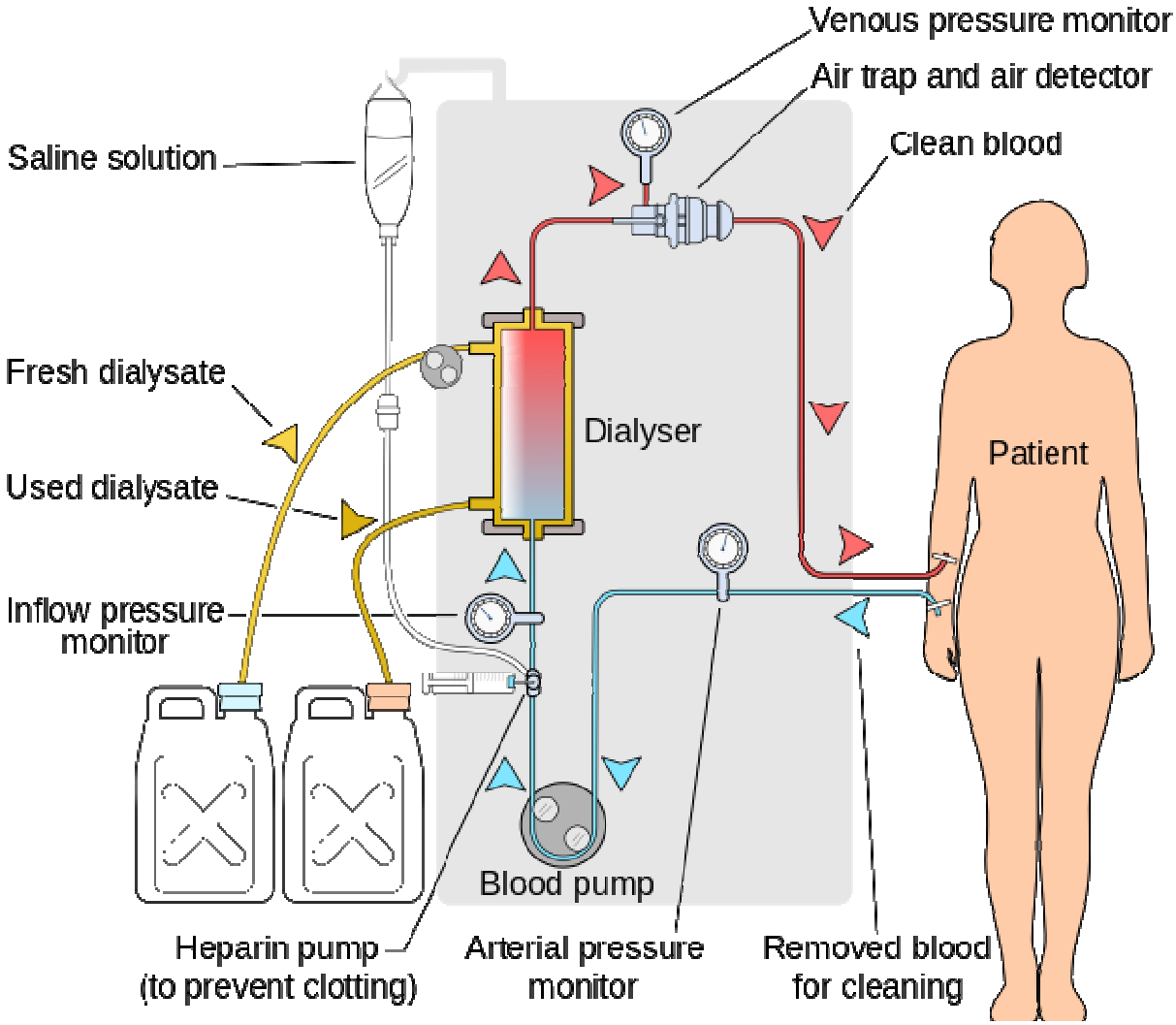

Fig. 14: Extracorporeal Blood Sonication. Hemodialysis requires the patient's blood to be pumped outside of the body into an extracorporeal setting. This provides an opportunity for leukemia cells to be sonicated without sound attenuation from anatomical structures. Sound intensities would likely be reduced as there is only a tube standing in the way between the US waves and the patient's blood. Published in [50].

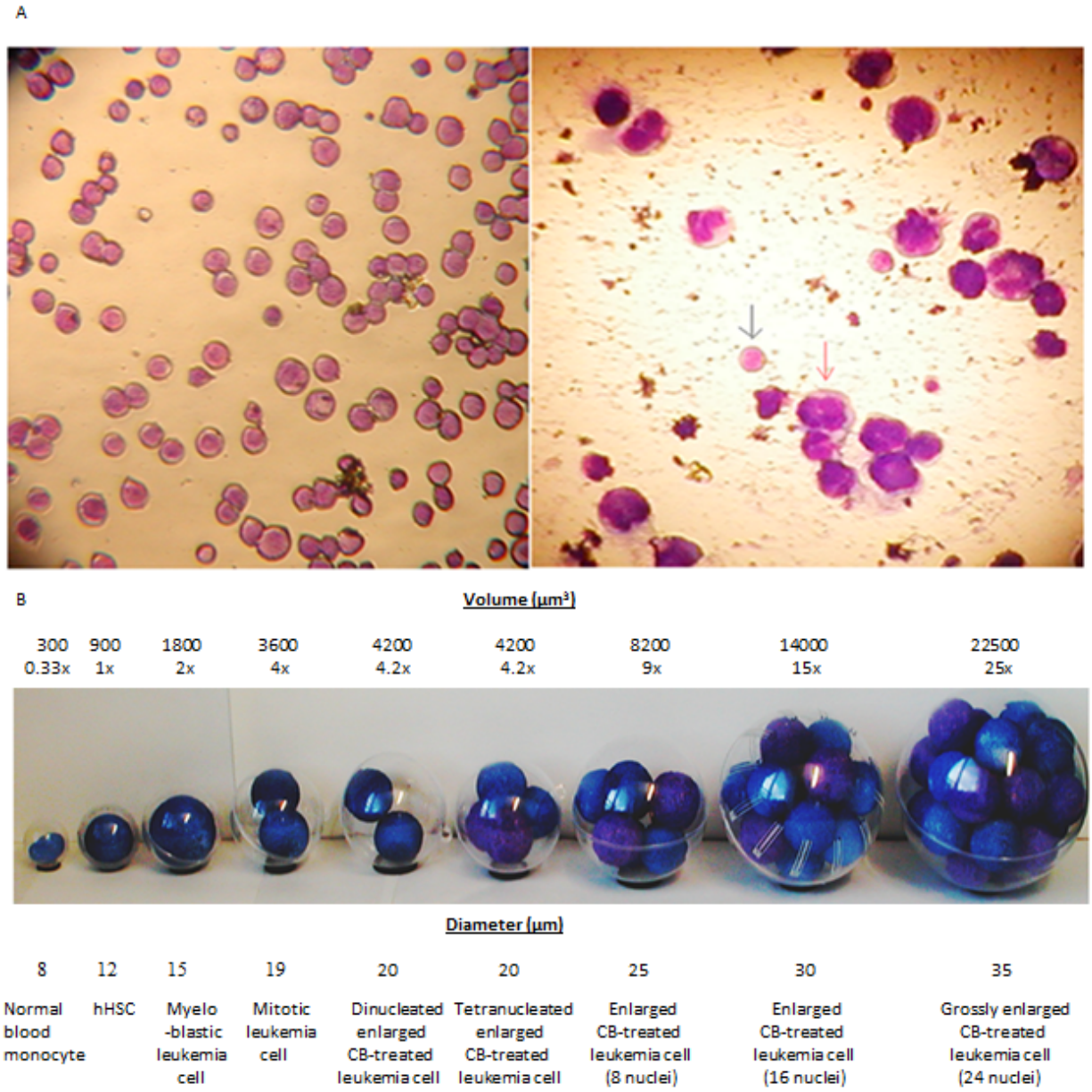

Fig. 15: Comparison of U937 cells after treatment with cytochalasin B. Image to the top left: Typical U937 cells that have not been exposed to any agents (13-18µm in diameter). Image to the top right: U937 cells treated with cytochalasin B at 1.5µM. The cells become grossly enlarged and multinucleated (19-40µm in diameter) Image on the bottom: A model of the size differential between blood cells and leukemia cells treated with cytochalasin B. While normal leukemia cells are approximately 15µm, leukemia cells treated with cytochalasin B can grow to 35µm or larger. Such cells are substantially more sensitive to US than normal leukemia cells. The additional nuclei suggest that nucleic acid agents could be coupled with cytochalasin B to further increase the efficacy of US treatments in the clinical setting. Nuclei were visualized with Wright-Giesma stain at 100x magnification. Published in [44].

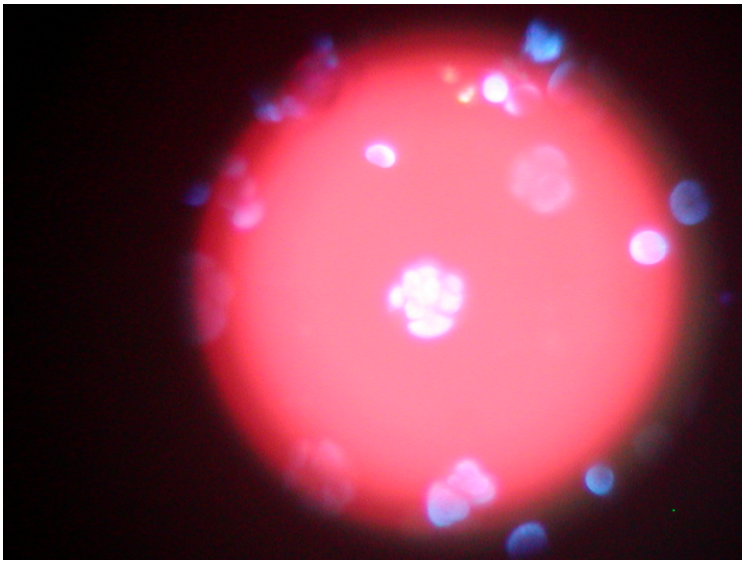

Fig. 16: DAPI staining of cytochalasin B treated U937 cells 48 hours after administration. DAPI was chosen for nuclei analysis as it passes through intact cell membranes. Therefore, it can be used to stain both live and fixed cells; necessary for visualizing the nuclei of cytochalasin B treated cells as they do not readily undergo apoptosis in the absence of ultrasonic irradiation. DAPI staining confirmed the extent of multinucleation in treated cells. Published in [44].

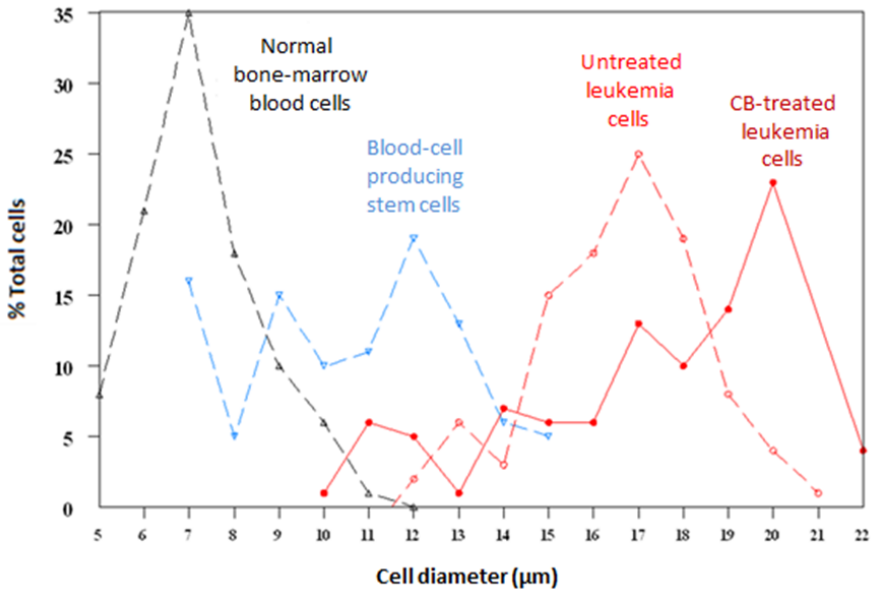

Fig. 17: Size distribution of blood cells. Flow cytometry and both cell counters confirmed a significant shift in U937 cell size 48 hours post-cytochalasin B (CB) administration. Therefore, the already significant difference in size between leukemia cells and normal blood cells becomes exceedingly amplified. Note: the cytochalasin B cells were still undergoing mitosis after 48 hours suggesting the size differential could be further increased if the cells were incubated further before sonication. Further incubation periods have produced U937 cells in excess of 40µm. Published in [44].

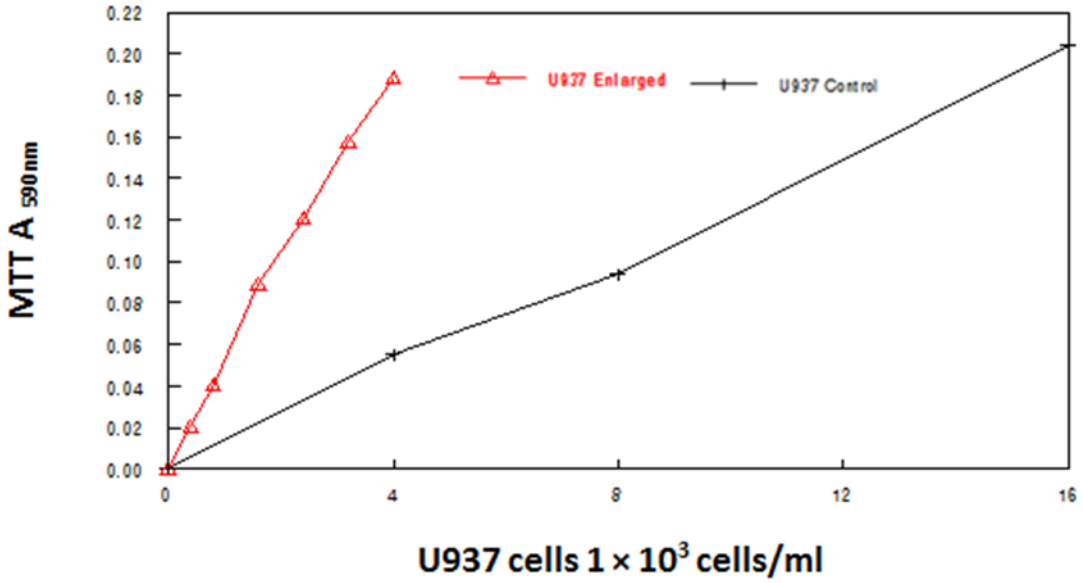

Fig. 18: MTT assay of U937 control and cytochalasin B treated cells. U937 controls (no cytochalasin B administered) and enlarged cells were assessed for mitochondrial activity using MTT assays. Cytochalasin B treated cells had about a 4-fold absorbance increase at 590nm, indicating enhanced number or activity of mitochondria. The increased mitochondrial activity coincides with the 4 times DNA content on average. U937 cells were seeded at 1 x 10^3 cells/ml for accurate MTT readings. Published in [44].

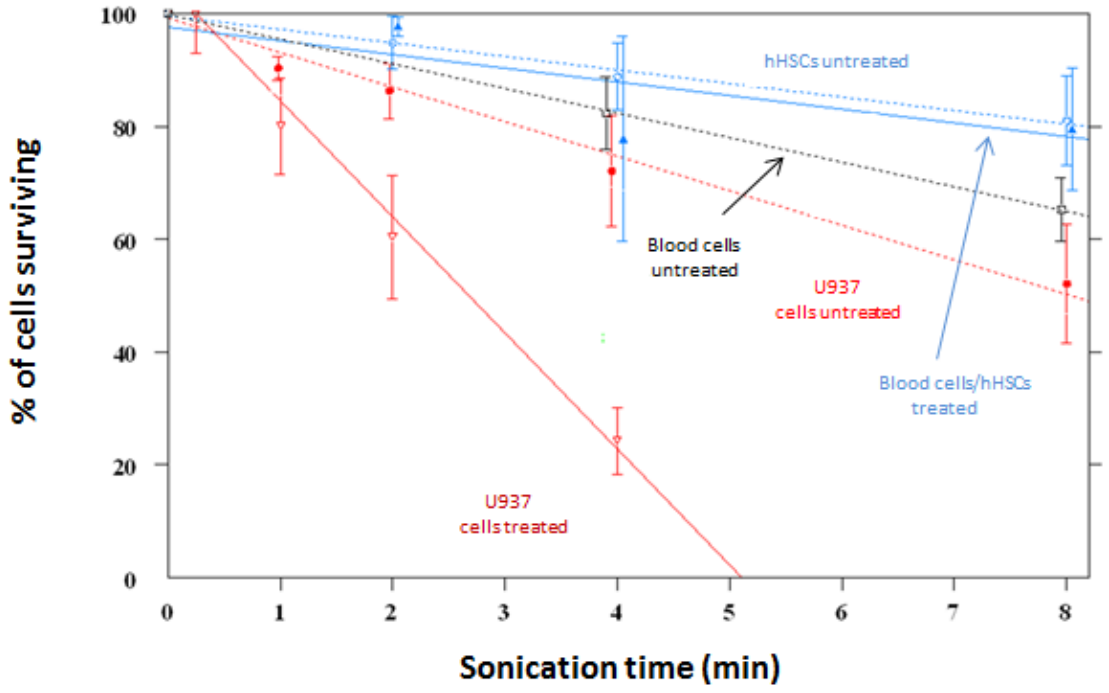

Fig. 19: Sonic sensitivity of cytochalasin B treated U937 cells. Although normal blood cells do have sensitivity to 3W/cm^2 of ultrasound, it was minor compared to the profound sensitivity of U937 cells. It is important to note that cytochalasin B drastically increased the efficacy of sonications as most U937 cells were deemed non-viable by Trypan Blue staining after 4min of sonication. Published in [44].

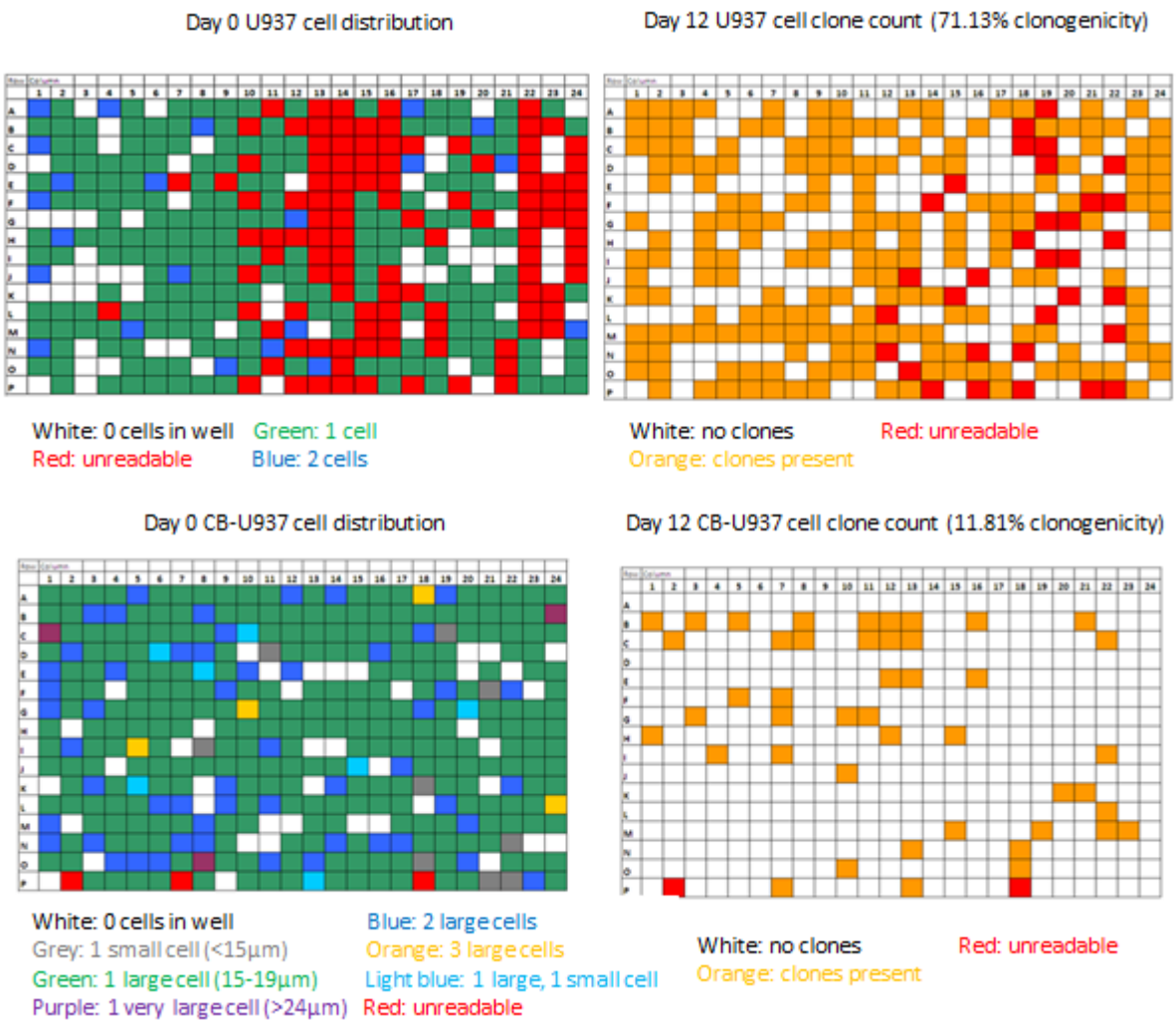

Fig. 20: The effects of cytochalasin B on U937 cell clonogenicity. Top row represents cells in the control, while the bottom row represents cells treated with cytochalasin B. U937 cells treated with cytochalasin B exhibited a markedly reduced ability to proliferate when compared to nontreated cells after the 12 day incubation period. Published in [44].