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Determining Therapeutic Efficacy of Low-frequency Ultrasound in Targeting In-Vitro Human Attached Cell Lines with Adjuvant Chemotherapy

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Determining Therapeutic Efficacy of Low-frequency Ultrasound in Targeting In-Vitro Human Attached Cell Lines with Adjuvant Chemotherapy

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Honors Thesis Project in Biochemistry

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Abstract

Research in the Fondy laboratory at Syracuse University has shown that in free-moving cancer cell lines, such as leukemia and lymphoma, larger cells are preferentially targeted by ultrasound therapy and have reduced viability as a result. Little time with respect to ultrasound, however, has been dedicated to the study of cell lines such as colorectal carcinoma and glioblastoma, which require connective tissue to grow.

Our research involves the low-frequency ultrasound treatment of attached carcinoma cell lines with adjuvant chemotherapy to evaluate an effective regimen for reducing viability of cancer cells. Prior research with human glioblastoma has shown that cells attached to a polystyrene plate are readily detached from their connective surface if provided with enough cavitation power from sonication. We plated, sonicated, and determined cell viability and number of RKO (human colorectal cancer) and hLN18 (human glioblastoma) in the 20kHz frequency range to evaluate effectiveness of sonodynamic therapy. These procedures were augmented by treatment with cytoskeleton-directed agent cytochalasin B (CB) either several days prior to or immediately following treatment to determine if sonication improves the chemotherapeutic properties of CB and vice versa. We additionally compared the sonication of RKO to cell detachment with trypsin, the latter of which is a technique commonly used in passaging attached cell lines, to determine if a statistically significant difference between the techniques implies a usefulness for sonodynamic treatment.

Early results imply that ultrasound significantly reduces cell viability when concentrated over a small region of cells. While the risk of metastasis is not known, we have assayed for apoptosis in sonicated cells to determine the proportion of cells at risk for metastasis. Trypsin treatment is near statistically significantly different for reducing viability compared to sonication with a relatively small sample size.

Executive Summary

Ultrasound has a storied history, both in nonmedical and medical use. From submarine warfare to fetal imaging, and in animal echolocation, the uses of ultrasound are well-known to most. As new applications are developed for ultrasound via biological research, the Fondy laboratory at Syracuse University has been conducting its own investigation in the efficacy of low-frequency ultrasound and its medical applications.

Most of the research in the Fondy laboratory is dedicated to the investigation of low-frequency ultrasound's effect on unattached cell lines (most commonly leukemias and lymphomas). My research involves the low-frequency ultrasound treatment of attached carcinoma cell lines to evaluate an effective regimen for reducing viability of cancer cells. These lines include RKO (a human colorectal carcinoma line) and hLN18 (a human glioblastoma cell line). Prior research in the Fondy laboratory with hLN18 has shown that cells attached to a well are readily detached from their connective surface if provided with enough energy from sonication. It is likely that cavitation bubbles from the ultrasound are responsible for damaging cells as well as this detachment.

Additionally, as cell detachment removes cells from its point of attachment (that is, tissue required for growth) while increasing cell surface area for treatment, chemotherapy should be more effective if used directly following sonication of cells. To test these efforts, we have thus far been using cytochalasin B (CB), a chemotherapeutic agent that increases cell size and creates multinucleated cells by cytoskeleton binding. These cells, which are larger and irregular, are more sensitive to our low-frequency ultrasound treatment methods.

To maintain the cell lines involved in these experiments, we commonly use trypsin, a proteolytic enzyme, to temporarily detach cells for movement into new containers. This is incidentally also how we prepare sample wells for sonication. For our experiments we prepared five types of samples: untreated controls, controls treated with only CB, experimental samples treated with only ultrasound, experimental samples incubated with CB two days prior to ultrasound, and experimental samples treated with CB immediately following ultrasound. These samples would be detached again with trypsin after three days of incubation, allowing us to use a cellometer and take counts of cell number and cell viability (a ratio of living cells to total cell number). Cells are determined as living by their ability to pump out trypan blue, a common cellular stain (dead cells cannot remove the stain and remain colored). The main goal of these experiments was to determine at which power of ultrasound there would be a statistically significant difference between the experimental samples, especially between the ultrasound-only and the ultrasound plus CB samples.

Additional experiments were conducted to determine if there was a statistically significant difference between trypsin detachment and ultrasound sonication: naturally, if there is no difference in cell viability, there is no reason to sonicate.

At this point, samples have shown a degree of possible significance, albeit with relatively small sample size. If our continued research appears significant, we can proceed to implement ultrasound in 3D cellular culture and eventually in-vivo research. Given that other in-vivo clinical studies with magnetic resonance-guided focused ultrasound (MRgFUS) have produced exciting results, it is possible that the disintegration of tumors by ultrasound may be therapeutically possible and practical. To do so, we would need to ensure that our method of ultrasound does not significantly damage nearby cells and inhibit in vivo function. Such a

procedure would also be minimally invasive, and reduce the risk associated with major surgical procedures.

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Acknowledgements

I would like to acknowledge Dr. Thomas Fondy and Thomas Stack Jr. for their supervision and support of this project. I would also like to thank the Biology Department of Syracuse University and the Thomas P. Fondy Cancer Research Fund for continued funding and interest in this and similar projects.

Advice to Future Honors Students

If you plan to pursue a thesis in the biological or chemical sciences, don't be afraid. Many will tell you that you should enter a lab as early as possible, and this is good advice. The intimidating aspect of the research comes into play at this point. How does one reach out to find their way into a laboratory?

Most professors in biology and chemistry at Syracuse University, and at most research universities nationwide, are published researchers and actively research as part of their position. It can be intimidating, especially for students who suffer from anxiety, to reach out to these professors and attempt to become involved in their research. There are several steps that students can take to involve themselves in undergraduate research.

1. Do not be afraid to reach across the aisle. There is not an issue with a biology student completing capstone research in chemistry. The inverse may be somewhat more complicated, as there must be a significant element of chemistry involved in a biology-oriented capstone, but there is no reason that a student in chemistry cannot complete a capstone in a biology laboratory.
2. Read published work from professors who you may be interested in working for. This is an arduous process, as a freshman or sophomore student may not have the course prerequisites to fully understand what they read. That said, this is the best way to discover what may be personally interesting to a student.
3. Reach out to all professors that you may be interested in researching under. Not all professors will be able to take on a new undergraduate researcher due to the size of their lab or the constraints of their research. It is best to reach out early in the semester, especially in the fall, as this is when labs are least likely to have filled positions.
4. Most importantly, become involved in research as early as possible in your undergraduate career. Even if, as a freshman or sophomore, you do not fully understand the theory behind much of your research, you will become experienced and respected the longer you work in your laboratory. If you find you do not enjoy your research or find that it is not especially rigorous, you will have time to change. A good laboratory is one that conducts rigorous research, but not all labs and professor tailor well to all students. Research should be a unique experience that should not only be of use to academia or society: it should be interesting and educational for all parties involved.

Introduction

A Brief History of Ultrasound

Ultrasound is defined as any inaudible sound wave which has a frequency, and therefore energy, higher than that of the audible spectrum. Application of ultrasound as a medical therapy is not a new idea, and its applications cross a wide variety of disciplines. Implementation of medical ultrasound began in the early 19th century with the characterization of light phase shifts by Thomas Young [1]. The implementation of obstetrician Dr. Ian Donald's 2D imaging Diasonograph tool for abdominal scanning in the 1950s foreshadowed modern gynecological techniques as the first relatively practical diagnostic tool utilizing ultrasound [1]. While unwieldy, Young's affectionately known "Dinosaurograph" effectively produced moving 2D images of abdominal tumors and fetuses [1]. Since then, application of MHz range ultrasound as a diagnostic tool has expanded to cardiology, orthopedics, and several other fields of note [2].

In the Fondy laboratory at Syracuse University, our primary concerns with ultrasound are with its therapeutic capabilities rather than its history as a diagnostic tool. In this, too, there is a great deal of medical precedent. The noninvasiveness of high-intensity focused ultrasound (HIFU) make it an ideal target for clinical study [3,4]. A technique that supplements HIFU with magnetic resonance imaging (MRI), called magnetic resonance-guided focused ultrasound (MRgFUS), increases the ability of clinicians and researchers to target biological samples with greater specificity [4,5]. Research into this topic has generated a great deal of clinical possibilities, including the ablation of tumors, disintegration of kidney stones, and creating transient openings in the blood-brain barrier for treatment of neurologic disorders such as Alzheimer's disease [3–5].

While not relevant to our research, it is worth noting that ultrasound is certainly not novel to medicine. Paul Langevin applied the inverse of the piezoelectric effect in 1915 to generate ultrasonic waves intended to locate submarines for military purposes [1]. Neither is ultrasound even of human origin: bats and other animals are known to use ultrasound echolocation to improve identification of surroundings.

Research Background

Ultrasound has been observed to lyse cells in medium by one of two methods: thermal ablation and microbubble cavitation [3,6,7]. The former is of interest in most prior HIFU research, but the Fondy laboratory is primarily concerned with the latter. The primary concern with ultrasound cavitation is the introduction of stress in cytoskeletal filaments. Cavitation has been shown to disrupt the actin filament network transiently, increasing the ability of adjuvant chemotherapy targeting actin filaments to disrupt cellular activity. Additionally, the rearrangement of cytoskeletal structure and introduction of cellular damage, including loss of phosphatidylserine (PtdSer) asymmetry and decreased glutathione (GSH) levels, stimulate apoptosis in leukemia cells [6]. PtdSer, normally present in the interior side of cellular double membranes, is normally moved via flippase enzymes to signal phagocytosis of an apoptotic cell [8]. GSH is an amino acid trimer that is oxidized to a dimeric form (GSSG) by reactive oxygen species (ROS) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) synthesized via the pentose phosphate pathway [9]. ROS are known for their ability to interfere with cellular reactions by the introduction of free radical species, so the presence of GSH acts to protect against cellular oxidation, while its absence may increase risk of apoptosis.

Prior unpublished experiments in the Fondy laboratory have indicated a tendency of hLN18 glioblastoma to detach from its growth plate when subjected to enough power under ultrasound. This would imply that ultrasound disrupts the expression of E-cadherins without allowing cells to undergo the mesenchymal transition, which would normally increase the risk of metastasis [10]. Thus, any cells that are detached by method of ultrasound should theoretically not increase metastasis risk.

The detachment of attached cells may reduce cell viability by a few proposed mechanisms. One is that, given that attached cells do not grow freely without basilar tissue (unless they have expressed the mesenchymal transition and begin to metastasize), the formerly attached cells will no longer be able to grow properly and will cease to perform cellular activities at a rate that will sustain life. Bursting of cavitation microbubbles may also introduce cell damage and lysis on their own, independently of cell detachment. Lastly, with cells that are separate and moving apart from each other, there is an increased surface area present for uptake of chemicals in the cellular environment. While this does not directly affect cell viability, it does create a possibility of increased uptake of adjuvant chemotherapy. With these possibilities in mind, we began to treat RKO human colorectal carcinoma and hLN18 human glioblastoma with low-frequency ultrasound in determining efficacy and stability of ultrasound treatment.

As an added measure of efficacy, we have also considered that cavitation microbubbles ought to have a stronger effect in reducing cell viability than standard methods of detaching cells. For this purpose, we can compare the ultrasound treatment to cellular detachment with trypsin, a proteolytic enzyme common to all cells.

Materials and Methods

Materials

For application of ultrasound therapy, experiments were conducted with a 20 kHz sonotrode with an 8 cm horn and enclosure. This sonotrode has a variable amplitude that could be varied by the experimenter. Cell lines samples were plated in 6 cm polystyrene-treated single wells. To standardize sonication conditions, Spectra 360 electrode gel was placed between sonotrode horn surfaces and wells. Cellular imaging was accomplished using 100x light microscopy and microscopic cameras.

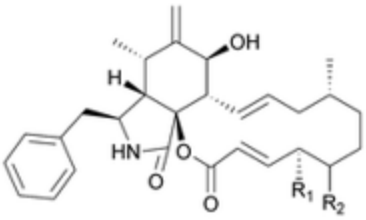
Cell lines evaluated include RKO colorectal carcinoma and hLN18 human glioblastoma. Cell lines were treated with cytochalasin B (CB), a cytoskeletal directed agent that interacts with the actin cytoskeleton. Cell counts and cell viability readings were taken with a Countess II Cellometer.

For apoptosis assay, we used an apoptosis detection kit containing 4X binding buffer, annexin-V fluorescein isothiocyanate (FITC) stain, and propidium iodide (PI) stain.

Important Biochemical Considerations

Our complete growth medium was comprised of Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum, L-alanyl L-glutamine, penicillin, streptomycin, amphotericin B, and gentamicin sulfate. The several antibiotics present in our medium either interfere with peptidoglycan formation, inhibit mRNA translation, or otherwise prevent bacterial growth from contaminating the plates [9].

Cytochalasin B (CB) is a member of a class of myocotoxins (polyketide toxins derived from fungi) with a wide variety of properties. CB was first described in 1966, isolated from *H. dematioideum*, containing a macrolactone ring, a hydrogenated isoindolone moiety and a benzyl group (Fig. 1).



Cytochalasin B: R_1 = OH, R_2 = H

Figure 1. The structural characterization of the mycotoxin CB [11].

By acting on cellular microfilaments and actinomysin, CB inhibits the elongation of cellular filaments, slowing the growth of filaments and preventing cell division. This also results in the creation of large multinucleated cells as the cells fail to divide. The cytotoxic effects of this inhibited growth make most of the cytochalasins, including CB, effective for study as anticancer agents [11]. The Fondy laboratory considers the size of CB-treated cells to be a target of low-frequency ultrasound. Prior studies in our laboratory consider cell size to be an important characteristic in affecting cells with low-frequency ultrasound, with larger cells being more easily targeted [6]. Considering this, CB is both a useful molecule in increasing the effectiveness of low-frequency ultrasound and determining if detachment via ultrasound has a greater effect on the chemotherapeutic cytotoxicity of CB.

Figure 2. The structural characterization of trypsin in the human brain. Note the polar interactions stabilizing the active site residues, His57 (center), Asp102 (left), and Ser195 (right) [PDB: 1H4W].

Trypsin is a serine protease in the chymoytrypsin family of enteropeptidases. It contains the serine catalytic triad common among this class of peptidases, consisting of a serine to attack a peptide carbonyl, a histidine to activate the serine for nucleophilic attack and an aspartate to stabilize the histidine residue. Trypsin initially has a negatively charged Asp-189 in its binding pocket, attracting positively charged arginine and lysine residues for proteolysis [9,12]. By proteolytic activity on

cadherins binding the cells to the polystyrene plate, trypsin temporarily disrupts cell attachment for the purpose of cell passaging [10].

Fluorescein isothiocyanate (FITC) is fluorescent dye modified with a reactive thiocyanate nucleophile. FITC is typically used as an antigen-binding protein, but it can be conjugated to other proteins and used for detection of the conjugated proteins, much like green fluorescent protein (GFP) and similar fluorescent labels [13]. Of importance to us is the ability of FITC to complex with annexin-V, a protein that bind to phosphatidylserine in the apoptotic response through a Ca^{2+}-mediated salt bridge [14]. Binding buffer facilitates this interaction, but the annexin-V FITC complex is essential to detecting the phosphatidylserine expressed in late-stage apoptosis.

Figure 3. The structural characterization of annexin V from rats. Phosphatidylserine interacts with exposed Ca^{2+} ions (grey), signaling an apoptotic cell [14; PDB: 1A8A].

Propidium iodide (PI) is useful for the detection of apoptotic cells. By intercalating in DNA of apoptotic cells with permeable membranes, PI dyes the DNA of these cells, making them readily detectible with flow cytometry. PI does have the disadvantage of being capable of invading late-stage necrotic cells and any other cells with disrupted membranes (including cells lysed by low-frequency ultrasound), but due to the short length of our experiments this should not be a significant concern [15,16].

Methods

RKO and hLN18 Cell Culture

Cell lines were initially incubated in a flask containing IMDM with 10% fetal bovine serum, kept in a cellular incubator at 5% CO_2 and 37^{o}C for several days. These cells were later passaged into wells using trypsin to detach and move the cells. After 24 hours, most cells

reattached to the wells and began to grow. Treatments began when the cells began to cover at least half of the bottom of each well (usually 3 to 4 days).

Sonication of Attached Cell Lines

Five sample types were used in this experiment as follows:

1. A control sample that remained untreated.
2. A control sample that was treated with 2 μM CB, but not sonicated.
3. An experimental sample that was not treated with CB but was sonicated at a variable amplitude at 20 kHz for 6 0.5s on/0.5s off pulses.
4. An experimental sample that was sonicated at the same conditions as the third sample, followed immediately by treatment with 2 μM CB.
5. An experimental sample that was treated three days prior to sonication with 2 μM CB, followed by sonication at the same conditions as the third sample.

To standardize cavitation, wells were sonicated with spectra gel placed between the sonotrode and well. Images were taken of samples before treatment, immediately after treatment, and 72 hours after treatment. After 72 hours of treatment, medium was removed from cell plates, plates were washed with phosphate-buffer saline (PBS) Cell counts were taken of samples 72 hours after treatment to evaluate cell viability.

Trypsin Detachment

A control sample was treated with 3.0 mL trypsin, while an experimental sample was sonicated with 80% amplitude (generating 0.26 W/cm^2 in cavitation) at 20 kHz for 6 0.5s/0.5s pulses. Images were taken of samples before treatment, immediately after treatment, and 72

hours after treatment. Cell counts were taken of samples 72 hours after treatment to evaluate cell viability.

Apoptosis Assay

Four sample groups were used for this experiment as follows:

1. A control sample of RKO that was detached using 3.0 mL trypsin.
2. A control sample of LN18 that was detached using 3.0 mL trypsin.
3. An experimental sample of RKO that was detached using sonication at 80% amplitude (0.26 W cm^{-2}) at 20 kHz for 6 0.5s on/0.5s off pulses.
4. An experimental sample of LN18 that was detached using sonication at 80% amplitude (0.26 W cm^{-2}) at 20 kHz for 6 0.5s on/0.5s off pulses.

Immediately after sonication, sonicated cell volume was balanced to 3.0 mL using Iscove's Modified Dulbecco's Medium (IMDM). All cells were centrifuged at 1000 rpm for 7 minutes and resuspended in 1.0 mL PBS in a washing step. Cells were again centrifuged at 1000 rpm for 5 minutes and resuspended in a mixture of 195 μL 1X binding buffer (created from a dilution of 4X binding buffer with distilled water) and 5 μL of FITC dye (a dye that binds proteins). Cells incubated in the dark for 10 minutes to react with the dye and centrifuged again at 1000 rpm for 5 minutes. Cells were then resuspended in 190 μL 1X binding buffer and 10 μL PI (a dye that binds DNA). The sample was read in a flow cytometer at Link Hall to determine the relative concentration of FITC and PI bound to free protein and DNA, respectively.

Results

Sonication of RKO Human Colorectal Carcinoma

Sonic treatment has the effect of reducing the regrowth of RKO colorectal carcinoma in medium. The cells are observed to detach from medium immediately after sonication, with observably reduced cell density (Fig. 4). Data from cell viability (the ratio of living cells to total cells) shows a downward trend of viable cells with increasing cavitation from sonication, but individual samples have variable results. (Fig. 5). Data from cell counts shows a downward trend in cell number with less variation between samples (Fig. 6). A graphical representation of these cell counts, with accompanying statistical analysis, shows a statistically significant difference between several experimental groups (Fig. 7, $\alpha = 0.05$).

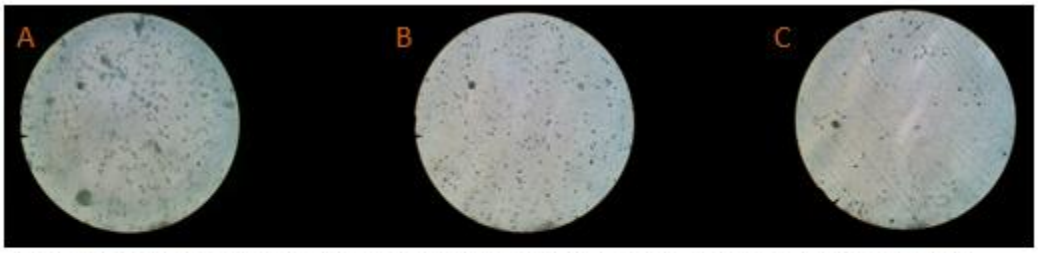


Figure 4. Imaging of RKO colorectal carcinoma with 100x light microscopy prior to ultrasound treatment (A), immediately following ultrasound treatment (B) and 72 hours after ultrasound treatment (C). Most cells are in motion immediately following sonication, indicating a detachment from the plate after sonication. Plate confluence is significantly lower three days after ultrasound treatment compared to the moments prior to sonication. Ultrasound was conducted with the 20 kHz sonotrode at 80% amplitude, producing 0.26 W cm^{-2} of cavitation.

Cell Viability of RKO Colorectal Carcinoma Samples	
Samples	Average Cell Viability (%)
Control (n = 9)	93.77319467
2μM CB only (n = 8)	88.00073738
Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) (n = 2)	95.663792
Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) -> CB (n = 2)	75.5991285
CB -> Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) (n = 2)	91.9791665
Sonic (0.26 W cm^{-2}, 60.5/0.5s) (n = 10)	79.1468639
Sonic (0.26 W cm^{-2}, 60.5/0.5s) -> CB (n = 8)	48.264107
CB -> Sonic (0.26 W cm^{-2}, 60.5/0.5s) (n = 4)	22.1153845

Figure 5. The average cell viabilities for all RKO colorectal carcinoma samples taken. Two sample readings were taken for each plate of RKO. A general trend of decreasing cell viability is present with increasing amplitude of sonication conditions as well as presence of CB treatment, though the viability of each individual sample tends to vary greatly.

Average Cell Number of RKO Colorectal Carcinoma Samples	
Samples	Average Cell Number
Control (n = 9)	1.71E+06
2μM CB only (n = 8)	1.55E+06
Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) (n = 2)	1.89E+06
Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) -> CB (n = 2)	1.79E+05
CB -> Sonic (0.195 W cm^{-2}, 6 0.5/0.5s) (n = 2)	4.08E+05
Sonic (0.26 W cm^{-2}, 6 0.5/0.5s) (n = 10)	8.02E+05
Sonic (0.26 W cm^{-2}, 6 0.5/0.5s) -> CB (n = 8)	1.39E+05
CB -> Sonic (0.26 W cm^{-2}, 6 0.5/0.5s) (n = 4)	3.81E+04

Figure 6. The average cell counts for all RKO colorectal carcinoma samples taken. Two sample readings were taken for each plate of RKO. A general trend of decreasing cell number is present with increasing amplitude of sonication conditions as well as presence of CB treatment.

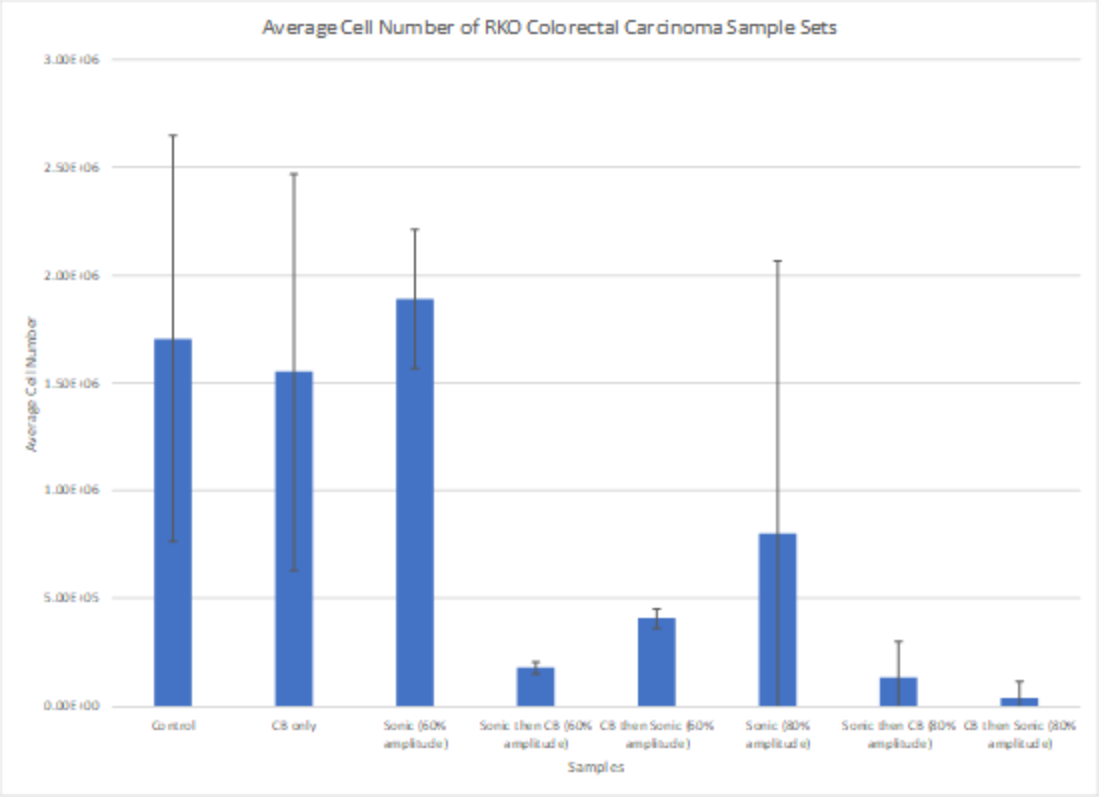


Figure 7. The average cell numbers for all RKO colorectal carcinoma samples taken. Sonication alone is a greatly variable treatment method, but with the exception of low-frequency ultrasound at 60% amplitude alone, all results are statistically significantly lower than their respective controls. Additionally, CB treatment after sonication is significantly preferred at lower amplitudes and CB treatment prior to sonication is significantly favored at higher amplitudes. Sonication before CB treatment is only significantly stronger than sonication after CB treatment at lower amplitudes.

Sonication of hLN18 Human Glioblastoma

Cell Viability of LN18 Glioblastoma Samples	
Samples	Average Cell Viability (%)
Control (n = 8)	94.7600155
2μM CB only (n = 4)	91.58401075
Sonic (0.195 W cm^{-2}, 60.5/0.5s) (n = 2)	95.8004095
Sonic (0.195 W cm^{-2}, 60.5/0.5s) -> CB (n = 2)	95.8333335
CB -> Sonic (0.195 W cm^{-2}, 60.5/0.5s) (n = 4)	90.71893325
Sonic (0.26 W cm^{-2}, 60.5/0.5s) (n = 2)	81.427305
Sonic (0.26 W cm^{-2}, 60.5/0.5s) -> CB (n = 2)	54.3367345

Figure 8. The average cell viabilities for all LN18 glioblastoma samples taken. Two sample readings were taken for each plate of LN18. A general trend of decreasing cell viability is present with increasing amplitude of sonication conditions as well as presence of CB treatment, though the viability of each individual sample tends to vary greatly.

Average Cell Number of LN18 Glioblastoma Samples	
Samples	Average Cell Number
Control (n = 8)	2.65E+06
2μM CB only (n = 4)	5.85E+05
Sonic (0.195 W cm^{-2}, 60.5/0.5s) (n = 2)	3.46E+05
Sonic (0.195 W cm^{-2}, 60.5/0.5s) -> CB (n = 2)	1.11E+05
CB -> Sonic (0.195 W cm^{-2}, 60.5/0.5s) (n = 4)	1.46E+06
Sonic (0.26 W cm^{-2}, 60.5/0.5s) (n = 2)	1.52E+06
Sonic (0.26 W cm^{-2}, 60.5/0.5s) -> CB (n = 2)	3.69E+05

Figure 9. The average cell counts for all LN18 glioblastoma samples taken. Two sample readings were taken for each plate of LN18. Sonicated cells demonstrated a decreased average cell number, but increased amplitude does not have a significant effect on this relationship.

Microscopic imaging on hLN18 human glioblastoma is not included, as results are similar to that of the RKO colorectal carcinoma. Data from cell viability similarly shows a

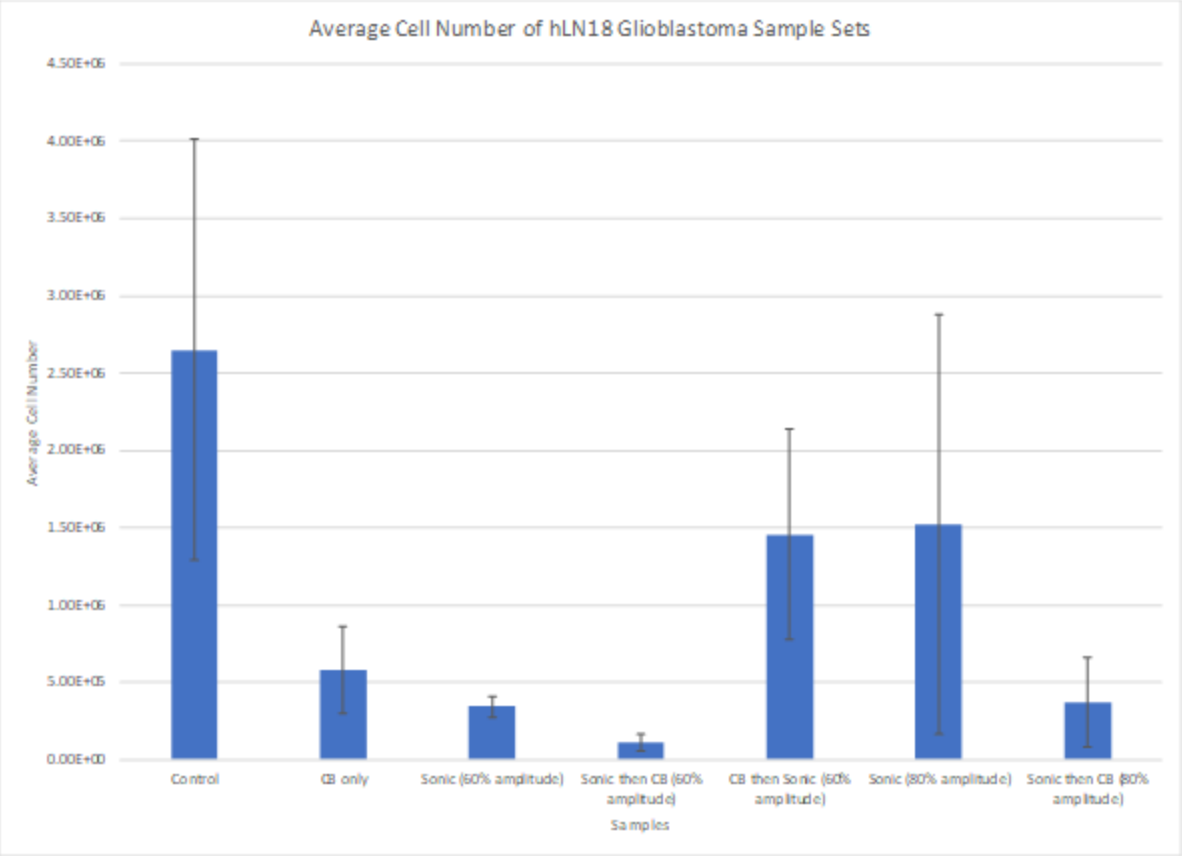


Figure 10. The average cell numbers for all hLN18 glioblastoma samples taken. Sonication, even in conjunction with CB treatment, appears far more variable and far less effective than is the case in RKO colorectal carcinoma. CB treatment prior to sonication is not significant, and other sonic treatments are only significant at lower amplitudes.

downward trend of viable cells with increasing cavitation from sonication, but individual samples have variable results. (Fig. 8). Data from cell counts shows a downward trend in cell survival, but the increasing amplitude of sonication does not significantly affect cell number as it does in the RKO (Fig. 9). A graphical representation of these cell counts, with accompanying statistical analysis, shows a statistically significant difference between the treatment of some of the experimental groups and their related controls (Fig. 10, $\alpha = 0.05$).

Comparison of Ultrasound Detachment and Trypsin Detachment

The experiments comparing ultrasound detachment and trypsin detachment show a near-statistically significant difference in cell counts after sample treatment (Fig. 11-12, $\alpha = 0.05$).

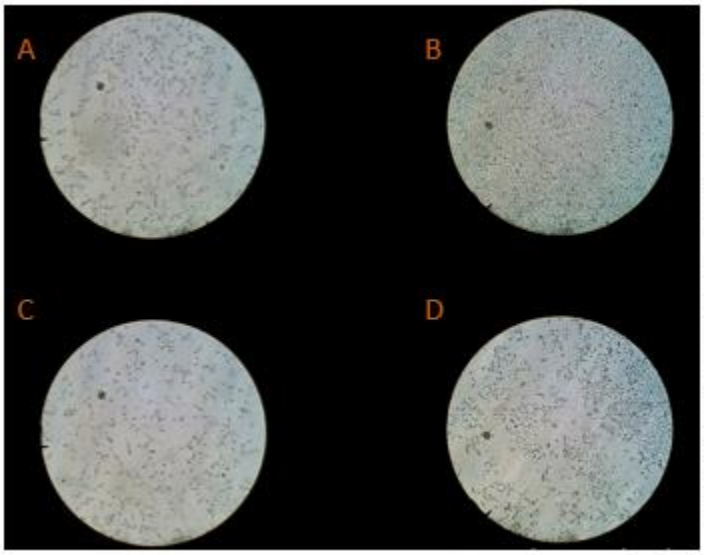


Figure 11. Imaging of RKO colorectal carcinoma with 100x light microscopy prior to trypsin treatment (A), 72 hours after trypsin treatment (B), prior to ultrasound treatment (C) and 72 hours after ultrasound treatment (D). Plate confluence is significantly lower three days after ultrasound treatment compared to three days after trypsin treatment. Ultrasound was conducted with the 20 kHz sonotrode at 80% amplitude, producing 0.26 W cm^{-2} of cavitation.

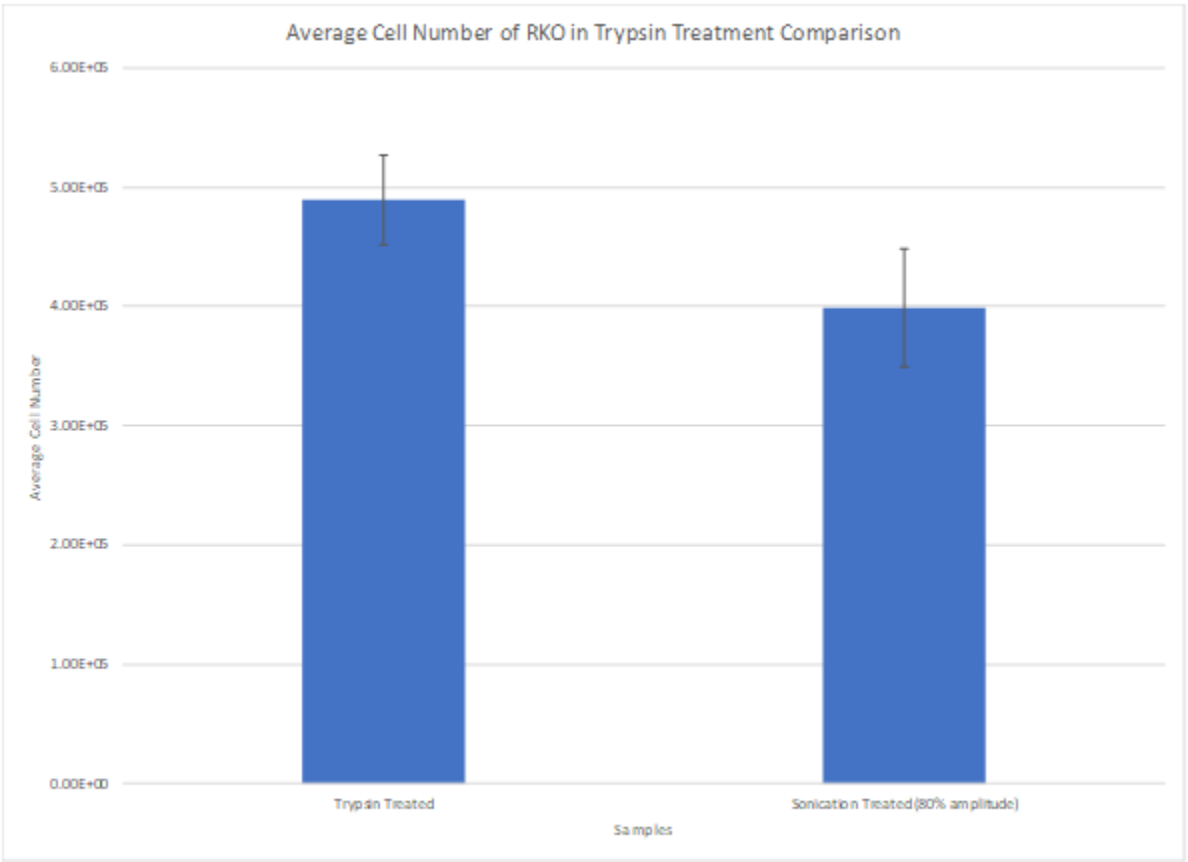


Figure 12. The comparison of trypsin treatment in RKO colorectal carcinoma to high-amplitude low-frequency ultrasound treatment. There is a small difference between populations that is near statistically significant.

Apoptosis Assay

Apoptosis assays were conducted to determine the risk of metastasis through the remaining proportion of apoptotic cells present after sonication. Our results show that under all circumstances, while sonication had the trend of decreasing cell number, it also decreased the proportion of apoptotic cells present (Fig. 13).

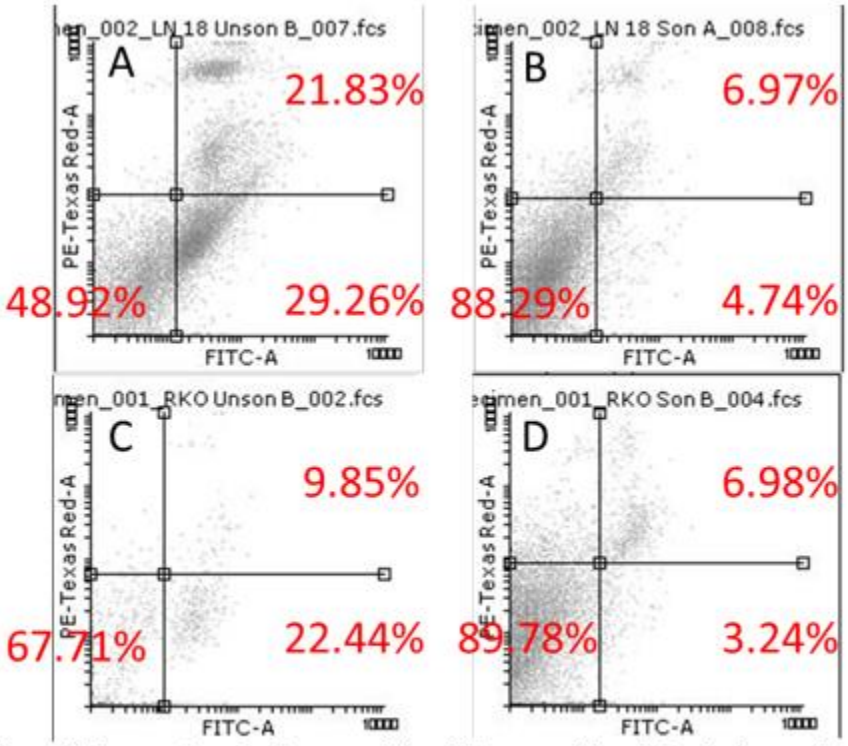


Figure 13. A comparison of cells untreated (A and C) or treated (B and D) with ultrasound, including both hLN18 (A and B) and RKO (C and D). Low-frequency ultrasound decreases both early-stage apoptosis (lower-right graph quadrants) and late-stage apoptosis (upper-right graph quadrants).

Conclusions

Sonication of RKO Human Colorectal Carcinoma

Significance of results indicates that low-frequency ultrasound alone is not necessarily significantly effective for the treatment of colorectal carcinoma. That said, in conjunction with existing chemotherapeutic agents, colorectal carcinoma can be effectively treated in vitro with low-frequency ultrasound. Part of the effectiveness of CB treatment prior to sonication is evidently due to the creation of large multinucleated cells that are more sonic sensitive compared to normal colorectal carcinoma cells. CB treatment after sonication may have been more effective due either to a greater exposed cellular surface area or weakened cell membranes for greater CB entry. The relative lack of preference for treatment before or after sonication indicates that all these possibilities may be occurring concurrently, and trials of similar cell lines is desirable for repetition. While we did not conduct experiments with other chemotherapeutic reagents, using another reagent, such as oxaliplatin, would eliminate the effect of increasing cell size and may show an increased preference for chemotherapeutic treatment following sonication. In future research we would like to include these methods, as well as 3D culture of carcinoma cells to increase simulation of in vivo conditions before progressing to animal models.

Sonication of hLN18 Human Glioblastoma

At a glance, low-frequency ultrasound treatment appears far less effective with respect to glioblastoma compared to colorectal carcinoma. Few treatments are actually significant, and those that are are non-intuitive, with lower amplitudes producing lower degrees of cavitation and greater cell damage. The implications are simply that low-frequency ultrasound is a far less

effective treatment for glioblastoma than it is for colorectal carcinoma for reasons we do not fully understand. This is interesting, as it implies low-frequency ultrasound is selectively useful in treating only certain types of tumors. This does not completely rule out low-frequency ultrasound in other brain targets, such as the blood-brain barrier, but it does imply that our LN18 cell line may not be useful for future studies.

Comparison of Ultrasound Detachment and Trypsin Detachment

Considering ultrasound must significantly reduce the viability of cells on its own, it should be distinct and significant compared to normal cellular methods of cell detachment. Our results showed that the relationship between ultrasound and trypsin detachment is near statistically significant, but not quite. While this means that ultrasound alone may not be an effective treatment method, it does open the availability of ultrasound for adjuvant treatment conditions that we have been studying.

Apoptosis Assay

The results of conducting apoptosis assays were unexpected, but certainly possible. In both RKO colorectal carcinoma and LN18 glioblastoma, cells appeared to be rescued from apoptosis because of ultrasound treatment. This implies an increased risk of metastasis due to our treatment conditions, which is a common issue with other physical methods of cancer treatment, such as surgery.

Future Goals

From our conclusions, we can draw that while both RKO colorectal carcinoma and hLN18 glioblastoma experience the phenomena of detachment when treated with low-frequency ultrasound, glioblastoma is more poorly reduced by ultrasound treatment. This does show an interesting preference for certain attached cell lines over others, and we do not fully understand the genetic basis of this preference. What role does gene expression play in sonication's ability to target colorectal carcinoma more preferentially over glioblastoma, and can we manipulate this gene expression to preferentially target carcinomas over normal attached cells? This is an important question for our research moving forward.

The results between CB effectiveness prior to and following sonication are not significantly different in most cases. This means that both proposed mechanisms (CB creating larger, multinucleated cells that are more susceptible to sonication as opposed to sonication yielding more surface area for CB treatment) are still possible and probable. To evaluate this possibility, I believe it would be useful to consider other common therapeutic reagents for the adjuvant treatment of colorectal carcinoma. Cisplatin analogues, such as oxaliplatin, seem favorable for these applications, and will be revealing as to the possibility of the latter mechanism of increased surface area.

Additionally, while our apoptosis results are not favorable for carcinoma treatment, there is likely a basis in the expression of cells that reduces this apoptosis in sonicated cells. It would be highly favorable for us to uncover this genetic basis to make sonication a more effective treatment in both our *in vivo* experiments and in clinical applications.

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