Treatment of brain cancer is limited in part by inefficient intracellular delivery of drugs and DNA for chemotherapy and gene therapy, respectively. This study tested the hypothesis that ultrasound may be used to enhance intracellular delivery and efficacy of chemotherapeutics and genes in glioma cells in vitro. First, suitable ultrasound conditions were identified by measuring intracellular uptake of calcein and viability of GS 9L rat gliosarcoma cells after a range of different ultrasound exposures. We selected sonication at 10 J/cm², which achieved intracellular delivery of ~10⁶ molecules/cell. Next, glial cells were sonicated with varying concentrations of model chemotherapeutics: BCNU and bleomycin. For both drugs, cytotoxicity was increased in a synergistic manner when accompanied by ultrasound exposure. Finally, expression of a plasmid DNA encoding a GFP reporter was increased up to 30-fold when exposed to ultrasound. Altogether, these findings suggest that ultrasound may be useful to increase the efficacy of chemotherapy and gene therapy of glioma cells.

Key words: Chemotherapy; Cytotoxicity; DNA transfection; Gene therapy; Gliosarcoma brain cancer; and Ultrasound.

Introduction

Malignant brain cancer afflicts more than 80,000 Americans and many more worldwide. In the United States, close to 18,000 new cases are diagnosed and 13,000 patients die each year (1-3). Treatment by surgery is preferred if the tumor is accessible. If not, radiation is often used. Chemotherapy has generally been found to be less effective against brain cancer, because gliomas are relatively slow-dividing tumors and the blood brain barrier hampers delivery of chemotherapeutics to the brain. Gene therapy may provide a new way to treat cancer, including suicide gene therapy (4), downregulation of oncogenes (5), and antiangiogenic therapy (6).

In all of these treatment options, one of the critical limitations of current therapy is insufficient targeting to cancer cells (7). In recent years, BCNU biodegradable wafers (Gliadel) implanted locally into brain have been introduced as a targeted therapy, which has been shown to increase life expectancy (8, 9). Despite moderate clinical success of Gliadel wafers, the prognosis for brain cancer patients generally remains poor and overall treatment of brain cancer, especially for high-grade gliomas, needs significant improvement (10).

This study addresses the hypothesis that ultrasound may be used to enhance intracellular delivery and efficacy of chemotherapeutics and genes in glioma cells. We and others have previously shown that under appropriate conditions ultrasound can drive large numbers of molecules into cells (11). In vitro studies
have demonstrated that ultrasound’s ability to transport molecules into cells appears to be ubiquitous to many different cell types, including cancer cell lines, primary cells, yeast (12), and bacteria (13), and a variety of different classes of molecules, including drugs, proteins, and plasmid DNA (14). Of direct relevance to this study, ultrasound has been shown to enhance transfection of rat (9L) and canine (J3T) glioma cells during exposure to complexes of DNA and cationic liposomes consisting of DOSPA/DOPE (15). It was also shown that ultrasound in the presence of microbubbles can enhance gene transfer in a variety of different cells types (16).

In vivo studies have demonstrated enhanced and targeted cancer chemotherapy and gene delivery/expression with the use of ultrasound in animals in more than a dozen different studies in a variety of different tissues (but not yet in brain tumors) (17). In related clinical trials, transcranial ultrasound was shown to enhance transport for thrombolysis in acute ischemic stroke, demonstrating the clinical feasibility of ultrasound-based therapy of the brain (18).

Ultrasound-mediated enhancement of chemotherapy has also received attention in a limited number of studies. For example, sonication of Chinese hamster ovary cells (HA1) and murine tumors in the presence of adriamycin was shown to produce a synergistic cytotoxic effect (19, 20), which is believed to be caused by increased intracellular uptake of the chemotherapeutic molecules. In a different study addressing “sonodynamic therapy,” certain drugs were shown to be activated by exposure to ultrasound by a mechanism believed to involve ultrasound-mediated production of highly active radicals that increase cytotoxicity (21).

Although ultrasound is commonly used in medicine for other applications, the ultrasound conditions needed to observe these therapeutic effects are different from those used, for example, in diagnostic imaging or tissue heating for physical therapy (22). Enhanced drug and gene delivery usually requires application of ultrasound at a high mechanical index (MI) and/or with the combined use of contrast agents, which serve as nucleation sites for cavitation. Conventional clinical applications of ultrasound seek to avoid cavitation, which is the creation, oscillation and, in some cases, collapse of gas bubbles in the alternating pressure field generated by ultrasound. Cavitation can have damaging effects on cells and tissues, such as the shattering of kidney stones by cavitation generated during lithotripsy. However, under controlled conditions, the mechanical effects of cavitation can be harnessed to drive molecules into living cells, as studied here.

The mechanism of intracellular delivery using ultrasound is believed to be initiated by local fluid dynamics generated by oscillating and/or collapsing cavitation bubbles (23). By either shearing or direct impact of fluid with the cell, its plasma membrane can be torn open to produce a hole of up to 1 μm in size (24, 25). Molecules can transport through this hole to gain access to the cytosol. By a mechanism involving transiently elevated intracellular calcium levels, cells traffic intracellular vesicles to the site of injury and actively patch the hole on a timescale of a few minutes. Under appropriate conditions, cells survive this process, retaining large numbers of intracellular molecules and appearing to regain normal function within hours. In other cases, cellular damage is too great and cells die by mechanisms that can involve necrosis or programmed death.

The effects of ultrasound on a population of cells are typically heterogeneous (26). After exposing a cell suspension to “mild” ultrasound conditions, almost all cells usually remain viable and a small fraction (e.g., 10%) show intracellular uptake of molecules. The remaining cells appear to be generally unaffected. After stronger ultrasound exposures, larger fractions of cells can show uptake (e.g., 10-40%), but cell viability correspondingly drops. At very strong ultrasound conditions, essentially all cells can be killed. This heterogeneity can be explained by the observation that cavitation bubble activity occurs at discrete times and locations (27, 28). Cells that are near a cavitation bubble upon, for example, its transient collapse, should be impacted more than cells located some distance away. In this way, a gradient of effects can be observed based on the distance that each cell is located from the nearest bubble.

Guided by this understanding of ultrasound mechanisms and previous successes with ultrasound for enhanced drug therapy, this study sought to determine if ultrasound could enhance cytotoxicity of chemotherapeutic drugs for brain cancer. Specifically, we looked for synergistic effects between ultrasound and exposure to BCNU (carmustine), which is an established chemotherapeutic for local treatment of brain cancer (8), and bleomycin, which is not typically used for brain cancer, but its efficacy is known to be limited by poor intracellular uptake (29), which could be improved by ultrasound. We addressed these questions using a 9L rat gliosarcoma model in vitro. To our knowledge, this is the first study to examine ultrasound’s effects on chemotherapy of glial cells. A related study showed that ultrasound increased blood-brain barrier permeability to improve Herceptin delivery into the brain (30).

This study also sought to assess the ability of ultrasound to facilitate gene transfer and expression in glioma cells. Nucleic acid therapeutics offer an exciting opportunity to efficiently target therapy to cancer cells, but require intracellular delivery of genetic material, which is notoriously inefficient using nonviral methods. In recent studies, it was shown that gene transfection of prostate cancer cells may be dramatically increased using optimized ultrasound protocols (31, 32).
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This study builds off those observations to partially optimize transfection of gliosarcoma cells.

Materials and Methods

Cell Culture

Rat gliosarcoma cells GS 9L (courtesy of John Hopkins University) were cultured as monolayers in a humidified atmosphere of 95% air and 5% CO₂ at 37 ºC (Incubator model 3110, Forma Scientific, Marietta, OH) in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) (33). After reaching 80-90% confluence, cells were harvested under sterile conditions prior to each experiment by trypsin digestion for 2 min (0.25% trypsin, 0.1% EDTA, Cellgro, Herndon, VA). Trypsin was inactivated by addition of five parts full media to one part trypsin. Cells were then centrifuged (1000 × g, 6 min, model GS-15R, Beckman Coulter, Fullerton, CA) and resuspended in RPMI-1640 media at a concentration of 10⁶ cell/ml, as determined by a Coulter Multisizer II (Beckman Coulter), unless otherwise noted.

BCNU, Bleomycin, DNA, and Calcein

Concentrated stock solutions of each chemotherapeutic were first created. 1,3-Bis(2-chloroethyl)-1-nitrosourea (also known as BCNU or carmustine, Sigma Chemical, St. Louis, MO) was dissolved in 95% ethanol immediately prior to use. Bleomycin (containing a mixture of bleomycin sulphate B₁ and A₂, Gensia Sicor Pharmaceuticals, Irvine, CA) was dissolved in sterilized DI water prior to use. The stock solutions were diluted into the culture media to concentrations of 0-1 mM BCNU or 0-100 μM bleomycin immediately before application of ultrasound. After addition of BCNU, the final concentration of ethanol in the cell suspension culture was kept below 1:1000 (v/v), which has been shown to be nontoxic for cells (34).

For transfection experiments, a concentrated stock solution of DNA plasmid coding for green fluorescent protein (GFP) (gwiz pgFP (5,757 bp), Aldevron, Fargo, ND) was added to cell suspensions immediately prior to application of ultrasound at a final concentration of 0-30 μg/ml.

Calcein, which is a green fluorescent molecule that cannot cross intact cell membranes, was used to quantify molecular uptake into viable cells. Calcein (Molecular Probes, Eugene, OR) was added to cell suspensions at a concentration of 10 μM before application of ultrasound.

Ultrasound Apparatus

The ultrasound apparatus and its characterization have been described in detail previously (35, 36). Briefly, 1.1 MHz ultrasound was produced using an immersible, focused, piezoceramic transducer (model H-101, Sonic Concepts, Woodinville, WA). The transducer has a 70 mm diameter, a 52 mm focal length, and a 1.5 mm focal width at half amplitude (-6 dB).

A pulsed rectangular signal was produced by a first programmable waveform generator (model DS345, Stanford Research Instruments, Sunnyvale, CA) and modulated by a 1.1 MHz waveform from a second waveform generator (model 33120A, Agilent, Austin, TX). This approach let us control the number of pulses, pulse length, frequency and peak-to-peak voltage. The waveform was then amplified by an RF broadband power amplifier (model 3100LA, Electronic Navigation Industries, Rochester, NY) before passing through an impedance matching network (model H-101, Sonic Concepts) and applied to the transducer.

The transducer was housed in a polycarbonate tank (30.5 × 29 × 37 cm) containing approximately 26 L of deionized, distilled, and partially degassed water at room temperature (21-23 ºC). A 5 cm thick acoustic absorber (acoustic rubber SC-501, Sonic Concepts) was mounted opposite the transducer to minimize standing wave formation. A three-axis positioning system (10 μm resolution, Velmex, Bloomfield, NY) was mounted on top of the tank to position samples and a hydrophone, discussed below, at desired locations in the tank.

To map and calibrate the acoustic field produced by the transducer versus the peak-to-peak voltage signal provided by the function generator, a PVDF membrane hydrophone (model MHA200A, NTR Systems, Seattle, WA) was used to measure spatial-peak-temporal-peak negative pressure.

Ultrasound Treatment

Before each experiment, the hydrophone was used to position the cell sample approximately 1 cm out of the ultrasound’s focus toward the transducer on the main axis of the transducer. The acoustic pressure was calibrated versus the peak-to-peak voltage of the signal created by the function generator using the hydrophone at the desired location. This out-of-focus location was used because it had a broader acoustic beam than at the focus, which was approximately 10 mm wide at half amplitude (-6 dB). This broader acoustic beam enabled a more uniform acoustic exposure across the sample. Vigorous mixing caused by cavitation during ultrasound exposure further facilitated a uniform average exposure of the sample.

To promote cavitation, albumin-stabilized perfluoropropane-gas bubbles (Optison, Mallinckrodt, St. Louis, MO) were slowly added to the cell suspension in all experiments using a 22-gauge flat needle (17 μl/ml, approximately 1.1
× 10^7 bubbles/ml, with average diameter of 2.0-4.5 μm, according to the manufacturer).

The sample chamber was a 0.4 ml polyethylene transfer pipette (catalog no. 293, Samco Scientific, San Fernando, CA), from which the bottom tab was removed and filled with silicone glue (Silicone II, GE Sealants & Adhesives, Huntersville, NC) several days prior to the experiment. The upper stem was cut to 4 mm length. Chambers were sterilized by placing under UV light in a biological safety cabinet for 30 min prior to each experiment. Each chamber was filled with cell suspension up to the top of the pipette stem and an aluminum rod of 3.5 mm diameter was inserted 2 mm into the stem. Overflow of solution was allowed to ensure no visible air bubbles were trapped in the sample.

Ultrasound exposures at 1.1 MHz were performed at a burst length of 1 ms, duty cycle of 1% (i.e., 10 pulses per second), peak negative pressures of 1.2, 1.7, 2.0, 2.5, 3.0, and 3.5 MPa, and exposure times of 3, 10, 30, 100, 300, 1000, and 3000 ms. Note that exposure time refers to the amount of time ultrasound was actively applied. Given the 1% duty cycle, the length of each experiment ranged from 300 ms to 300 s. This low duty cycle was used to minimize heating, which was < 1 °C for all conditions studied.

For each experiment, at least three samples were not exposed to ultrasound, but were subjected to all other procedures. These "sham" samples were used as negative controls for viability and transfection measurements.

After ultrasound application, cells were incubated at 37 °C for 5 min to allow cell recovery. Cell samples were then washed once by centrifugation (1000 × g, 6 min, Eppendorf 5415 C, Brinkmann Instruments, Westbury, NY), resuspended in full growth medium (2 ml for each sample) and placed into six-well cell culture plates (Corning Inc., Corning, NY) maintained in the cell culture incubator under cell growth conditions for five days when assessing BCNU or bleomycin cytotoxicity and 24 h when assessing DNA transfection. When calcein uptake and cell viability were determined, samples were not placed in the incubator, but were washed three times and maintained on ice for up to 1 h before analysis.

Transfection, Uptake, and Viability Measurements

Expression of GFP, uptake of calcein, and cell viability were quantified by flow cytometry. Cells were harvested from six-well plates by trypsinization and centrifugation, as described above, and then suspended in phosphate-buffered saline (PBS, Cellgro). Propidium iodide (Molecular Probes) was added to cell suspensions at a final concentration of 1 μg/ml to label non-viable cells. After 10 min incubation at room temperature, cells were placed on ice until analysis.

We also added 2.5 μm fluorescent beads (LinearFlow Green Fluorescence Flow Cytometry Intensity Calibration Kit L-14821, Molecular Probes) to cell samples at a concentration of 10^6 beads/ml for volumetric calibration, as previously described (37). Briefly, cells that were destroyed or otherwise lost during the protocol were identified by comparing the ratio of cells-to-fluorescent beads in each sample to that of control samples. Especially after extended incubation (i.e., for five days in the context of chemotherapeutic experiments), this measure of cell viability accounted for possible cell death due to the treatment, as well as decreased cell concentration due to impaired cell division.

For each sample, 20,000 viable cells were analyzed by flow cytometry (BD LSR Flow Cytometer, Becton Dickinson, San Jose, CA), as described previously (33), which determined the concentration of viable and dead cells in each sample. For DNA transfection and calcein uptake experiments, the intensity of green fluorescence (530/28 nm bandpass filter) was measured in viable cells. Cells were counted as GFP-expressing cells if they had a green fluorescence greater than that of 99.9% of cells in control samples. Transfection data are reported as the fraction of cells expressing GFP among all cells exposed to ultrasound, which provides the most rigorous assessment of transfection efficiency. This contrasts with some other methods used in the literature, in which transfection efficiencies have been reported as a fraction of cells remaining viable at the time of analysis (38, 39).

Intracellular calcein uptake was quantified by converting fluorescence measurements into absolute numbers of calcein molecules delivered into each cell by calibration relative to a panel of fluorescent microspheres of known fluorescence (Quantum MESF FITC high level, Bangs Laboratories, Fishers, IN) (37).

Data Analysis

Intracellular calcein uptake is reported as the average number of molecules per cell by calibrating fluorescence measurements relative to the calibration microspheres mentioned above. Cell viability is calculated as the percent of viable cells compared to sham-exposed cells in the absence of chemotherapeutic. However, for samples exposed to both ultrasound and chemotherapeutic, it is of special interest to identify synergistic effects of ultrasound and chemotherapeutics. For these dual exposures, viability measurements were corrected to subtract out the additive effects of ultrasound alone and report only the synergistically increased chemotherapeutic cytotoxicity induced by exposure to ultrasound. Thus, under the conditions used during experiments to assess BCNU cytotoxicity, ultrasound alone caused a 30% loss of viability (i.e., viability was 70%). To subtract out the effects of ultrasound alone, the reported viability was corrected by multiplying by the factor 1.0/0.7 = 1.43. Similarly, under
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the conditions used during experiments to assess bleomycin cytotoxicity, ultrasound alone caused a 60% loss of viability (i.e., viability was 40%). To subtract out the effects of ultrasound alone, the viability was corrected by multiplying by the factor 1.0/0.4 = 2.5. Although the same ultrasound conditions were used, cell viability from ultrasound alone was higher in the BCNU study than in the bleomycin study. This is probably an artifact created because we used a new batch of Optison for the bleomycin study, which probably showed more cavitation activity and thereby caused more cell death.

After exposure to DNA, transfection efficiency is reported (i) on a relative basis as the number of GFP-positive cells after ultrasound exposure relative to sham-exposed cells incubated in the same concentration of DNA and (ii) on an absolute basis as the GFP-positive cells as a percent of all exposed cells. Other measurements determined the amount of GFP expressed by cells, which is reported as the average fluorescence (530/20 nm bandpass filter) among GFP-positive cells relative to sham-exposed cells incubated in the same concentration of DNA. Finally, total GFP expression is reported as the product of the relative increase in the number of GFP-positive cells (i.e., transfection efficiency) and the relative increase in intracellular GFP among GFP-positive cells.

Experiments at all conditions were performed at least three times (n ≥ 3), each on a different day. Student’s t-test was used to identify differences between pairs of data points, whereas Analysis of Variance (ANOVA) was employed to compare three or more data points using StatView statistical software (SAS Institutes, Cary, NC). A value of p < 0.05 was interpreted as significant. Data on graphs are reported as the mean ± SEM (standard error of the mean).

Results

Intracellular Uptake and Cell Viability

After Ultrasound Exposure

We hypothesize that ultrasound may be used to enhance intracellular delivery of chemotherapeutics and genes into glioma cells. As a first test, we examined the effect of ultrasound on intracellular uptake of calcein, which is an inert, fluorescent marker that is not normally taken up into cells. Gliosarcoma cells were exposed to ultrasound in vitro over a range of acoustic pressures (1.2-3.5 MPa) and exposure times (8-1480 ms) that corresponded to acoustic energies between 0.4-600 J/cm². After exposure to ultrasound, intracellular uptake of calcein and cell viability were quantified.

As shown in Figure 1A, cells exposed to ultrasound exhibited high levels of intracellular uptake. At the weakest conditions used (i.e., ~1 J/cm²), uptake averaged between 10⁴ and 10⁵ molecules per cell. At larger energies, average uptake exceeded 10⁶ molecules per cell. Dividing this number of molecules by the average cell volume of 1600 μm³ (determined by a Beckman-Coulter Multisizer II) yields an intracellular calcein concentration on the order of 10% of the extracellular concentration, which indicates extensive uptake. These results are similar to those reported previously for other cell types (11).

Achieving intracellular uptake in some cells was often associated with loss of viability in other cells. As shown in Figure 1B, cell viability decreased with increasing ultrasound energy dose. At the weakest conditions (< 1.7 J/cm²), cell viability did not change significantly from 100% (ANOVA, p = 0.11). However, at larger energies, cell viability dropped to 75-80% viability at intermediate energies and down to 20-30% viability at the strongest conditions used (ANOVA, p < 0.0001 for E > 200 J/cm²).

Guided by these findings, we selected ultrasound conditions that applied 10 J/cm² using a pressure of 2 MPa and an exposure time of 10 ms for subsequent experiments to...
deliver chemotherapeutic agents. Under these conditions, there was extensive intracellular uptake and limited cell viability loss due to the ultrasound.

**Synergy of Ultrasound and BCNU Cytotoxicity**

To identify possible synergy between cell death caused by ultrasound and BCNU, we first measured the cytotoxicity of BCNU alone as a function of BCNU concentration. BCNU was selected as a model chemotherapeutic because of its established use in brain cancer therapy (40). As shown in Figure 2 (white data points), BCNU at concentrations ≤ 30 μM had no significant effect on cell viability (ANOVA, p = 0.32). At 100 μM BCNU, cell viability dropped to 50%. At larger BCNU concentrations, cell viability dropped still further.

Cytotoxicity was significantly greater when cells were additionally exposed to ultrasound. As shown in Figure 2 (black data points), the synergistic effects of ultrasound to increase BCNU cytotoxicity were evident at all BCNU concentrations studied (two-way ANOVA, p < 0.0001). At small concentrations of BCNU (i.e., 0.1-10 μM), BCNU alone did not kill significant numbers of cells, but the addition of ultrasound increased BCNU cytotoxicity to reduce cell viability to approximately 75%. Larger BCNU concentrations increased cytotoxicity still more (ANOVA, p = 0.003). In this way, the initial increase of BCNU cytotoxicity was observed at ≥ 1000-fold lower concentration (i.e., from 100 μM to < 0.1 μM) and the dose to kill 50% of cells (LD50) was reduced by more than a factor of 3 (i.e., from 100 μM to 30 μM). We hypothesize that this increased cytotoxicity is due to increased intracellular uptake of BCNU due to ultrasound.

**Synergy of Ultrasound and Bleomycin Cytotoxicity**

We next looked for possible synergy between cell death caused by ultrasound and bleomycin using an approach similar to that used for BCNU. Although bleomycin is not commonly used to treat brain cancer, we selected it as a model chemotherapeutic because it is known to have difficulty crossing the cell membrane, but is highly cytotoxic once it reaches its intracellular target (37). For this reason, cytotoxicity of bleomycin has been shown to be synergistically increased when combined with electroporation, which is known to transiently increase cell membrane permeability and thereby increase intracellular delivery (42).

As shown in Figure 3 (white data points), bleomycin alone was increasingly cytotoxic to gliosarcoma cells at increasing drug concentrations. When combined with ultrasound, cytotoxicity was significantly increased (Figure 3, black data points, two-way ANOVA, p = 0.015). The difference was statistically significant, even when the outlier point at 1 μM concentration is included.

**Effect of Ultrasound Conditions on DNA Transfection**

Gene-based therapies offer exciting prospects to treat brain cancer, but have been limited by inefficient gene delivery and expression. To test the hypothesis that ultrasound can be used to increase gene transfection of glioma cells, we measured the effects of ultrasound parameters on the transfection efficiency of 9L gliosarcoma cells by plasmid DNA encoding green fluorescent protein (GFP). As shown in Figure 4, transfection efficiency increased with ultrasound pressure over the range of conditions tested (two-way ANOVA, p < 0.0001). Transfection also increased with ultrasound exposure time at short exposures (≤ 15 ms; two-way ANOVA, p = 0.01), but showed no significant change at longer exposures (≥ 75 ms; two-way ANOVA, p = 0.24). The best transfection
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Ultrasound-enhanced chemotherapy efficiency obtained in this set of experiments increased the number of cells expressing detectable amounts of GFP by 30-fold, which corresponded to 2.5% of treated cells. The maximum transfection efficiency is often limited by cell death at stronger conditions. In these experiments, cell viability was found to decrease with increasing pressure and exposure time (two-way ANOVA, p = 0.008 and p < 0.0001, respectively) to values as low as 60% at the strongest conditions used (data not shown). Thus, the plateau and possible decrease in transfection efficiency at the strongest ultrasound conditions used can be explained by the competing effects of ultrasound more effectively driving DNA into cells, but also more extensively killing cells, such that the net effect is a leveling off or reduction in overall transfection efficiency.

Effect of DNA Concentration on DNA Transfection

The effect of DNA concentration on transfection efficiency is shown in Figure 5A. Although the effect was small, increasing DNA concentration increased the number of transfected cells (two-way ANOVA, p = 0.0005). One might also expect, however, that DNA concentration could additionally affect the amount of GFP expressed by each transfected cell. Consistent with this expectation, Figure 5B shows that increasing DNA concentration increased the intensity of GFP expression by each cell (two-way ANOVA, p < 0.0001). In contrast, increasing ultrasound energy exposure did not affect the average intensity of GFP expression (two-way ANOVA, p = 0.75).

Often, the total amount of protein expression is more important that the number of cells expressing the protein. To assess this metric, the total amount of GFP expressed was determined by multiplying the number of GFP-positive cells (Figure 5A) by the average cellular intensity of GFP expression (Figure 5B). As shown in Figure 5C, DNA concentration increased total GFP expression (two-way ANOVA, p < 0.0001) but increase in energy exposure above 1 J/cm² did not change it significantly (p = 0.52, respectively). At the optimal conditions, GFP expression was increased by 30-fold relative to sham-exposed controls.

Discussion

Prospects of Ultrasound-enhanced Chemotherapy

To study the ability of ultrasound to increase chemotherapeutic cytotoxicity, we first needed to identify suitable ultrasound parameters. Initial studies identified a trade off between high pressure and exposure time.
levels of intracellular uptake and high levels of cell viability. Because ultrasound is intended to facilitate delivery of chemotherapeutics that specifically target cancer cells, it is not desirable for ultrasound to indiscriminately kill cells by itself. As a compromise, we selected ultrasound conditions that loaded cells with millions of molecules per cell and maintained 70% viability. If higher viability were important, then weaker ultrasound conditions could have been selected to deliver on the order of 10^6 molecules per cell with minimal cell loss. Moreover, it is likely that cell viability would be higher in vivo, where cells are supported by their natural extracellular environment, rather than suspension in media (43). For this proof-of-principle study, ultrasound conditions were not fully optimized.

Chemotherapeutic cytotoxicity was first measured using BCNU either with or without exposure to ultrasound. As expected, BCNU alone was effective at killing gliosarcoma cells. However, the addition of ultrasound increased cytotoxicity in a synergistic way. Our proposed hypothesis is that ultrasound facilitated greater intracellular delivery of BCNU into cells and thereby increased BCNU efficacy. However, BCNU is a small (214 Da), lipophilic (XlogP = 1.26) molecule that should cross cell membranes fairly well. Thus, an alternative hypothesis is that ultrasound otherwise sensitized cells to BCNU. For example, a previous study showed that exposure of cells to ultrasound in combination with another chemotherapeutic, quercetin, synergistically increased cytotoxicity by a mechanism believed to involve depletion of a heat shock protein by ultrasound (44). In a separate study not involving ultrasound, topoisomerase I inhibitors were shown to sensitize cells to BCNU (45).

We also examined the effects of ultrasound on cytotoxicity of bleomycin. This drug provides a useful contrast with BCNU, because bleomycin is large (1.4 kDa) and hydrophilic and, therefore, cannot easily cross cell membranes (41). Nonetheless, ultrasound also increased the cytotoxicity of bleomycin in a synergistic way. In this case, the proposed mechanism involving increased intracellular delivery of bleomycin caused by ultrasound is more plausible.

When considered all together, this study suggests that ultrasound may be a useful adjunct to chemotherapy of brain cancer. Ultrasound was shown to synergistically increase cytotoxicity of two chemotherapeutics with very different physicochemical properties. This observation should motivate further studies of protocol optimization, in vivo efficacy and safety, and clinical considerations.

**Prospects of Ultrasound-enhanced Gene Therapy**

Ultrasound may also be useful to facilitate gene therapy as a nonviral approach. This study showed that increasing the strength of ultrasound increased the number of transfected cells and increasing the DNA concentration increased the expression level in each transfected cell. The best conditions tested in this study found a 30-fold increase in total expression levels over non-sonicated controls.

Despite large increases in transfection efficiency, the observed increases may not be enough for some applications. At partially optimized conditions, just 2.5% of cells showed increased expression above background. For applications in which, for example, transfection needs to be achieved in most, if not all, cells for efficient tumor removal, further enhancement of expression is needed. In contrast, other cancer gene therapy approaches require only a subset of cells to be transfected so that the expression products can be distributed among neighboring cells via extracellular or intracellular pathways. For example, in a previous study, suicide gene plasmids were shown to be effective when transfected into just 2% of the glioma cell population (46).

**Conclusion**

This study assessed the prospects of using ultrasound to enhance treatments of brain cancer. Guided by initial experiments to identify suitable ultrasound conditions for intracellular delivery to glioma cells, we found that ultrasound synergistically increased in vitro cytotoxicity of two chemotherapeutics with very different physicochemical properties: BCNU and bleomycin. This suggests the possibility that chemotherapeutics could be administered to the brain in combination with ultrasound focused on the tumor to increase efficacy by a mechanism believed to involve increased intracellular uptake of drug. We also found that ultrasound increased in vitro glioma cell transfection with a GFP reporter DNA plasmid by 30-fold. This similarly suggests that ultrasound could facilitate gene therapy in the brain, especially for indications where the dangers of virus-based delivery are undesirable and moderate transfection efficiency is sufficient.

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