Investigating Compounds to Target Glioblastoma Progression

by

Emily Elizabeth Cade

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics
(Honors Scholar)

Presented May 21, 2015
Commencement June 2015
AN ABSTRACT OF THE THESIS OF

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Abstract approved:

Juliet A. Greenwood

Glioblastoma multiforme is a devastating primary brain tumor with a median five year survival rate of less than 5%. It is a cancer that is resistant to current cancer treatments, such as surgery and chemotherapy, as well as one that is prone to recurrence after surgical removal. This highlights the importance of discovering or improving upon treatments for glioblastoma. Thus, the goal of this research is to investigate the anti-cancer properties of two compounds: zinc-oxide nanoparticles and synthetic calpain-2 inhibitors. In the first part of this research, the xCELLigence system was used to assess the anti-proliferative and cytotoxic effects of zinc-oxide nanoparticles in a cell culture model of glioblastoma. From the results gathered, it was concluded that, although a 200 uM solution of ZnO NP dispersed in DMSO had a significant cytotoxic effect on GBM cells, zinc-oxide nanoparticles would not make an effective glioblastoma treatment at this time. In the second part of this research, an embryonic zebrafish xenograft model of GBM was used to test the anti-invasive properties of synthetic calpain-2 inhibitors. Preliminary results from this model are
inconclusive and necessitate further testing. Future work regarding both parts of this research are also discussed.

Key Words: glioblastoma multiforme, zinc oxide nanoparticles, calpain-2 inhibitors, xCELLigence system, embryonic zebrafish model, cancer treatments

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

________________________
Emily Elizabeth Cade, Author
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I also would like to acknowledge Dr. Alex Punnoose from the Boise State University Department of Physics for providing zinc oxide nanoparticles, Dr. Isaac Donkor from the University of Tennessee Health Science Center for providing synthetic calpain-2 inhibitors, and the Sinnhuber Aquatic Research Laboratory in Corvallis, Oregon for providing embryonic zebrafish specimens.

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INTRODUCTION

Glioblastoma multiforme (GBM) represents 53.9% of primary brain gliomas\textsuperscript{1} and 20% of all primary brain tumors\textsuperscript{2}. Having a five-year survival rate of less than 5\%\textsuperscript{3,4}, it is one of the most common and devastating malignant tumors occurring in the central nervous system. Currently, treatment for GBM involves surgical removal of the main tumor followed by chemotherapy, radiotherapy, or a combination of both\textsuperscript{5}. However, GBM is resistant to conventional treatment methods such as surgery, chemotherapy, and radiation therapy\textsuperscript{6,7}, which may be due to minor subpopulations of cancerous stem cells within GBM tumors able to avoid and repair damage caused by these treatments\textsuperscript{8}. Additionally, GBM tumors are highly angiogenic and demonstrate a propensity to grow invasively throughout surrounding brain tissues\textsuperscript{9,10}, making surgery impractical as pockets of cells are left behind inside the brain, which lead to tumor recurrence. Thus, the development of GBM treatments that target its progression is a priority.

Zinc oxide nanoparticles (ZnO NPs) are a metal oxide compound measuring less than 100 nanometers in at least one spatial dimension, comparable to the size of many biological structures\textsuperscript{11}. While the more well known uses of NPs include applications in electronics, sunscreens, and drug delivery systems, ZnO NPs have previously
demonstrated selective toxicity to cancerous eukaryotic cells. This selective toxicity was explored previously in Premanathan et. al, where it was found that ZnO NPs induced apoptosis in leukemia cells while having little to no effect on non-cancerous blood cells. ZnO NPs were also found to generate greater amounts of reactive oxygen species in U87 glioma cells than in normal human astrocytes. This suggests that ZnO NPs may make an effective GBM treatment.

However, using these nanoparticles for a cancer treatment, necessitates the use of a carrier compound or dispersal solvent for delivery to the treatment site. In this study, the effects of three solvents on the anti-proliferative properties of ZnO NPs were compared at different concentrations; these solvents were dimethyl sulfoxide (DMSO), water (H2O), and fetal bovine serum (FBS). DMSO was chosen for its common use as a medical solvent and topical treatment vehicle, H2O was chosen for its non-toxicity to biological systems, and FBS was chosen for its bovine serum albumin content, which was thought to coat the nanoparticles, keeping them dispersed.

The xCELLigence System, a system facilitating label-free dynamic monitoring of cell proliferation in real time, was used to determine the most effective dispersal solvent for use with the ZnO NPs. Cultures of cells were exposed to various concentrations of ZnO NPs suspended in the three different dispersal solvents, and cell proliferation was monitored to determine which of the concentration-solvent combinations were the most cytotoxic or the most effective at proliferation inhibition.
Cell migration and invasion depends heavily on cell motility, which itself requires the protrusion of leading lamellipodia and the formation of new attachments to the substratum, coupled with the release from the substratum at the trailing edge of the cell. An integral player in the cellular de-adhesion process is calpain-2, an isoform in the calpain family of cysteine proteases which are required for de-adhesion of the trailing edge of a cell during haptokinesis and chemokinesis, which enables the forward locomotion required for cell migration and invasion\textsuperscript{14}. Calpain-2 activity has been shown to be regulated by autocrine glutamate levels\textsuperscript{15,16,17}. Autocrine glutamate activates α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors on GBM cells, stimulating an influx of calcium that is required for calpain-mediated disassembly of focal adhesion complexes, and promoting migration and invasion\textsuperscript{2,18,19}. The mechanism of calpain function and inhibition is shown in Figure 1. Because of this, calpain-2 is a promising target for inhibiting GBM progression. In previous studies, inhibitory compounds targeting calpain-2 were shown to decrease invasion of U87MG GBM cells, and the knockdown of calpain-2 within cells reduced GBM invasiveness by 90\%\textsuperscript{2}. 


Figure 1: This diagram illustrates the pathway by which calpain-2 is controlled by the stimulation of calcium ion influx by autocrine glutamate levels. A synthetic calpain-2 inhibitor would theoretically inhibit invasion by preventing calpain-2 from cleaving focal adhesion complexes.

An embryonic zebrafish xenograft model of GBM was employed to test the anti-invasive properties of the calpain-2 inhibitors in vivo, the live brain microenvironment of the embryonic zebrafish providing a conducive environment for human cancer cell migration, proliferation, and angiogenesis. Because of their small size, vertebrate biology, and ease of maintenance, embryonic zebrafish offer a cost-effective, simple way of testing the effectiveness of anti-cancer compounds in vivo, acting as an intermediary
step between *in vitro* methods and larger mammalian models.

In summary, both *in vitro* and *in vivo* methods were employed in order to test two compounds which could have major significance in future GBM treatments, either used singularly, alongside current treatments, or together in a combined treatment in order to more comprehensively target GBM progression.
MATERIALS AND METHODS

ZnO NPs and Dispersal Solvents

ZnO NPs (batch NCBA 030) were obtained from Dr. Alex Punnoose of the Department of Physics at Boise State University. These nanoparticles were synthesized in diethylene glycol by the forced hydrolysis of zinc acetate\textsuperscript{11}.

DMSO (J.T. Baker, 99.9%), Millipore filtered H\textsubscript{2}O, and FBS (Corning, 100%), were used to disperse the NPs in solution.

Cell Line and Culture

U251 GBM cells (Sigma?) were used in this investigation due to their observed mimicking of the salient features of GBM, including infiltrative patterns of invasion, and the physical similarities of mouse xenografts to tumors seen in human patients\textsuperscript{21}. Cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM) containing 1% L-glutamine and 10% FBS (SCM) at 37 °C, 100% humidity, and 5% CO\textsubscript{2}.

\textit{xCELLigence System and Plate Preparation}

The \textit{xCELLigence} system operates on the properties of electrical impedance to monitor cell proliferation. The analyzer sends an electrical current along gold electrodes lining the bottom of each cell plate and measures the impedance caused by the cells attaching to them (Figure 7). A parameter called the cell index is then calculated from these impedance values using the following equation:
\[ CI(t) = \frac{[R(f_n,t) - R(f_n,t_0)]}{Z_n} \]

Where \( f_n \) denotes the frequency of the electrical pulse used to carry out the impedance measurement, \( R(f_n,t) \) denotes the measured impedance at frequency \( f_n \) and time \( t \), \( R(f_n,t_0) \) denotes the measured impedance at \( t_0 \), which is the background impedance taken with no cells present in the cell plate, and \( Z_n \) denotes the frequency factor, in Ohms, of \( f_n^{22} \).

As seen from the above equation, an increase in cell index suggests cell proliferation, due to the increase in impedance over the background. Conversely, a decrease in cell index suggests either cell death and release from the cell plate, or a shrinkage of the cells still attached to the plate.

Ninety microliters (uL) of SCM were added to each well of an E-16 cell plate. The plate was then loaded into the RTCA DP Analyzer to record a background cell index reading. U251 glioblastoma cells were suspended in FBS-containing medium at a concentration of 50,000 cells per milliliter, and 100 uL of this cell solution (5,000 cells) were added to each well. The RTCA DP Analyzer was then set to monitor the cells for 144 hours, collecting electrical impedance data from each well every 30 minutes.
Figure 2: The xCELLigence system functions by measuring the impedance caused by the adhesion of living cells to the gold electrode lining the bottom of each well of the 16-well cassette plate. From this impedance, the system calculates the cell index, which corresponds to the amount of living cells in each well. As cells proliferate, the cell index increases due to the increase in impedance. When cells die, they detach from the plate, decreasing impedance and cell index as a result.
**NPs Preparation, Dilution, and Addition**

For DMSO trials, 122.8 millimolar (mM) solutions were prepared. For H₂O and FBS trials, 12.28 mM ZnO NPs solutions were prepared. The NPs solutions were then sonicated at 20-22 °C for 20 minutes to evenly disperse them in solution, and diluted to 20X working concentrations of 4000, 2000, 1000, and 500 uM with SCM.

Approximately 24 hours after experiment initiation, and after the cells had reached an approximate cell index of 1, the plate was removed from the analyzer, and 10 uL of SCM, 10 uL of NPs solution, or 10 uL of 4% dispersal solvent were added to the wells, resulting in final NPs solution concentrations of 200, 100, 50, and 25 uM, and the final dispersal solvent concentration of 0.2%.

**Qualitative Post-Assay Plate Imaging**

After assay completion, all wells were emptied, and any remaining cells attached to the plate were fixed and stained using a Diff-Quik Stain Set (Dade Behring) and corresponding protocol. Cells were fixed with 1.8 mg/L Triarylmethane dye in methyl alcohol, and were stained with both 1 g/L Xanthene dye, which stained the cytoplasm, and 1.25 g/L Thiazone dye, which stained nuclei. Plates were then examined under a microscope to qualify the amount of cells still attached to the well bottoms.
**Zebrasfish Preparation**

A solution of 1X embryo media was prepared from a 20X stock solution containing 17.5 g/L sodium chloride, 0.75 g/L potassium chloride, 2.9 g/L calcium chloride, 0.41 g/L mono potassium phosphate, 0.142 g/L anhydrous sodium phosphate, and 4.9 g/L magnesium sulfate.

Freshly fertilized zebrafish embryos were obtained from SARL and stored at 28 °C on a 14 hour light/10 hour dark photoperiod in embryo media (EM) containing 1X embryo media solution and 1X sodium bicarbonate. The media was changed daily.

To inhibit melanogenesis in the embryos, which begins approximately 24 hpf\(^2\), they were treated daily with 75 uM 1-phenyl-2-thiourea (PTU). Continual treatment with PTU results in transparent fish, which is optimal for the xenograft imaging protocol, since pigment can interfere with clear image acquisition and cell counting.

**Fluorescent Cell Preparation**

U251 glioblastoma cells at 80-90% confluence were dyed using carboxymethylbenzamido derivative of dialkyl-carbocyanine (CM-Dil) cell tracker dye (Molecular Probes/Invitrogen). The dye excites at 553 nm and emits at 570 nm (553-570 nm). After dyeing, the cells were suspended in serum-free media (SFM) at a concentration of 1x10\(^7\) cells per milliliter. One part phenol red was then added to every ten parts of this cell solution to darken the solution, aiding in injection needle loading and
visual confirmation of injection success without negative side-effects to the embryos. Cell-free SFM with phenol red added in the same 1:10 ratio was used as a control injection solution.

Cell Injections

Injection needles were loaded with 1 microliter of cell solution or SFM control solution and placed in a MPPI-2 Pressure Injector (API). Embryos at 3 dpf were anesthetized with 0.2 milligrams per milliliter tricaine solution in EM. Embryos were then injected into the top of the cranium with approximately 0.01 microliters of solution, or approximately 100 cells. After injection, the embryos were placed in fresh EM for overnight recovery at 33 °C. For this procedure, 25 embryos were injected with SFM control solution and approximately 175 embryos were injected with cell solution.

Calpain–2 Inhibitor Preparation and Exposure

Calpain-2 inhibitors were obtained from Dr. Isaac Donkor from the University of Tennessee Health Science Center. Inhibitors at a stock concentration of 50,000 micromoles per liter (uM) in DMSO were diluted to 2X working concentrations of 160, 80, 40, 20, and 10 uM in a solution of 1X EM, 75 uM PTU, and 0.4% DMSO (well solution). After the initial dilution, the solution was sonicated for three minutes to break apart aggregates and disperse the inhibitors evenly throughout solution.

After the first round of imaging (see Embryo Imaging), two 96-well plates were
prepared for the calpain-2 inhibitor exposures. Each well was filled with one hundred microliters of well solution, into which a single embryo was placed mirroring its initial location in the imaging plates. 100 microliters of calpain-2 inhibitor or DMSO control solution were then added to each well for final inhibitor concentrations of 80, 40, 20, 10, and 5 micromolar, and a final DMSO control concentration of 0.2%. The plates were then incubated for three days at 33 °C. To prevent evaporation of the well solution over the course of the treatment, water was added to the outer rim of wells, and the plates were covered loosely with parafilm.

Embryo Imaging

After overnight recovery, embryos were anesthetized with 0.2 mg/mL Tricaine in EM and loaded into 96-well clear-bottom imaging plates using 0.8% agarose gel. The embryos were imaged in a high-content imager using both a brightfield and a tetramethylrhodamine isothiocyanate (TRITC) fluorescence filter (541-572 nm) in order to locate the starting position and quantify the baseline population of the glioblastoma cells in their heads.

After imaging, the embryos were exposed to calpain-2 inhibitor solutions, 0.2% DMSO, or nothing for a three day treatment period, as described previously in Calpain–2 Preparation and Exposure. After the treatment period, the embryos were imaged a second time, using the same procedure as before.
Cell Dispersal Analysis

A 32 step z-stack image, with 8 um in between each step, was taken of each embryo cranium. Using the MetaExpress software, the z-stack images were flattened into best focus images. The software did this by determining the z-axis location of each in-focus fluorescent signal in a single embryo cranium and creating an amalgamated image of all the focused objects in the cranium.

Multi-wavelength cell scoring was then used to count and log the position of the cells in each embryo cranium. The application operates by using user-defined parameters for cell size and fluorescence intensity to mark objects in the best focus images as 'cells', allowing for measurements of cell number, location, and distance. Using the scored images, a tumor area was determined by drawing a circular perimeter around the outermost cells in each image (Figure 3).

Corresponding day 1 and day 4 post-injection tumor areas were then compared to determine an average change and percent change in tumor area.
Figure 3: Best focus images were processed by the MetaExpress software using a multi-wavelength cell scoring application, which marks objects within the image as ‘cells’ in accordance with user-defined parameters for cell size and fluorescence intensity above background. Tumor area was then determined by drawing a circular perimeter around the outermost cells in each image.
RESULTS AND DISCUSSION

*DMSO-Dispersed ZnO NPs Effectively Induced U251 GBM Cell Death in vitro*

As shown in Figure 4, the addition of a 200 uM solution of DMSO-dispersed ZnO NPs resulted in a significant decrease in cell index over a period of six hours post addition. Further analysis of the cell indices reported reveals an 80% decrease in cell index and cell population over that time period (Figure 5). Also observable in Figure 4 is the fact that, at approximately 48 hours post addition, the cell index of wells treated with 200 uM ZnO NPs began to increase again, indicating cell recovery.

![Figure 4: RTCA-Generated graph of cell index versus assay time for DMSO-dispersed NPs. NPs and control solutions were added at 24 hours. The 200 uM solution (shown in green) was the only one to have a significant effect on cell proliferation. The 100 uM (dark blue), 50 uM (pink), 25 uM (light blue), SCM control (red), and 0.2% DMSO (purple) solutions had no appreciable effect. Cell recovery in the 200 uM treatment can be seen as early as approximately 70 hours into the assay.](image-url)
Figure 5: DMSO-dispersed NPs treatments at 200 uM had a significantly negative effect on cell survival. Data points were collected at 30 hours (6 hours post solution exposure). As in the RTCA-generated graph in Figure 1, the 200 micromolar NPs solution demonstrated the only significant effect on cell proliferation (p < 0.05).

Culture plates were stained, fixed, and imaged following assay completion in order to determine if the decrease in cell index was due to cell death; few to no cells were seen in wells treated with 200 uM NPs solutions, indicating cell death (Figure 6).
Figure 6: At the endpoint of the real-time proliferation assay, the 16-well plates were fixed, stained, and examined to determine if any observed decrease in cell index was due to cell death, which would cause the cells to detach from the well bottom. The bottom two images are examples of a well treated with a 200 uM ZnO NPs solution (left), which had a final cell index of approximately zero at the time of fixing, and a DMEM control well (right), which had a final cell index of approximately four. Few to no cells can be seen between the gold electrodes (shown in black) in the left image, confirming that the
decline in cell index was caused by cell death and subsequent detachment from the well bottom.

No significant decrease in cell index was observed at concentrations of DMSO-dispersed NPs lower than 200 uM, or in any concentration of H2O-dispersed and FBS-dispersed NPs (Figure 7). H2O and FBS-dispersed NPs treatments showed a slight increase in proliferation compared to SCM and solvent controls.

![Figure 7: FBS- and H2O-dispersed NPs at 200 uM had no significant effect on cell survival. Data points were collected at 30 hours (6 hours post solution exposure). The 200 micromolar NP solution dispersed in DMSO was the only one to have a significant anti-proliferative effect on the cells (p < 0.05).](image)
These results suggest that DMSO increases or better facilitates the cytotoxicity of the ZnO NPs when compared to H₂O and FBS. However, the reason for this perceived effectiveness is suspect. It has been shown that stock solutions at higher concentrations are generally less stable than those prepared at lower concentrations, and that the hydrodynamic sizes of NPs in solution has a significant impact on bioavailability and toxicity. The hydrodynamic sizes of NPs dispersed in DMSO range from 1000 to 4000 nanometers, compared to the sizes of NPs dispersed in water, which range from 250 to 1250 nanometers. This large hydrodynamic size, coupled with the instability of NPs at higher concentrations may have caused the NPs in DMSO to agglomerate and fall out of solution, coming into direct contact with the cells. The significant toxicity delivered by this contact could be causing the observed cell death. The fact that large agglomerates of NPs induce less cytotoxicity than small agglomerates supports this idea. In contrast, the NPs in FBS likely remained in solution, as FBS-dispersed stock solutions of NPs demonstrated a relatively stable hydrodynamic size and slower sedimentation rates, an effect hypothesized to be the result of bovine serum albumin (BSA) proteins in the FBS coating the NPs, preventing agglomeration. The lack of toxicity observed in the FBS trial would then be due to the NPs remaining in solution, but not being at a high enough concentration to have any noticeable cytotoxic or anti-proliferative effect. Regarding the lack of toxicity observed in the H₂O trials, the smaller hydrodynamic sizes might have been enough to keep the NPs in solution, despite the lack of stability and possible agglomeration. In an assay of U87 glioblastoma cells exposed to ZnO NPs suspended in
phosphate-buffered saline, significant apoptosis was seen at concentrations as low as 1000 uM. However, the highest concentration used in this assay was 200 uM, indicating that a high concentration is crucial to the cytotoxicity of NPs suspended in aqueous solutions, as a lack of agglomeration seems to render them effectively non-toxic at low concentrations. Additionally, as the dilution of the NPs solutions was done using SCM, the BSA in the SCM might have coated the NPs, which, in the case of the H₂O solutions, may have been enough to keep the NPs completely in solution, accounting for the lack of observed toxicity.

A particularly troubling observation is the fact that, over the 114 hours following exposure to the 200 uM solution of DMSO-dispersed NPs, the cell index began to rise again, indicating cell recovery. Whether this is due to cellular endocytosis and metabolism of the NPs in lysosomes or another mechanism of NPs dissolution, it suggests that the cytotoxic effects of NPs are time-limited and that re-treatment may be necessary for more effective targeting. This issue with this need for re-treatment is the unfeasibility of opening up a patient’s brain periodically for direct application. Unless a treatment of this nature is proven to be effective when administered intravenously, this limitation precludes the use of ZnO NPs as is for use as a treatment for GBM.

Previous studies demonstrate the selective toxicity of ZnO NPs and suggest their potential for use as a treatment for a variety of cancers. However, preliminary results suggest that the NPs, dispersal solvents, and application methods used here present a GBM treatment that may cause healthy cell death or other non-specific cytotoxic effects.
Batches of NPs may be inconsistent in their physical and chemical properties due to the nature of the synthesis process, making the mass production of a ZnO NPs-based treatment difficult. This, combined with the delicacy of brain tissue and the unfeasibility of constant re-treatment needed to suppress cell recovery, makes the use of these NPs potentially dangerous for GBM patients. In another study, these NPs were used in the embryonic zebrafish xenograft model of GBM, and an increase in cell proliferation was observed, further supporting the unfeasibility of using these NPs as a GBM treatment. Ultimately, these particular NPs and dispersal methods cannot be recommended for use in a live animal model, and no further experiments involving these NPs are planned at this time.
The Effects of Calpain-2 Inhibitors on GBM Invasion and Migration In Embryonic Zebrafish

The results from the zebrafish xenograft model are shown in Table 1 and Figures 8 and 9 below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1 Post-Injection Tumor Area (um)</th>
<th>Day 4 Post-Injection Tumor Area (um)</th>
<th>% Change in Tumor Area</th>
<th>% Fish Survival</th>
<th>% Cell Count Change</th>
<th>Fish Tested</th>
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<tr>
<td>0.2% DMSO</td>
<td>127015.76 +/- 18892.69</td>
<td>255759.97 +/- 19086.85</td>
<td>101.36%</td>
<td>61.76%</td>
<td>-8.18%</td>
<td>14</td>
</tr>
<tr>
<td>05 uM</td>
<td>152573.30 +/- 7498.42</td>
<td>259336.08 +/- 12078.78</td>
<td>69.97%</td>
<td>55.88%</td>
<td>17.79%</td>
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<tr>
<td>10 uM</td>
<td>131804.12 +/- 10423.82</td>
<td>263072.39 +/- 17293.70</td>
<td>99.59%</td>
<td>70.59%</td>
<td>13.59%</td>
<td>20</td>
</tr>
<tr>
<td>20 uM</td>
<td>112732.05 +/- 10015.24</td>
<td>247186.15 +/- 18094.08</td>
<td>119.27%</td>
<td>50.00%</td>
<td>12.21%</td>
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<tr>
<td>40 uM</td>
<td>125933.13 +/- 8930.52</td>
<td>252602.32 +/- 16426.03</td>
<td>100.58%</td>
<td>64.71%</td>
<td>36.00%</td>
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</tr>
<tr>
<td>80 uM</td>
<td>151662.19 +/- 6943.53</td>
<td>274970.34 +/- 15723.30</td>
<td>81.30%</td>
<td>38.24%</td>
<td>44.06%</td>
<td>12</td>
</tr>
<tr>
<td>Untreated</td>
<td>130653.48 +/- 16298.41</td>
<td>213327.64 +/- 33844.71</td>
<td>63.28%</td>
<td>100.00%</td>
<td>5.88%</td>
<td>4</td>
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Table 1: Quantitative results from the embryonic zebrafish model, including day 1 and day 4 post-injection tumor areas, percent change in tumor area, percent of surviving fish following calpain-2 inhibitor exposure, and percent cell count change from day 1 to day 4 post-injection. Numbers of fish tested are combined over two separate assays.
Figure 8: All treatment groups demonstrated an increase in tumor area from day one post-injection (1dpi) to day four post-injection (4dpi). Percent changes in tumor area from 1dpi to 4 dpi are shown in Figure 9.
Figure 9: Percent changes in tumor area are shown here, according to the results presented in Table 1.

It was expected that the percent change in tumor area would demonstrate an inverse relationship to calpain-2 inhibitor concentration; an increase in inhibitor concentration would result in a decrease in percent change in tumor area, indicating an inhibition of cell invasion. However, as seen in Table 1 and Figure 9, there is no significant difference between the 0.2% DMSO control and the 10 uM, 20 uM, and 40 uM calpain-2 inhibitor treatments. The 05 uM calpain-2 inhibitor treatment and untreated fish group demonstrated a reduced percent change in tumor area compared to the previously mentioned inhibitor treatments and carrier control. This was likely a result of
error, as the decrease cannot be correlated to fish survival or to percent cell count change in both cases.

The only treatment for which the relative reduction in percent change in tumor area can be correlated to fish survival is the 80 uM treatment. Only 38.24% of fish survived exposure to this treatment, in comparison to fish survival percentages ranging from 50.00% to 70.59% in the other calpain-2 inhibitor treatments. It could be the case that the increased concentration of inhibitors had a slightly toxic effect on the zebrafish embryos, creating a non-ideal environment for cell growth and invasion, and resulting in the percent change in tumor area of 81.30%.

It was expected that the percent change in tumor area and percent cell count change of the untreated fish would be approximately 100% and 150%, respectively. However, the percent change in tumor area of the untreated fish was only 63.28%, while the percent cell count change was only 5.88%. This discrepancy could have been caused by a group of untreated fish that was too small, producing values that were likewise small compared to expected values. A possible fix for this would be to screen more untreated fish in following experiments, as well as to repeat the entire experiment multiple times to counter the natural variance in data. If the discrepancy remains after extensive re-screening, then a more definite conclusion as to the accuracy of the imaging procedure, chosen parameters for multi-wavelength cell scoring, and overall analysis procedure can be made.

Overall, cell count change percentages seem to be indicative of an imaging and
cell scoring procedure that is not optimized for the accurate detection of U251 GBM cells within the embryonic zebrafish cranium. Future work with this model should first involve the optimization of the multi-wavelength cell scoring application parameters of the MetaExpress software for use with this particular cell line. This could involve embedding a known number of cells into a medium mimicking the optical properties of a zebrafish embryo cranium, agarose or matrigel, for example, and imaging them immediately. Since the number of cells initially plated is known, the parameters for cells size and fluorescence intensity used in the multi-wavelength cell scoring application can be optimized for U251 GBM cells embedded in a zebrafish cranium, allowing for more accurate cell counts and scoring, as well as a reduction in the chance for accidental scoring of auto-fluorescence or fluorescent reflection. With the optimized settings, further experiments using U251 GBM cells can be performed and more accurate data can collected, allowing for a more concrete conclusion on the effectiveness of these synthetic inhibitors to be made.

An alternative to the optimization of the MetaExpress software would be to image the injected embryos using a confocal microscope and quantifying cell invasion and migration in real time and in three dimensions. Another possible future experiment might involve comparing cells treated with synthetic calpain-2 inhibitors to calpain-2 knockdown cells and cells treated with the natural calpain-2 inhibitor calpastatin as control groups to determine a relative effectiveness.

Another problem highlighted by these results is the uncertainty as to whether or
not the inhibitors are entering the embryo cranial cavities and actually reaching the cells. To remedy this, antibodies designed to target cleaved spectrin protein, for example, could be synthesized and applied to the GBM cells during initial inhibitor exposure. In this case, if the spectrin is cleaved, it can be concluded that the inhibitors are not reaching the cells, as calpain-2 is still active. If need be, the inhibitor solutions can be applied through direct injection, for example, to ensure optimal exposure.

Some aspects of the embryonic zebrafish xenograft model, such as the cranial injections and embryo plating for imaging, are highly skill dependent, allowing for a significant amount of human error to be present in the data. As these preliminary results are the combined results from two assay attempts, the experiment would need to be repeated multiple times in order to overcome the natural error generated by the assay procedure.

A more definite analysis of the cause behind the observed increase in invasion seen in the calpain-2 inhibitor treatments could be done if the structure of the inhibitors was made public, as the cause behind this increase may be structural in nature. However, as the inhibitors are patent pending, this analysis cannot be made at this time. Ultimately, the preliminary results presented above do not allow for definite conclusions on the effectiveness of the synthetic calpain-2 inhibitors at this time. Further experiments need to be performed before a final conclusion can be made.
CONCLUSION

In conclusion, the 200 uM treatment of NPs suspended in DMSO was the most effective inhibitor of cell proliferation out of all the methods tested, and NPs suspended in H2O and FBS had no effect on the cells at any concentration. While a 200 uM solution of ZnO NPs dispersed in DMSO had a significant negative effect on cell growth, the hypothesized mechanisms behind this effect and the issues behind application and use in a human patient highlight its unfeasibility as a GBM treatment in its current state. Barring further research into the effect of these nanoparticles on normal human astrocytes and potential application procedures that do not endanger a patient, DMSO-dispersed ZnO NPs cannot be recommended as a GBM treatment at this time.

Preliminary results from the embryonic zebrafish model are inconclusive as to whether the synthetic calpain-2 inhibitors had a significant effect on cell invasion and migration. As a result, further experiments, as well as repeats of previously described experiments, need to be undertaken in order to determine this effect. After this is done, a decision can be made as to whether the inhibitors should be tested in a larger mammalian model.
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