Glioblastoma, which occurs in adults primarily between ages of 40 to 70 years and accounts for 15% of all brain tumors, is a devastating diagnosis with a median survival rate of less than a year. Invasiveness of glioblastoma cells significantly reduces the effectiveness of current treatments, highlighting the importance of understanding the characteristics of the invasive population. In glioblastoma cells, influxes of Ca\(^{2+}\) activate Ca\(^{2+}\)-dependent signaling pathways, which promote cell invasion. Calpain 2 is a calcium dependent protease. Previous studies have shown a 90% reduction of glioblastoma cells invasiveness by knocking down calpain 2, demonstrating calpain 2 expression is required for glioblastoma cell invasion.

Understanding the mechanism through which calpain 2 promotes cell invasion may lead to an effective treatment for glioblastoma. In collaboration with Dr. Issac Donkor, we are screening novel synthetic calpain 2 inhibitors for the ability to inhibit glioblastoma cell invasion in culture. Four synthetic calpain 2 inhibitors were examined. Initial screening identified one calpain 2 inhibitor (LJ351) that blocked cell invasion at concentrations of 50-100 µM. Our morphology assay showed that LJ351 does not have an effect on cell morphology. We are currently in the process of testing these inhibitors in the zebrafish xenograft model, which is a rapid and
inexpensive method for measuring glioblastoma invasion in a dynamic brain microenvironment. Use of the xCELLigence system combined with the zebrafish xenograft model provides an efficient approach for identifying calpain 2 inhibitors with the potential to inhibit tumor cell invasion in glioblastoma. Identifying molecular based treatment can reduce the global burden for cancer. At the same time, the socioeconomic differential in cancer survival should also be considered and addressed.

Key Words: synthetic calpain 2 inhibitors, glioblastoma, xCELLigence system, cancer burden, cancer inequality

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Fighting the Global Cancer Burden: a Promising
Molecular Based Cancer Therapy

By
Selina Qiuying Liu

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

____________________________
Selina Qiuying Liu, Author
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DEDICATION

This thesis is dedicated to my beloved mother and father, thank you for giving me strength to always strive for better, giving me a chance to improve myself through my walks of life and helping to make me who I am.
Chapter I:
Calpain 2 inhibitors: a promising molecular based cancer therapy for glioblastoma

INTRODUCTION:

Glioblastoma: A Highly Invasive and Refractory Brain Tumor

Gliomas are the most common primary brain tumors of adults, with an estimated 2.5% of all cancer deaths in the United States \(^1\). The most common and aggressive form of glioma is the glioblastoma multiforme, representing as much as 53.9% of all primary brain gliomas \(^1\). Approximately 66,290 primary brain tumors are diagnosed in the United States each year, 16% of which are glioblastomas \(^1\). Despite decades of research on tumor biology and treatment, glioblastoma remains to have a poor prognosis \(^2,3\). The median survival of glioblastoma is 15 months after diagnosis and the five year survival rate is less than 5% \(^1,3,4\). The standard treatments include surgery to remove as much tumor tissue as possible followed by radiotherapy combined with chemotherapy using the drug temozolomide (TMZ) \(^3,5\). Surgical removal of tumor often leaves residual tumor cells on the edge. These residual cells are exceptionally infiltrative and are able to invade into normal brain tissue, which pose the primary challenge to treat glioblastoma successfully \(^6\). Surgeries intend to remove as much tumor tissue with as little damage to normal brain tissue as possible. However, there are residual tumor cells on the edge of the resected cavity. These tumor cells are highly resistant to chemotherapy \(^7\) and continue to invade into surround tissues, leading to death of the patient. Therefore, it is crucial to understand the molecular mechanisms that are involved in the regulation of glioblastoma cell migration and invasion. Moreover, it remains a high priority for researchers and clinicians to discover new
targets and therapeutic strategies to increase the survival rate and improve the clinical outcomes of glioblastoma\textsuperscript{1,2}.

**Glutamate and calpain 2 are required for glioblastoma cell invasion**

Studies done by Lyons et al. demonstrated that glioblastoma cell invasion and migration are regulated by autocrine glutamate\textsuperscript{8–10}. Glutamate activates AMPA receptors on glioblastoma cells, which stimulate calcium influxes and promote cell migration\textsuperscript{11,12}. Moreover, studies by Giannone et al. have shown that focal adhesion disassembly in glioblastoma cells requires calcium oscillation\textsuperscript{13}. The role of glutamate induced calcium fluxes in tumor cell invasion is unique to glioblastoma cells. Although the target of calcium influx required for glioblastoma cell invasion is not known, calpain 2 is a candidate.

Calpain is a class of intracellular signaling cysteine proteases\textsuperscript{11}. Calpain 2, a ubiquitously expressed isoform of the calpain family\textsuperscript{14}, has been shown to regulate cell migration. Calpain 2 activity is required for both the assembly and disassembly of the focal adhesion complexes which aids cell migration\textsuperscript{15}. Shiba et al. was the first group identifying the correlation between calpain 2 and cancer development where they reported increased calpain activity in breast cancer tissue\textsuperscript{16}. More recent studies have further established the correlation between calpain 2 and tumor progression\textsuperscript{17–19}. Mamoune et al. demonstrated that inhibition of calpain activity decreased the invasiveness of human prostate tumor cells by 50\textsuperscript{20}. These results collectively identify calpain 2 as a potential target for molecular-based therapies.

Previous studies in our laboratory examined the role of calpain 2 in glioblastoma cell invasion. In the study, both chemical calpain inhibitors and shRNA based knockdown of calpain 2 were used. The results indicated that glioblastoma cell invasion was reduced 90 percent by
knockdown of calpain 2\textsuperscript{21}, suggesting that calpain 2 activity is required in glioblastoma cell invasion. To examine whether calpain 2 is required for dispersal of glioblastoma cells in a dynamic brain microenvironment, Lal \textit{et al} transplanted human glioblastoma cells and calpain 2 knockdown cells into the brain of zebrafish\textsuperscript{21}. A 2.9-fold decrease in human glioblastoma cell invasion in zebrafish brain was observed for calpain 2 knockdown cells. This suggests that dispersal of glioblastoma cells in a living brain microenvironment requires the expression of calpain 2. The findings from this study indicate that calpain 2 expression is required for glioblastoma cell dispersal within the dynamic microenvironment of the brain. Moreover, findings from this study suggest that zebrafish are an effective model for brain tumor invasion studies. Based on the findings by our laboratories, as well as others, we proposed a working model for the role of calpain 2 proteolysis in glioblastoma cell invasion (Fig. 1). Although the mechanisms through which calpain 2 promote cell invasion are unclear, inhibition of calpain 2 activity will reduce glioblastoma cell invasion. Therefore, identifying effective calpain 2 inhibitors may provide promising therapeutic molecules to inhibit glioblastoma cell migration and invasion.
Screening of novel calpain 2 inhibitors for inhibition of glioblastoma cell invasion

In collaboration with Dr. Issac Donker from the University of Tennessee Health Science Center, we have received four novel synthetic calpain 2 inhibitors (LJ351, LJ222, LJ2265, and LJ2169). Real-time monitoring of cell invasion and migration of cultured cells was done using an xCELLigence Real-Time Monitoring System. U251 glioblastoma cells were used in the screening protocol because these cells mimic the salient feature of human glioblastoma\textsuperscript{22}. In addition U251 cells in mouse xenograft models to recapitulate histological and immunohistochemical features of human Glioblastoma, U251 cells also show genetic alterations (such as alterations in key tumor suppressor and oncogenic pathways) consistent with human glioblastoma. In this study, cell invasion and migration assays were performed \textit{in vitro}. The invasion and migration potentials of control glioblastoma cells (not treated with calpain 2 inhibitor) were compared with the glioblastoma cells treated with calpain 2 inhibitors. Synthetic calpain 2 inhibitors can inhibit calpain 2 activity, glioblastoma cell invasion will be reduced.

\textbf{Figure 1: Working Hypothesis for role of calpain 2 in glioblastoma cell invasion.} The diagram illustrates how glioblastoma cell invasion can be promoted by glutamate stimulation of calcium influxes. If a synthetic calpain 2 inhibitor can inhibit calpain 2 activity, glioblastoma cell invasion will be reduced.
calpain 2 inhibitors that are effective at reducing cell invasion and migration will be introduced to a zebrafish xenograft model to examine their effect in a brain microenvironment. The primary purpose of this study is to identify synthetic calpain 2 inhibitors that are effective at reducing glioblastoma cell invasion. In addition, I am proposing an effective yet low-cost drug screening protocol including in vitro screening using the xCELLigence system and in vivo screening using the zebrafish model.

EXPERIMENTAL PROCEDURE:

Cell Culture

U251 cells, a cell line of human glioblastoma, were obtained from NCI repository (National Cancer Institute, Frederick, MD) and cultured in DMEM containing 2mM L-glutamine and 10% fetal bovine serum (FBS). Continuous cultures were plated in T-75 flasks. The cell culture is maintained at 37°C, 100% humidity, 95% air, and 5% CO₂.

Calpain 2 Inhibitors

We have received four synthetic inhibitors developed by Dr. Issac Donkor: LJ351, LJ222, LJ2265, and LJ2169. The inhibitors are active (catalytic) site directed reversible competitive inhibitors of calpain. The compounds are soluble in DMSO but not in water. Compounds chemically similar to the four tested inhibitors are found to be membrane permeable. Due to patenting considerations, the structures of these compounds were not disclosed. The inhibitors
were resuspended to 50 mM stock solutions using DMSO. The stock solutions were stored in 5μl aliquot at -80°C.

**Matrigel Concentration and Dilution**

For the invasion assays, Matrigel was used as an artificial extracellular layer for cells to invade through. Matrigel was obtained from BD Biosciences with an initial concentration of 8.6mg/ml. In order to obtain the optimal Matrigel concentration for the invasion assay, three different Matrigel concentrations were tested with the xCELLigence system in absence of the calpain 2 inhibitors. The Matrigel was thawed overnight at 4°C and diluted to the appropriate concentration with ice-cold serum-free DMEM to the desired concentration. All material used to handle the Matrigel (pipet tips, CIM-Plates 16, and eppendorf tubes) were pre-cooled overnight because Matrigel forms a gel at temperature above 10°C.

**xCELLigence System**

The xCELLigence system from Roche Applied Science and ACEA Biosciences was used to measure invasion and migration. It consists of four main components: “the RTCA analyzer, the RTCA SP station, the RTCA computer with integrated software, and disposable CIM-16 plates” 23. The RTCA SP station is placed inside of a standard tissue-culture incubator, connected to the analyzer and the laptop computer with software, which are on the outside. The CIM-16 plates are disposable device used to perform invasion or migration assays using the RTCA SP instrument. The CIM-16 plate is composed of an upper chamber and a lower chamber, as shown in figure 2. The bottom of the upper transwell membrane is integrated with gold cell sensing microelectrodes. These microelectrodes maintain electrical impedance when the apparatus is connected to electricity.
The electronic impedance of the microelectrodes is measured to allow real-time monitoring of cell movement. When a cell invades through and reaches the lower side of the membrane, the impedance across the membrane changes. Cells attached to the microelectrodes act as insulators and thereby lead to an increase in impedance. Thus, the more cells that move from upper chamber to the lower chamber and are attached to the microelectrodes, the larger the value of electrode impedance. Cell index (CI) was derived to express the relative change in the impedance between measurements at any time (t) and the background value (t₀), which provides real-time quantitative information about the number of invading cells. Cell index is directly proportional to the number of the cells that move from the upper chamber to the lower chamber. The higher the cell index, the more the cells are migrated/invaded, i.e. the higher the migration/invasion potential.

**Figure 2: Transwell Setup.** Cells are suspended in serum free media (SFM) in the upper chamber. Serum containing media on the bottom of the well act as chemo-attractant for cell invasion. As more cells attached to the Gold Electrodes, the cell index increases.
Real-Time Invasion Assay

Real-time monitoring of U251 cell invasion was performed using the xCELLigence system. This assay allows us to examine how glioblastoma cells navigate a three-dimensional environment. The Matrigel layer resembles the complex extracellular environment in tumor tissues.

The invasion assays were performed following instructions provided by the manufacturer\textsuperscript{24}. Briefly, Matrigel was diluted in cold serum-free media and then used to coat the upper chamber of CIM plates. Preparation of a Matrigel layer on the CIM-Plate was carried out by adding 50μl of the dilution sequentially on top of the membrane of the upper chamber followed by immediate removal of 30μl, leaving a total of 20μl Matrigel dilution. The coated membranes in the upper chambers were incubated at 37°C for at least 4 hours to ensure formation of homogeneous Matrigel layer. The lower chamber is filled with 160 μl of DMEM containing 10% FBS and the lower and upper chamber plates were assembled. Serum-free DMEM (30μl) was added to each well in the upper chamber. The assembled plate was placed in a 37°C incubator for one hour. Immediately before adding cells, the background impedance of the media was measured and recorded. Twenty-thousand cells suspended in serum-free medium were added to the Matrigel coating in the upper chamber. The 10% FBS in the lower chamber is the chemo-attractant stimulating the cells to invade.

Inhibitors were diluted to the proper concentrations using serum-free DMEM and added to both the upper and lower chamber (upper chamber has the same concentration as the lower chamber). The tested concentrations of synthetic calpain 2 inhibitors were 10μM, 50μM, and 100μM. After allowing cells to settle for 30min at room temperature, inhibitors were added to each well right before the CIM plate assembly was placed in the RTCA-DP analyzer. The
The electrical impedance of the membrane was recorded at an interval of 15 minutes for 150 hours. The data obtained from invasion assays were analyzed using RTCA software provided by the manufacturer and Microsoft Excel.

**Real-time Migration Assay**

For the *in vitro* assays, cell migration is different from cell invasion in that it does not require degradation of extracellular matrix. Real-time monitoring of U251 cell migration was performed using the same xCELLigence system as for invasion assay. The only differences in experimental setups between these assays were that in the migration assay, the upper chamber was not coated with Matrigel, allowing the cells to migrate to the lower chamber without having to invade through an artificial membrane. Cells in the upper chamber were suspended in serum free DMEM with three different concentrations of synthetic calpain 2 inhibitors: 10 µM, 50 µM, and 100µM. The electrical impedance of the membrane was recorded at an interval of 15 minutes for 150 hours. The migration data were analyzed using RTCA software provided by the manufacturer.

**Cell Morphology Assay**

For cultured cells, changes in adhesion and morphology provide sensitive outputs for detecting cellular stress and cytotoxicity. To determine whether the calpain 2 inhibitors change the morphology of U251 cells, μClear® Black 96-well plates from Greiner Bio-one were used to plate the U251 cells treated with synthetic calpain 2 inhibitors. In order to promote cells attachment to the bottom, each well of the 96-well plate was coated with 100µl of DMEM containing 10% FBS and placed in 37°C incubator for 1 hour. Twenty-thousand cells suspended
in serum-containing media were added to each well of the 96-well plate. The plate was kept in 37°C incubator for 4 hours to ensure a uniform cell adhesion. Then, the plate was imaged with ImageXpress Micro and MetaXpress High Content Image Acquisition and Analysis Software (Molecular Devices). This set of images was used as the control (before treatment).

Synthetic calpain 2 inhibitors were diluted to the proper concentrations using serum-free media. 20μl of calpain 2 inhibitors or DMSO with various concentrations was added to each well. For control well, 20μl of DMEM was added. The final concentrations of calpain 2 inhibitors were 10μM, 50μM, and 100μM. The corresponding concentrations of DMSO (0.2%, 0.5%, and 1%) were set as vehicle control.

Immediately after addition of calpain 2 inhibitors and DMSO, images were captured every 30 minutes for 16 hours. During the course of the time–lapse assay, the plate was kept at 37°C with the standard conditions of 100% humidity, 95% air, and 5% CO₂. Another set of images was taken 24 hours after administration of calpain 2 inhibitors. All the images taken were using a 10X objective and three images were taken per well.

RESULT:

Glioblastoma Cell Invasion Dependent on Matrigel Concentration

Previous studies in our laboratory showed that U87 cells (another glioblastoma cell line) are able to invade through Matrigel at 0.2mg/ml, 0.4mg/ml and 0.8mg/ml concentrations. To optimize the Matrigel concentration for invasion assays in this study, Matrigel concentrations of 0-1mg/ml were tested using 10% FBS in the lower chamber as chemoattractant for cell invasion. The impedance of microelectrodes on the bottom of the upper chamber was measured every 15 minutes for 150 hours and represented as cell index. The impedance reading changes when cells
traverse the Matrigel matrix and reach the lower chamber of the CIM-16 plate. Higher cell index implies that more cells have invaded through the Matrigel layer.

Figure 3a is a representative graph of time vs. cell index generated by the RTCA software. The trend of cell invasion over time can be broken down into three phases: lag phase, exponential phase, and a plateau. The lag phase is where cells are moving through the Matrigel and increase in cell index is minimal. During the exponential phase, increased number of cells invades to the lower chamber of the CIM-16 plate, which causes an increase in cell index. Finally, the maximum cell index was reached and a plateau was observed thereafter (Fig. 3a).

As seen in figure 3a, the rate of invasion through 0.25mg/ml Matrigel was almost identical to the control group saturating at approximately 95 hours. For cells separated by 0.5mg/ml Matrigel, all three phases are present. The maximum cell index, which is lower than that for the control group, was achieved around 100 hours. For the group with 1.0mg/ml Matrigel, no increase in cell potential was observed until 80 hours. Moreover, the cells were still in exponential phase at 150 hours when the assay was stopped. Wells with no FBS serve as negative control. FBS act as chemical attractant for cell invasion. Without it, minimal cells invaded through the Matrigel layer; minimal increase in cell index was observed.

To analyze the invasion potential for each group at a particular time point, average cell index for each Matrigel concentration at time point 80 hours after starting invasion assay was graphed (Fig. 3b). The time point of 80 hours was chosen because it is near the end of the exponential phase and is used for analyzing all future invasion and migration assays. As seen in figure 3b, cell index for wells coated with 0.25mg/ml Matrigel was almost identical to the cell index for the control group and they have the highest cell index among all groups tested. This suggests that Matrigel did not provide an effective barrier for cell invasion at concentration of
0.25mg/ml. In other word, using Matrigel at 0.25mg/ml is the equivalent of a migration assay. The group with Matrigel at 1.0mg/ml had the lowest cell index, suggesting that Matrigel at 1.0mg/ml was too thick for cells to invade through within a reasonable assay time. The wells with Matrigel layer at 0.5mg/ml had average cell index that was higher than wells with 1.0mg/ml but lower than 0.25mg/ml Matrigel and the control group. Our result showed that Matrigel at 0.5mg/ml was effective to provide a barrier for cell invasion and the assay was finished within a manageable period. Therefore, we used 0.5mg/ml Matrigel for all invasion assays in this project.

![Figure 3: Invasion of U251 cells through different concentrations of Matrigel.](image)

Real-time measurement of invasion over time (A). Cell index at 80hrs was graphed for comparison. Cell index was measured every 15min, n=1 ± SD in duplicate. 0.5mg/ml was selected as the optimal Matrigel concentration because it was an effective barrier yet allowed the assay to be finished in a timely manner.

**U251 cell invasion was reduced by calpain 2 inhibitors LJ351 and LJ222**

Our previous studies have showed that calpain 2 activity is required for glioblastoma cell invasion\(^\text{21,24}\). We first investigated whether exposing human glioblastoma U251 cells to the inhibitors would reduce their invasion potential. U251 cells were treated with calpain 2
inhibitors at three concentrations: 10μM, 50μM, and 100μM. A control group was tested where cells were not treated with any synthetic calpain 2 inhibitor. Cell invasion through Matrigel was quantified in real time by using the xCELLigence system. In order to examine the effect of the solvent (DMSO), cell invasion assays were also performed using U251 cells treated with DMSO (vehicle control).

Fig. 4A shows the effect of calpain 2 inhibitor LJ351 on U251 cell invasion. The highest cell index was seen in control group (0μM LJ351). The cell index has an inverse relationship with the concentration of LJ351. The cell index was the lowest for cells treated with 100μM LJ351 (Fig. 4A). Cell index for the group treated with 1% DMSO was not significantly different than the control group, suggesting that 1% DMSO had minimal effect on cell invasion. Cell invasion was significantly reduced for cells treated with 50μM and 100μM LJ351 (Fig. 4A). This demonstrated that LJ351 inhibitor is effective at reducing cell invasion at concentrations of 50μM and 100μM.

Fig. 4B shows the effects of calpain 2 inhibitor LJ222 on U251 cell invasion. The control group with 0μM of LJ222 had the highest average cell index (Fig. 4B). Although cell index was not reduced by treating cells with 10 μM or 50 μM LJ222, treating cells with 100 μM LJ222 reduced the cell index by almost 80%. Cell index for the group treated with 0.2% DMSO was not significantly lower than the control group, suggesting a minimal effect of 0.2% DMSO on cell invasion (Fig. 4B).

Fig. 4C shows the effect of calpain 2 inhibitor LJ2265 on U251 cell invasion. No significant difference was observed comparing the treatment groups with the control group. The differences in cell index among different LJ2265 concentrations were minimal. This may suggest that LJ2265 is not effective at reducing cell invasion potential. Fig. 4D shows the cell
index for cells treated with calpain 2 inhibitor LJ2169. The cells treated with higher concentration of LJ2169 had lower average cell index (Fig. 4D). Treating cells with 100μM LJ2169 reduced the average cell index by about 20%.

**Figure 4: Calpain 2 inhibitors decrease U251 cell invasion.** 20,000 cells suspended in serum free DMEM were added to each well of the CIM-16 plate coated with 0.5mg/ml Matrigel. Calpain 2 inhibitors designated LJ351 (A), LJ222 (B), LJ2265 (C) and LJ2169 (D) were diluted with serum free DMEM and added to both the upper and lower chambers as described in experimental procedure section. The cell index after 80 hrs of invasion was graphed, n=1 in triplicate. DMSO, the solvent for the inhibitors, was tested as a control.

**U251 Cell Migration was Reduced by Calpain 2 Inhibitors LJ351, LJ222 and LJ2265**

Calpain 2 inhibitors can reduce glioblastoma cell invasion through two mechanisms: inhibiting cell migration, and/or inhibiting the degradation of the extracellular matrix. By
investigating the inhibitor’s effect on glioblastoma cell migration, we propose reduced cell migration as a potential mechanism through which the tested calpain 2 inhibitors are inhibiting cell invasion. We performed migration assays to examine whether the calpain 2 inhibitors reduce cell migration. Migration assay is a more sensitive approach to examine the effect of the inhibitors since there is no Matrigel layer blocking the inhibitors from diffusing into the upper well. The setup for migration assays was identical to the setup for invasion assays except Matrigel was not used here. One migration assay was performed for each inhibitor (n=1). Within the same migration assay, triplicates (three wells) were done for each concentration and the average cell index was graphed (Figure 5). The error bars in the graph represent standard deviation within the same experiment.
Fig. 5a shows effect of LJ351 on cell migration. Cells treated with 50 μM and 100 μM LJ351 had the lowest cell index. This suggests that 50 μM LJ351 effectively reduce cell migration. The control group had the highest average cell index. Cells treated with 1% DMSO had lower average cell index than the control group, suggesting that 1% DMSO may reduce cell migration. Fig.5b shows the effect of LJ222 on cell migration. Only cells treated with 100 μM LJ222 had significantly reduced cell index compared to the control group. LJ222 is effective at

Figure 5: Calpain 2 inhibitors decrease U251 cell migration. 20,000 cells suspended in serum free DMEM were added to each well of the CIM-16 plate with no Matrigel coating. Calpain 2 inhibitors designated LJ351 (A), LJ222 (B), LJ2265 (C) and LJ2169 (D) were diluted with serum free DMEM and added to both the upper and lower chambers as described in experimental procedure section. The cell index after 80 hrs of invasion was graphed, n=1 in triplicate. DMSO, the solvent for the inhibitors was tested as a control.
reducing cell migration potential at concentration of 100µM. Cells treated with 0.2% DMSO did not have lower cell index than the control group. Therefore, the effect of 0.2% DMSO on cell migration potential is minimal.

Fig. 5c shows effect of LJ2265 on cell migration. Cells treated with higher concentration of LJ2265 had lower average cell index. Cells treated with 100µM of LJ2265 had the lowest average cell index, indicating that LJ2265 is effective at reducing cell migration at 100µM. Fig. 5d shows the effect of LJ2169 on cell migration. Only the cells treated with 100µM LJ2265 had significantly reduced cell index compared to that of the control group. However, the variation within the triplicates was large for cells treated with 100µM LJ2265 so the difference may not be significant suggesting that LJ2265 may not be effective at reducing cell migration at concentrations between 10µM to 100µM.

**Inhibitor LJ222 Caused Changes in Cell Morphology**

Our result from invasion and migration assays indicated that some calpain 2 inhibitors were effective at reducing glioblastoma cell invasion and migration. It is important to investigate the effect of these inhibitors on cellular cytotoxicity. To investigate this, we observed how the cell morphology in the presence of each calpain 2 inhibitor. Cell detachment and change in cell morphology were used as indicators of cell stress and toxicity. 20,000 U251 cells were plated in each well of the 96-well plate and 20 µl of synthetic calpain 2 inhibitors at different concentrations were added to each well as described in Experimental Procedure.

Immediately after addition of the inhibitors, some cells rounded up. Since inhibitors were added at room temperature, the change in cell morphology may be due to the change in
temperature. Moreover, most of the cells recovered soon after the plate was placed under 37°C. Cells were placed in 37°C incubator for 24 hours and then imaged under 10X magnification as described in the experimental procedure section.

Before receiving treatment, cells were flat and well attached to the bottom of the wells (Figs. 5A-5D). LJ351 had minimal effect on cell morphology at all three concentrations tested. The cells were flat and well attached to the bottom of the well 24 hours after inhibitor was added (Figs. 5E, I, M). For the wells with 100µM LJ351, fibrous substances were observed (Fig. 5M). These may be precipitation, suggesting that the solution may be saturated at 100µM. This may also suggest that the inhibitors are too hydrophobic. At 24 hours, drastic change in cell morphology was observed in cells treated with LJ222. Change in cell morphology was not observed in cells treated with 10µM of LJ222 (Fig. 5F). However, cells treated with 50µM and 100µM LJ222 had dark appearance, dark nuclei, and jagged membranes (indicated by red arrows). These observations are consistent with apoptosis. LJ222 at 50µM and 100µM may be toxic to cells.

For cells treated with 10µM and 50µM LJ2265, no change in cell morphology was observed after 24 hours (Figs. 5G, K). However, drastic morphological changes were observed in cells treated with 100µM LJ2265. Majority cells were round with dark nucleus and jagged membrane (Fig. 5O). The dark nucleus may indicate chromosomal defragmentation, which is a phenomenon for cells undergoing apoptosis. For cells treated with LJ2169, no change in cell morphology was observed at all concentrations (Figs. H, L, P); cells were flat and well attached.
DISCUSSION

Glioblastoma is one of the most lethal forms of cancer. Despite chemotherapy, radiation and surgical technique, the median survival for patients remains poor\(^5,7\), with the median survival of 15 months after diagnosis. It has been shown that Calpain 2 activity is required for glioblastoma invasion and migration\(^21,24\). Previous work in our laboratory has demonstrated that...
knockdown of calpain 2 in glioblastoma cells reduced invasion by 90\%^{24}. Therefore, targeting calpain 2 may provide novel therapeutic strategies for glioblastoma patients.

In this project, I screened four novel synthetic calpain 2 inhibitors: LJ351, LJ222, LJ2265, and LJ2169. Our results showed that inhibitors LJ351 and LJ222 are effective at reducing cell invasion. LJ351, LJ222, and LJ2269 are effective at reducing cell migration.

We first determined the effects of calpain 2 inhibitors on glioblastoma cell invasion. In order to perform invasion assays, we used Matrigel as an artificial barrier (see Experimental Procedure section for detail). We found that 0.5mg/ml Matrigel was effective at providing a barrier for cell invasion and a plateau phase was achieved within a timely manner (Fig. 3a). The results from Real-time invasion assays showed that LJ351 and LJ222 were effective at reducing cell invasion rate at concentrations of 50\mu M and 100\mu M (Figs. 4A, 4B). Then we investigated whether the inhibitors also block glioblastoma cell migration because calpain 2 is involved in both cell migration and invasion, and migration assay is a more sensitive approach to examine the effect of the inhibitors. Our results from migration assays suggest that LJ351, LJ222, and LJ2265 were effective at reducing migration potential at concentrations between 50\mu M and 100\mu M (Figs. 5A, 5B, 5C). The results for LJ351 and LJ222 are consistent with the result from the invasion assay, suggesting that LJ351 and LJ222 are fairly specific for calpain 2. However, invasion assay with LJ2265 did not show reduction in invasion rate even at the 100\mu M concentration. This may be due to technical error and another invasion assay should be done with LJ2265 in order to determine its effect on invasion rate.

In addition to the cell invasion and migration, we examined the effect of synthetic calpain 2 inhibitors on glioblastoma cell morphology using 96-well plates. The conditions used for this assay were very similar to the conditions used in invasion and migration assay in order to
examine the effect of the inhibitors on cell morphology. Cells were passed the day prior to the starting of this assay and 20,000 cells were plated in each well of the plate. Our result showed that the morphology of U251 cells was changed dramatically by 50µM and 100 µM LJ222 displaying morphologies consistent with that of apoptosis. This suggests that LJ222 at 50 µM and 100µM concentrations may be toxic to cells. LJ351 and LJ2159 both had minimal effect on cell morphology.

Implications and Future study

The selective and effective calpain 2 inhibitors can be used in studying the mechanism through which calpain 2 promotes cell invasion. Identifying selective calpain 2 inhibitors may lead to novel treatments for glioblastoma that prolong and save thousands of people’s lives each year. Our result suggests that LJ351 is effective at reducing cell invasion and migration at concentrations of 50µM and 100µM. Moreover, LJ351 had no effect on cell morphology at all concentrations tested. We are currently in the process of testing LJ351 in the zebrafish xenograft model, which is a rapid and inexpensive method for measuring glioblastoma invasion in a dynamic brain microenvironment. Concentrations of 50µM and 100µM are high partially due to the conditions in which the assays were done. The cells were not pre-incubated in the calpain 2 inhibitor and the diffusion rate of the inhibitors is unknown. The effective concentrations can be lowered by incubating cells with calpain 2 inhibitors overnight before the assay. The proposed protocol can be used to screen more calpain 2 inhibitors. Inhibitors effective in the cultured cells and zebrafish models will be advanced for testing in a rodent xenograft model. This study is a significant step forward in glioblastoma research. Findings from this study showed that use of the xCELLigence system combined with the zebrafish xenograft model provides an efficient
approach for identifying calpain 2 inhibitors with the potential to inhibit tumor cell invasion in glioblastoma. This screening protocol can be implemented in the Cell Imaging and Analysis Facility at Oregon State University and be used to study the effect of drugs and environmental toxins on normal or cancer cells.
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Chapter II:
Cancer burden and inequalities in South Africa

INTRODUCTION

Last spring, I had the opportunity to be on an international internship in South Africa. The internship involved rotating through both urban and rural medical facilities. I worked with physicians in an array of specialties. While actively engaging with patients, I also observed the individual, cultural and economical challenges faced by local medical facilities due to the ongoing epidemic of Human Immunodeficiency Virus (HIV) / Acquired Immunodeficiency Syndrome (AIDS) and tuberculosis (TB). Surprisingly, I encountered a large number of patients with chronic diseases such as diabetes and cancer; diseases that I never associated with South Africa. I began to wonder whether the prevalence of chronic disease is increasing in South Africa. If so, how does the national health care system responds to this shift of health demand? Many studies published recently indicate that the incidence rate of cancer is on the rise in South Africa\textsuperscript{25,26}, putting more demand on the underdeveloped cancer care system. In response to this increase in demand, members of biomedical field suggest to increase the funding for cancer treatment/prevention. Such changes in policy will inevitably affect the degree of health inequality in South Africa. By analyzing the cancer disparities in other countries (mostly European countries), I wish to draw attention to the bioethical consequences of increased funding in cancer treatment/prevention. By discussing the factors that contribute to cancer inequalities, I advice the policymakers to integrate the aspect of health inequality into their discussion of new cancer care policies.
INCIDENT RATE OF CANCER IS INCREASING DRASTICALLY IN AFRICA

According to the International Agency for Research on Cancer, there will be more than 21 million new cases of cancer and 13 million deaths annually worldwide by the year of 2030\textsuperscript{27}. While the global cancer burden continues to rise, a majority of it will fall on countries that still suffer from epidemics of infectious diseases\textsuperscript{27}. It is predicted that in 2030, African continent will have 1.27 million cases of cancer and 0.7 million of cancer related death\textsuperscript{27} (see figure 7). Although malnutrition and starvation are still main concerns in some parts of Africa, other areas, such as South Africa, are suffering from increasing incidence of non-communicable chronic diseases such as diabetes, cancer and cardiovascular diseases\textsuperscript{27,29}. In 2008, there were roughly 715,500 new cancers cases and 540,000 cancer-related deaths in Africa\textsuperscript{30}. The World Health Organization (WHO) foresees that non-communicable diseases will account for 46% of mortality and 40% of total disease burden in Africa\textsuperscript{31}. Clearly, African countries are faced by not only the persisting HIV epidemic, but also the unprecedented growth in cancer burden.


HEALTH CARE SYSTEM IN SOUTH AFRICA

South Africa has a history of racism. The recent history of the apartheid left people who had to suffer under it with terrors deeply inscribed in their minds\textsuperscript{32}. Moreover, it left the country
with social inequalities in income and employment status, which are powerful predictors of health condition and access to health care. South Africa does not have universal health care\textsuperscript{26}. The current health care system is divided into two sections: a private system that is based on private insurances and a public sector that relies on government funding. The private health care system is highly efficient and is comparable with some of the best system in developed countries. The public system, on the other hand, is poorly organized and constantly lacking resources. The annual expenditure on health care in South Africa is 8.3\% of its Gross Domestic Product (GDP). Half of it provides care for 16\% (about 8 million individuals) of the population through private insurances, while the other half funds the 84\% of the population who is poor and relies heavily on public health care\textsuperscript{26}. In most countries, income level is the most influential factor for health care disparities. For South Africa, however, race is also an important indicator for health care inequalities. The white tend to use the private health care system whereas the blacks, or the “colored” (as referred by the South Africans), are more likely to use the public health care system.

**SOUTH AFRICAN GOVERNMENT’S EFFORT TO IMPROVE CANCER CARE**

South Africa is well known for their continuous epidemic of infection diseases such as HIV/AIDS and TB. The westernization of lifestyle (such as increased consumption of tobacco, alcohol, and food with high fat content)\textsuperscript{33} makes the country vulnerable to the upcoming cancer epidemic. However, South Africa is also the least prepared for the cancer epidemic because of the lack of cancer care facilities and prevention programs. In response to this emergence of cancer burden, South African government and the medical community are taking actions to strengthen cancer prevention and treatment programs. South African government proposes to
provide national health insurance over the next 14 years\textsuperscript{26}. In addition, private oncologist organizations such as the Independent Clinical Oncology Network (ICON) have initiated a cost-effective cancer treatment protocol that has shown promising results\textsuperscript{25}. ICON has also launched an integrated continuum of cancer care program with special emphasis on cancer prevention, early detection, the use of multidisciplinary approach, follow-up and terminal care guidelines. Unfortunately, the efforts taken by ICON only represent the private sector instead of the entire oncological community in South Africa. Although the private sector has much more resources, they have limited access to good practice. On the other hand, the public sector is composed of highly skilled consulting physicians who can provide quality care even with limited resources. This is because formal training programs for all the future medical oncologists in South Africa are provided only by the public sector. Owing to the high level input from the academic oncology staff, the oncologists in the public sector are indeed very skilled\textsuperscript{25}. Due to the difference in demand, collaboration among oncologists in the private and public sectors can be beneficial and is highly recommended. Good relationship between the two sectors can improve quality of the provided cancer care. For example, the private sector can share their resources with the public sector and share their knowledge on how to provide the most cost-effective cancer care. In 2003, the South African Oncology Consortium gave an opportunity for all oncologists in the nation to come together and work with other professional association, in an effort to develop a model, which can increase the accessibility and quality of cancer care in the country. The model is based on evidence-based guidelines and is supported by all oncologists in South Africa\textsuperscript{25}. The success of this model relies heavily on the collaboration between the public and private sections.
SOCIO-ECONOMIC INEQUALITIES IN CANCER SURVIVAL

As cancer is becoming a major health problem in South Africa, voices in biomedicine demand more funding for cancer research, prevention and treatment. In the midst of the discussion on improving cancer care, the bioethical consequences of such policy change are being ignored. As Dr. Paul Farmer mentioned in his book *Pathologies of Power*, the discussion of medical ethics focuses too much on the problems at the individual level, such as improving the quality of cancer care. However, limited attention is paid to the issues at the social level, such as increasing access to cancer care. In South Africa, for example, quality cancer care is available in the private sector, the health care that majority of the population don’t have access to. The oncology ward in the public hospital is often lacking the vital machines and bed-space. Instead of a place of healing, it is a place where patients receive some degree of comfort from the medical staff and await for death.

Cancer is a unique chronic disease where the condition can only be managed with long-term treatment and medication. Cancer in Africa is often associated with chronic infections, excruciating pain, and disfigurement; factors that cause the cancer patients to distant themselves from the society. The oncology ward provides a temporary network for the cancer patients to share their experience and support each other. The nurses working in oncology ward show empathy toward the patients and “reaffirm the humanity of patients who are decomposing”. Oncology is essential for cancer patients because it eases pain, offers care, increases quality of life and extends life. However, these rewards only come with surgeries, chemotherapy, radiation, and life-long medication, which all require strong financial and family support. Improved oncology can be redemptive. At the same time, the existing health inequality may be exacerbated in a historically unjust country, such as South Africa. Effective cancer prevention
programs require frequent physical checkups by medical professionals. Unfortunately, many patients living in poor communities cannot afford to travel to the clinics on a monthly basis. Patients undergoing surgery and chemotherapy require hospitalization. Yet, many public hospitals are forced to avoid giving anesthesia with minor procedures because of the lack of hospital beds. Before the effort to improve quality of the cancer care, the health disparity between rich and poor needs to be lessened and equal access to general health care should be guaranteed.

The comorbidity of HIV and TB has been the main disease burden in South Africa. An increase in funding for cancer care may have an impact on the current epidemic of infectious diseases. Majority of the government funding for health care in South Africa comes from foreign funding. Currently, most of the foreign funding is directed toward infectious diseases such as HIV and TB. For example, United States National Institute of Health distributes more than $200 million dollars to research groups in Africa annually. About 95% of the funding is for the three infectious diseases (HIV, TB, and malaria), leaving 5% that is designated for non-communicable diseases. A decrease in funding for HIV will be required to compensate for the increase in funding for cancer treatment/prevention. Although the HIV/AIDS epidemic is fairly controlled with the current health care system, a shift in health care focus from infectious disease to cancer may result in a rise in the incidence rate of infectious disease.

There is an increase in awareness about cancer inequality around the world. Unfortunately, literature on cancer inequality within South Africa is very limited. Numerous studies, mostly done in European countries, present compelling evidence for the presence of socio-economic differences in cancer incidence, survival and mortality. This socioeconomic difference in cancer survival is present in all countries, varying in magnitude. For all types of
cancer, the five-year survival rate differs significantly between the most affluent and the most deprived groups. The magnitude of the difference inversely correlates to the prognosis of cancer. Cancers (such as brain cancer) with poor prognoses tend to have moderate level of inequality. However, for cancers with good prognoses, such as breast cancer, the magnitude of differences is the widest\(^\text{38}\). Multiple factors may contribute to the high correlation between socioeconomic status (SES) and cancer survival. They can be grouped into three general groups: cancer stage at diagnosis, characteristics of the patients, and access to health care services\(^\text{39}\).

**Cancer stage at diagnosis:**

Early stage at diagnosis is the most important prognostic factor for survival\(^\text{36}\). Regardless of the type of cancer, earlier diagnosis usually leads to better treatment outcome. Studies have shown that patient with lower SES tend to have greater delay in seeking medical care\(^\text{39}\). As a consequence, cancers are diagnosed at a later stage, resulting in lower levels of response to treatment. Several social constraints contribute to this delay in seeking medical care. First of all, regular health care services may not always be available for people with lower social status. Clinics are not always built in places where it is needed the most; people living in poor communities often have to travel long distance to the closest clinic. In South Africa, for example, there is a huge imbalance between demand for care and available resource in the public sector. It is common that a patient wait in the clinic for the whole day without being seen by a medical professional. Secondly, people with low social status have limited knowledge about cancer symptoms. A survey conducted by Union for International Cancer Control (UICC) revealed that more than a quarter of the African population who participated in the survey believed that cancer has no cure and only 36% referred to cancer as an important health issue\(^\text{27}\).
The lack in availability of health care in addition to the limited knowledge of cancer lead to seeking medical help in later stages of cancer, which greatly reduces cancer survival.

**Patient’s characteristics**

Another potential socioeconomic determinant of the cancer disparity is that socially disadvantaged patients may be more susceptible to the aggressiveness of cancer\(^{36}\). People living in poor communities may have higher chance of being malnourished, which contributes to lower level of immunity. Damaged immunity makes the individual more vulnerable to diseases. People of lower SES may also be exposed to higher level of carcinogens. Studies indicate that behavioral risk factors for cancer, such as smoking or alcohol consumption, increases as one’s SES decreases. Blue-collar worker also may experience higher level of occupational exposure to carcinogens. Moreover, high co-morbidity is known to be associated with low social status, which can contribute to reduced cancer survival\(^ {39}\). Sanitation continues to be a problem in poor communities, especially in the shantytowns where people live in improvised dwellings made from scraps. Infections is a common comorbidity and/or cause of cancer; studies have shown that 36% of cancers in Africa are due to various forms of infection\(^ {35}\). Shantytowns are usually over-crowded and lack basic sanitation and sewage system. Dwelling in shantytowns contributes to not only increased HIV infections but also increases the risk for co-morbidities such as cancer. Investment in improving standard of living such as access to clean water and improved sanitation can improve health condition drastically and reduce the incidence of cancer as well as infectious diseases.

**Differential Access to Quality Cancer Care**

This factor presents the role health care plays in modifying the probability of cancer survival. Even in countries where national health care is free and includes cancer care, there is a
differential in access to cancer care and treatment outcome. Studies indicate that different treatments are offered to patients in different socioeconomic groups\textsuperscript{39}. For example, in the United States, women with breast cancer with low SES are less likely to receive breast-conserving surgeries than those with higher SES. In addition to the difference in treatments offered, there is also difference in access to quality treatment and medication. Since neither the United States nor South Africa have universal health care, not all the people have equal access to necessary medications. Once an individual is diagnosed with cancer, the survival rate is greatly linked to access to treatment and medication. People with lower social status may not be able to afford the suggested treatment or required medication.

**DISCUSSION:**

A number of studies done indicate that the incident rate of cancer is increasing in South Africa. Professionals in the field predict that the rate will continue to climb over the next several years. In response to this growing demand, discussions within the biomedical communities in South Africa demand a shift in the focus of medical care from HIV/TB treatment to cancer treatment/prevention. Redistributing funding may result in a decrease in funding for HIV/TB treatment. This shift in health care focus may result in less people receiving proper HIV/TB treatment, which can lead to another uproar of HIV/TB epidemic.

South Africa has a history of racism that gives rise to inequality between white and black, rich and poor. The two-tiered health care system gives opportunities for health care inequalities among people with different SES. Professionals are focused on improving quality of cancer care while ignoring the importance of equitable health care access. The majority of people in South
Africa rely on the public health facilities funded mostly by foreign funding. An increase in funding for cancer care may pose more constraints to the public sector, which is always faced with the challenge of limited resources.

The molecular based therapy proposed in the first part of this document may be more suited for high-income countries instead of countries like South Africa. The development of such therapy is time consuming, which may result in higher cost. As the prevalence of cancer continues to rise, new health care policies that target cancer care need to be implemented. However, there are several aspects that the policymakers should consider when proposing new health care policies. First of all, effort should be put in educating the general public about cancer prevention and early signs of cancer. Moreover, understanding the disease and knowing how to prevent them can improve health conditions of the population with relatively small investment. Investment in improving living standard such as access to clean water and improved sanitation can improve health condition drastically and reduce the incidence of cancer as well as infectious diseases. The local government should take leadership and demand international donors to distribute their funding among different area (both targeting medical areas and other areas such as improving sanitation) instead of competing among themselves in single area. More importantly, the issues around health care inequality need to be acknowledged and addressed. General health care should become more accessible for people living in the poor communities. As the discussion on increasing funding for cancer treatment unfold, the policymakers should also consider ways to reduce the existing health care disparities.
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