

Aptamer conjugated gold nanorods for targeted nanothermal radiation of Glioblastoma cancer cells (A novel selective targeted approach to cancer treatment)



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ABSTRACT

Selectively targeted nanothermal radiation differs from traditional cancer treatment in two main ways: by targeting specific cancer cells, and localizing treatment. This research focused on the ability to selectively target cancer cells (and cancer stem cells) by targeting the cancer with an aptamer/gold nanorod conjugate. This nano-bio molecule emits heat when excited by a harmless near infrared laser and kills cancer cells, allowing for a selective and targeted treatment. My research showed that selective targeting and killing of aptamer conjugated gold nanorods was possible to kill Glioblastoma cancer cells.

Introduction

The number of cancer patients who are currently living with a diagnosed primary or metastatic brain tumors (or gliomas) in the United States is 350,000. Mortality rate due to gliomas is almost 100%, with most patients living less than a year.^[1] About 50% of gliomas are glioblastoma multiforme (GBM) tumors, one of the most dangerous and aggressive types of tumors.^[2] As a prominent example, Senator Edward Kennedy died on August 27, 2009 of a Glioblastoma tumor, 15 months after the initial diagnosis.

As the fight to cure cancer continues, more treatments have become available, and mortality rates have declined for some forms of cancer. However, brain cancer mortality has declined only by

5% in the last 50 years.^[3] Despite substantial research efforts over the years, surgery and systemic therapy are still the most common treatments used. These treatments are painful, expensive, and have many side effects such as burns, hair loss, fatigue, nausea, and immunosuppression (<http://www.cancer.gov/>). Furthermore, they don't target cancer stem cells (CSC). The "cancer stem cell hypothesis" suggests that a tumor harbors multiple types of cells, including cancer stem cells. Although they are not actual stem cells, they mimic many stem cell properties; they are resistant to traditional cancer treatments and also exhibit slow division rate.^[4,5] CSC are resilient, and difficult to kill, especially because most cancer drugs only target fast dividing cells. The resistance of CSCs against traditional treatments has led scientists to believe that surviving CSCs are the cause of cancer relapses and metastasis.

A second reason for the low efficacy and severe side effects of conventional cancer treatments is that they kill the cancer and healthy cells that exhibit rapid cell division rates (e.g., bone marrow, blood cells, hair follicles, GI cells). Treatment efficacy could be maximized and consequently, side effects minimized if treatment specifically targets cancer cells and CSC with minimal adverse effects on healthy cells using aptamers. Aptamers are oligonucleic acid molecules with a complex 3D structure that bind to a specific target molecule and can recognize virtually any class of target molecules with high affinity and specificity.^[6-10] In lay language, aptamers are human synthesized strands of DNA and have the ability to target any specific cell marker.

Nanosized particles have chemical and physical properties different from their larger counterparts.^[11] Gold nanorods (GNR) in particular have the potential to kill cancer cells using hyperthermia. Hyperthermia is the old practice of using heat to kill cancer cells though it is difficult to heat a tumor without damaging nearby tissues.^[12] In addition to hyperthermia killing cancer cells directly, studies have shown it sensitizes cancer cells (which are susceptible to heat) for other systemic therapies.^[13] When GNRs are excited by a low energy Near Infrared (NIR) laser, they heat up a large area. If a specifically targeted aptamer is bound to the GNR, the aptamer conjugated gold nanorod (A-GNR) is delivered straight to the cancer cell. Upon NIR laser treatment, the GNR releases heat causing cancer cells to die, and leaving surrounding healthy cells unaffected.

The overall goal of my project was to develop a framework for treating tumors and metastasis of cancer (Specifically GBM) that addresses the shortcomings of current therapy. My specific objectives were to: (1) Synthesize and characterize gold nanorods; (2) Evaluate effects of gold nanorods (GNR) and laser on cells, and determine safe concentration of GNR; (3) To determine if nanothermal radiation is capable of killing tumor cells; (4) To investigate if aptamers can be used to target GNR to specific cells; and (5) Investigate efficacy of targeted nanothermal radiation to kill targeted tumor cells. My project is designed to target and personalize cancer treatment and to kill fast and slow growing cells by using aptamer-conjugated GNRs as part of the cancer treatment scheme [Figure 1]. I hypothesized that the use of the selectively targeted nanothermal radiation using an aptamer conjugated gold nanorod would lead to a

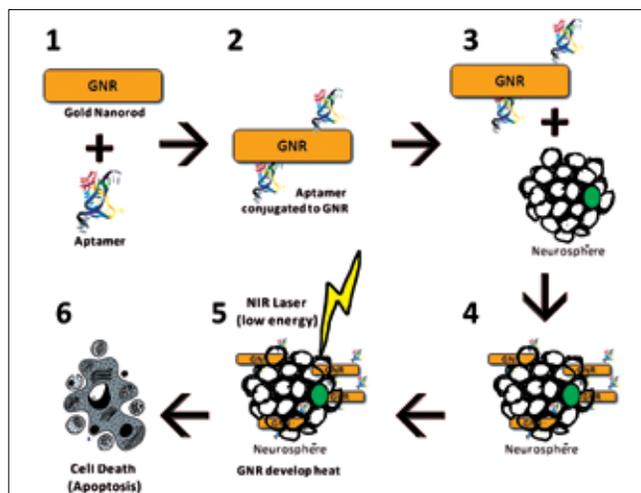


Figure 1: An overview of the aim of the project. Conjugate aptamers and antibodies, expose to cells, heat with a NIR last, and selectively kill cancer cells

superior treatment option that targets cancer cells, and does not affect healthy cells.

Methods

This experiment required numerous materials and methods, some of which are quite complicated. I have not included them in this paper due to complexity and length.

Results

Gold nanorods synthesis and characterization

I synthesized 20nm x 50nm gold nanorods and coated them either with PEG or left them in CTAB, the latter is necessary for covalent modification of the nanorods like conjugation to the aptamers. I characterized the GNR by TEM and UV VIS spectroscopy TEM pictures demonstrate uniformity and even dispersion of nanorods and confirms their size [Figure 2a]. UV VIS analysis showed that the GNR absorbance maximum was at 736nm [Figure 2b]. This analysis was performed every few months to ensure that the GNR did not aggregate or change in size or shape.

GNR emission of heat when exposed to a NIR laser

Gold nanorods emission of heat when exposed to a near infrared (NIR) laser was measured using an agar phantom setup.^[14] As can be seen from Figure 3, the temperature of agar was measured over 900 seconds (15min). The graph shows the steady temperature

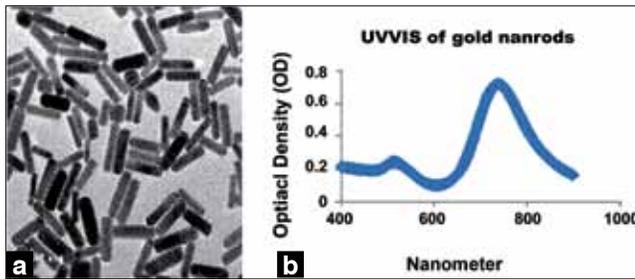


Figure 2: (a) A transmission electron microscope (TEM) image of freshly synthesized gold nanorods was taken. The dimensions of these nanorods were approximately 20nm x 50nm. It also ensures uniformity and even dispersion of GNR. (b) UV-VIS analysis of GNRs. Optical density of nanorods was measured from 400nm-900nm. The peak resonance absorption band was at 736, clearly in the near infrared (NIR) range

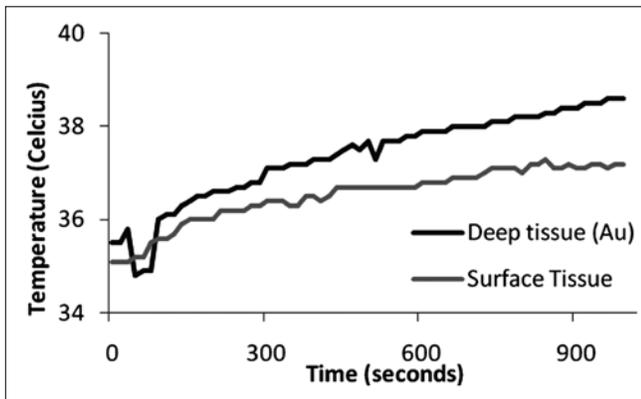


Figure 3: Comparison of temperature (°C) increase in 15min of surrounding agar (surface tissue) vs. agar with gold nanorods (Deep tissue). After about 2 minutes a temperature difference between deep tissue and surface tissue is seen. By the end of 15min, deep tissue is still increasing while surface tissue plateaued

increase over 15 minutes. It is evident that the agar with the GNR (deep tissue) heats up more rapidly than the surrounding agar (surface tissue). After 15 minutes there is an increase of over 2 °C by the deep tissue, and about 1 °C increase by surface tissue. All experiments were performed in at least triplicates.

GBM cells exposure to GNR

A titration series was performed to determine a safe concentration of GNR to cells without being lethal or toxic to the cells. Figures 4 a and b show a visual and statistical representation of the effects of various gold concentrations on cell growth.

Figure 4A shows the drastic decrease in number of neurospheres at a concentration of 1:100 or higher. Statistical significance and IC 50 was measured using the program Excel. 4B shows statistical data of the cell counts. The stars represent a statistically significant decrease in number of cells due to the concentration of GNR. It can be seen that at any concentration 1:100 and higher, the gold has obvious

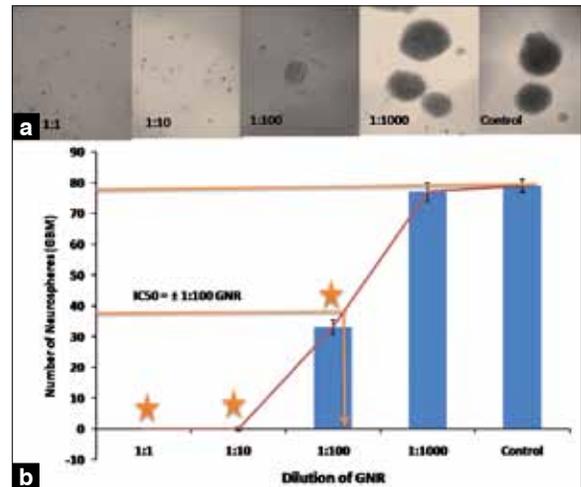


Figure 4: Effect of gold nanorods at various concentrations on neurosphere growth. (a) Light microscope pictures of neurospheres after 7 days of exposure to cells. The only concentration that did not have any significant effect on the cells was at 1:1,000. All higher concentrations of GNR killed cells. IC50 was at the concentration 1:100, meaning that concentration killed 50% of the cells. (b) This graph shows neurosphere count after 7 days of exposure to gold nanorods. The toxic effect of higher concentrations is great enough to be considered statistically significant correlates identically with the graph. This experiment was done in replicates of three, and the data are displayed as mean ± SEM and p<0.01

and severe effects of the cells. It was therefore determined the concentration of 1:1,000 PEGylated GNR should be used. Figure 5 uses a nuclear stain (DAPI) and an apoptotic marker (Annexin V) to look at the health of cells when exposed to the predetermined concentration of GNR to ensure cell health was maintained.

Exposing cells to laser

Figure 6A shows the long term effects from the laser by performing a neurosphere assay and counting the number of neurospheres seven days after exposure. The cells with the laser had about twice as many neurospheres as the control cells. Figure 6b shows almost identical health in populations between control cells and cells that were exposed to the laser, indicating that the laser did not harm the cells.

Cells exposed to GNR and laser

Seen from Figure 7, there was an obvious difference between all the samples except the one where cells were exposed to gold and the laser. It should be noted that the left graph (annexin V) shows an obvious decrease in cells. While the intensity of the cells with the gold and laser are not at a higher intensity, the percent of max cells, also known as the number of cells counted was about a fourth of the original number [Figure 7]. This shows that the method

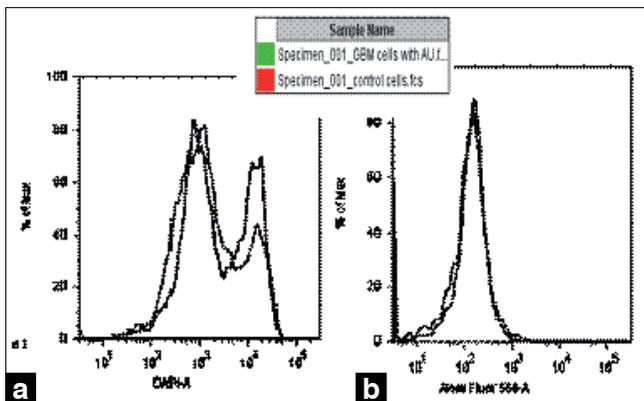


Figure 5: Flow Cytometry using Florescent Activated Cell Sorting was used to collect data on percent of cells that showed markers of apoptosis after 12 hours. (a) Used DAPI stain to look at nuclear staining. Cells exposed to gold look almost identical to control cells. The same thing can be seen with (b), which looked at Annexin V (conjugated to Alexafluor 568), an apoptotic marker. Samples in both graphs look almost identical to control, further proving that the nanorods have no effect on cells

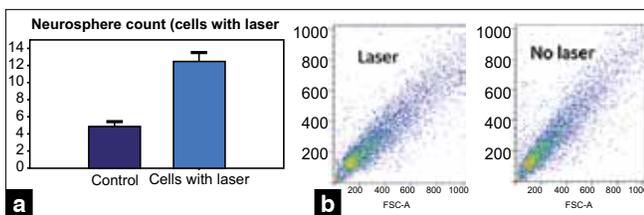


Figure 6: (a) Neurosphere formation assay-number of neurospheres were counted seven days after exposure to the laser. Sample exposed to the laser had twice as many neurospheres as the control sample. (b) Raw Florescent Activated Cell Sorting data comparing control cells to cells exposed to laser after 24 hours. FSC on the x-axis and SSC on the y-axis shows the size and complexity of sample cells. The two samples look almost identical, indication there are no significant effects on the cells when exposed to laser

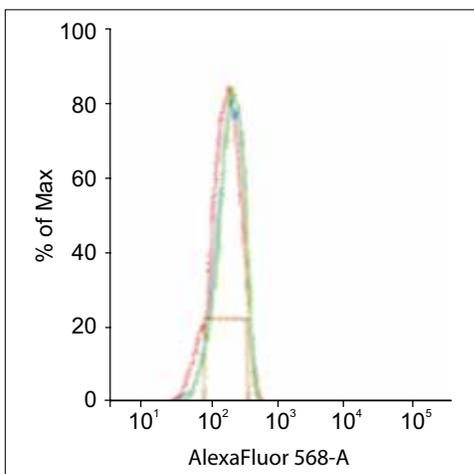


Figure 7: An analyzed series of samples from control cells to cells with gold and laser. Annexin V (AlexaFluor 568) used to analyze nuclear staining and apoptosis. The low peak show that the cells were killed when exposed to gold and laser. The higher peaks represent the control and cells exposed to one part of the method (ex: gold, laser, etc)

kills cells extremely quickly. Dead cells decay, and are spun out during procedures preceding FACS analysis, which accounts for the low numbers.

Analysis of cancer stem cells

This experiment was performed to observe cancer stem cells (CSC) within neurospheres by using carboxyfluorescein succinimidyl ester (CFSE), a proliferation indicator. Using confocal microscopy, CSC were observed after seven days of growing (Figure 8). Bright green dots inside the neurosphere are cells that have not divided as many times as the rest of the cells, a strong characteristic of CSC.

Aptamer targeting of GBMs

Specific targeting was done by attaching an aptamer to the GNR. Because an aptamer specifically synthesized for GBM cells was not available, a different aptamer was used. Sgc8 is a leukemia marker, but the same marker is commonly found on other cells, and is slightly more general than other aptamers, which is why it was chosen. This experiment was performed to measure the preference of aptamer binding to the GBM cells. Affinity was measured using flow cytometry [Figure 9A]. Since a flourescein molecule was attached to the aptamer, anything bound to the aptamer would fluoresce at 488, (excitation wavelength of fluorescein). FACS data here shows the control (left) compared to cells exposed to the aptamer (right). Cells showed a 99.09% binding affinity to the aptamer, proving that the aptamer/gold conjugate is extremely effective in binding to cells. 9B shows a confocal image of a GBM cell. The green dots on the outer edge of the cell are aptamers fluorescing.

Aptamer specificity of GBM compared to mouse cells

This experiment was performed to measure the specificity of the aptamer to the GBM cells compared to the binding of aptamers in other cells [Figure 10]. The results showed that at 1:3 aptamer/nanorods to cell ratio (compared to the original concentration used), the GBM has a much stronger signal of the aptamer than the mouse cells.

Efficient killing of all GBM cells after aptamer targeted GNR treatment and laser exposure

This portion of the research was to see if the A-GNR would be a reliable method for killing the cells. Analysis was performed visually (under a microscope) and numerically. Results showed immediate death of cells [Figure 11 a and b] shows visual and numerical data of cells after incubation. The picture in Figure 11a of experimental cells shows complete

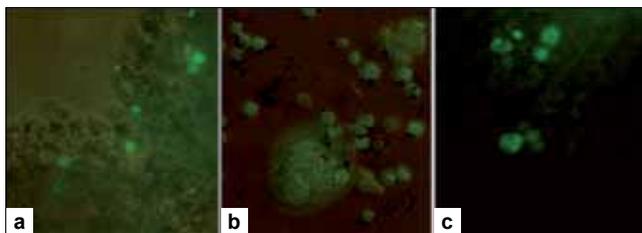


Figure 8: GBM cells exposed to CFSE and examined after 7 days growing in culture. Fast growing cells (not green) and slow growing (green) cells are indicative of population variations within GBM sphere. A control, B cells exposed to GNR, C cells exposed to aptamers.

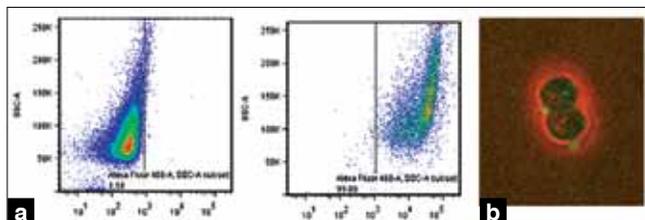


Figure 9: (a) Aptamer exposed to cells and analyzed using FACS. The graphs have the fluorescence intensity on the x-axis (There would be high intensity if aptamers were bound to cells), and side scatter on the y-axis. The left graph is the control, with no fluorescence as expected. The right graph shows cells exposed to aptamers. The high fluorescence intensity indicates a strong binding of aptamers to cells (Control). (b) A layered confocal image of two GBM cells together. The large, bright green fluorescent dots show the aptamers that have targeted and stuck onto the cell)

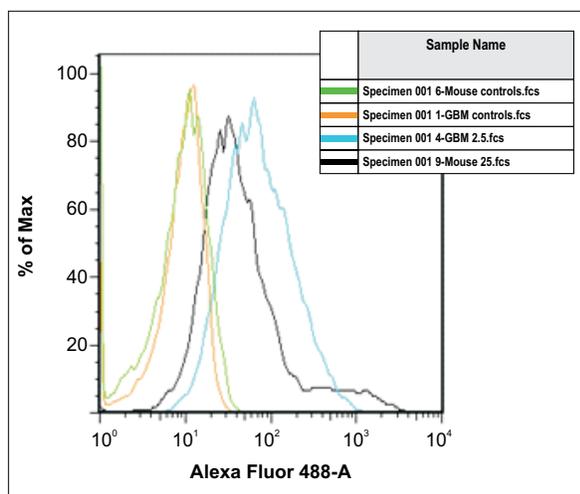


Figure 10: Affinity of Sgc8 aptamer to different cell types. The FACS analysis data show that the cancer specific aptamers bind preferentially to GBM cells (blue) and less to a non-cancerous mouse neuronal cell line (black) at a 1:3 ratio.

destruction of cells after being exposed to the laser.

The graph in Figure 11b shows the number of live and dead cells. For each sample, the left column is cell count after two hours, the right column in cell count after 16 hours. A dramatic decrease in overall

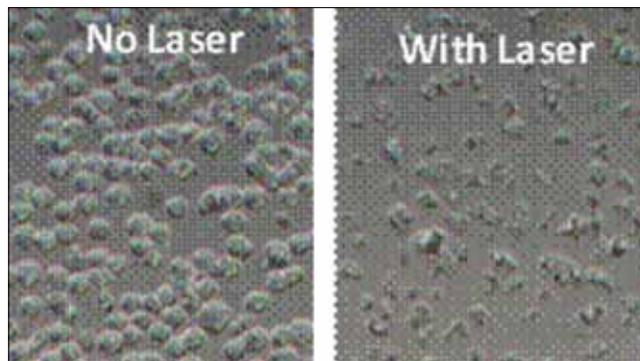


Figure 11a: Aptamer conjugated gold nanorods were exposed to cells and either exposed to laser or not (control). Cells that were incubated with A-GNR but not exposed to the laser were healthy, while the same treatment but exposure to the laser killed all cells efficiently. Image on the right shows dead cells

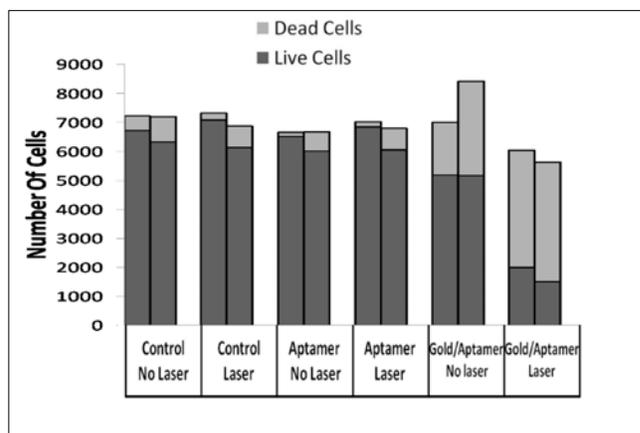


Figure 11b: This bar graph shows cell counts of all the samples, from control, to cells exposed to A-GNR and laser. Cells exposed to A-GNR and laser showed an overall decrease in cell count, and a dramatic decrease in number of living cells. Control cells were hardly affected

cell count and rapid decrease in number of live cells is obvious from both the graph and microscopy.

Discussion

All parts of the original hypotheses were supported by the data and results. The results indicated that this method is credible and could potentially be used as a new treatment option for patients.

Synthesis and characterization of gold nanorods. I have demonstrated that the GNR I made were uniformly and evenly dispersed, shown by a tight peak in the UV VIS experiment and the EM analysis. The characterization of GNR is limited due to the fact that the particles were rod shaped and not spherical. I was able to effectively and specifically conjugate the GNRs to specific aptamers which I used for targeted

nanothermal radiation therapy on the GBM cells.

GNR emission of heat when exposed to a NIR laser: As discussed previously, cancer cells are known to be sensitive to increased heat (hyperthermia), which is key to the success of nanothermal radiation. I have shown in an *in vitro* experiment [Figure 3] that GNRs cause local heat emission and that the surrounding areas plateau, after an increase of about 1-2°C, at a temperature of ~37°C, which is not harmful to healthy tissue, but causes apoptosis in most cancer cells.^[15]

Observe effects of GNR, laser and aptamers on cells. Before I tested targeted nanothermal radiation therapy, the exposure of cells to A-GNR and the NIR laser, I wanted to ensure that there were no negative side effects of the gold, the aptamer or the laser by itself. My results show that, at the ideal concentration, GNRs (1:1000), aptamers and the NIR laser gave no negative effect on the health of the cells. Too high concentration of GNR (<1:100) was shown to be toxic to the cells but aptamers at any concentration were not found to be toxic. Toxicity of GNRs could be caused by residue of CTAB, which is known to be toxic to cells.^[16,17]

The exposure of cells to NIR laser or to GNR and the laser has resulted in slight increase of cell numbers in 3 different experiments in the nanosphere survival assay. This can possibly be attributed to the heat from NIR laser, stimulating growth of the cells. Although this could indicate some toxic processes in the light of the hypothermic nanothermal radiation treatment, the cells should become more sensitive to heat induced killing, chemotherapy and radiation therapy after the exposure to hypothermia.^[15,18]

Expose cells to gold and laser - examine cell death. Though the GNR and aptamers had no effect on the cells individually, when combined, cell death was obvious. The cells were exposed to gold for a period of time, washed, and analyzed using various apoptotic markers and DNA stains. Seen on the FACS graphs, there was no obvious difference between all the samples except the one where cells were exposed to gold and the laser, and the cells exposed to just the laser. It should be noted that the left graph (annexin V) shows an obvious decrease in cells. While the intensity of the cells with the gold and laser is not at a higher intensity, the percent of max cells, also known as the number of cells counted was about a fourth of the original number. This shows that the method kills cells extremely quickly. Dead cells decay, and are spun out during procedures preceding FACS analysis, which accounts for the low numbers.

Analyze the presence of cancer stem cells within GBM neurospheres. Analysis of cancer stem cells: Carboxyfluorescein succinimidyl ester (CFSE) is a common dye used to look at division rate of cells. In this study, the dye was used to attempt to distinguish cancer stem cells from the "normal" fast growing tumor cells. Using a confocal microscope with a green laser, the difference between cells was very obvious. Within neurospheres, there were a few bright green cells. This is evidence that the cell did not divide as rapidly as the majority of cells, a major characteristic of cancer stem cells. These cells were the minority of the population, only about 1% of the cells.

Selectively target A-GNR to GBM cells and measure cell death: However, because there has not been an aptamer specifically synthesized for GBM cells, a different aptamer had to be used. Sgc8 is a leukemia marker, recognizing protein tyrosine kinase 7,^[9] but the same marker is commonly found on other cancer cells. The process of detecting and synthesizing an aptamer specific to GBM is currently in progress and I will repeat certain experiments once I have the GBM specific aptamer. This experiment was performed to measure the specificity of the aptamer to the GBM cells. Cells were exposed to A-GNR conjugates, incubated, and washed.

Aptamer specificity of GBM compared to mouse cells: This experiment was performed to measure the specificity of the aptamer to the GBM cells compared to the binding of aptamers in other cells. This data demonstrates the specificity of the aptamer. If an aptamer specifically used for GBM was used, the binding affinity would have been much higher. It would also show a higher binding affinity if the concentration was higher, but that would not necessarily indicate specific binding affinity.

Efficient killing of all GBM cells after aptamer targeted GNR treatment and laser exposure: This portion of the research was to see if the A-GNR would be a reliable method for killing the cells. The experimental cells completely disintegrated after being exposed to the laser. This data further proves the idea of cell selectivity, absorption of A-GNR into cell, and the efficient killing of the cancer cells.

Problems

CTAB: Though there are multiple methods to synthesize nanorods, the most stable and simple method involves making seed particles and

allowing them to grow. This process requires cetyltrimethylammonium bromide (CTAB) for the nanorods to grow. However, CTAB is known to be cytotoxic. Because gold nanorods were functionalized in various methods, the stock solution was kept in .2M CTAB. Despite the fact that nanorods were washed repeatedly before being exposed to the cells, some residue still remained. This may have accounted for some of the cell death. However, the results have shown that the death of cells due to the treatment method described are fall to significant to be completely explained by CTAB.

Functionalized GNR – clearance from body; Although previous studies have been done showing that gold nanorods are not toxic, the rods can still be easily excreted through the body. A study done by Powers in 2009 showed the effect of PEGylated vs. “naked” gold nanorods in a rat. Using various techniques they concluded that naked rods were sent to the kidney within five minutes, while PEGylated rods stayed suspended through the body for about 24 hrs. All nanorods in this study were PEGylated, but this could still play a factor in specificity or absorption of GNR into cells.

Finding the right concentration for gold treatment: A large problem often encountered when working with nanosized material is consistency and the spontaneous changes in the properties of the particles. Each batch of gold nanorods made will be slightly different, no matter how precise the method is carried out. If one batch is made, and then stored, the properties could change over time. The nanorods were kept in .5M CTAB which is known to keep nanorods stable, but analysis of the nanorods batch was performed multiple times to ensure consistency in size.

Specificity of the aptamer: as previously mentioned, due to time restraints, costs, and expertise in the field, I have not yet been able to create a specific aptamer that will bind to GBM. However, this process is very feasible, and in progress. Usually this process takes about three months. However, after visiting the Burnham Institute in Orlando, Florida, they introduced me to a robot that collects data points for an experiment. This extremely complex machine can remove an ELISA plate from the incubator, analyze and replace it, without human interference. At optimal use, the robot can collect 2 million data points a day. If access to this machine were granted, the aptamer selection method would be reduced greatly.

Studies ongoing and future plans

This research project has been a continuation for the last year, and has kept evolving into a larger and more complex study [Figure 12]. One large aspect of this project is to be able to image and treat the tumor at the same time, thus saving time, money, and stress of the patient and health care system. This can be accomplished by attaching a fluorochrome to the aptamer which will be picked up on the imaging device.^[19] By attaching the proper fluorochrome to the aptamer, a radiologist would be able to see exactly where the tumor is, and the size of it. This has an additional benefit of being able to detect or diagnose metastasis of cancer.^[20]

Another important aspect that is currently being researched in this field is tumor population dynamics. Contrary to popular belief, a tumor is made up of multiple types of cells, not just one: an example being tumor stem cells. It is a growing belief in medical field that in order to treat cancer in the best possible way, an understanding of the tumor on the micro and macroscopic level is needed. A tumor is much like a mini ecosystem, with different populations, needs, etc. In order to understand how a tumor grows and reacts, and how therapy affects these populations, modeling can be used to determine and hypothesize how a tumor will react to specific disturbances.

The ability to have a specific aptamer for the specific type of cancer is essential.^[21] There have been a few studies finding a specific aptamer for GBM. I am planning to find a specific aptamer for the exact cell line I'm using. I would also like to synthesize an aptamer specifically for the GBM CSC.

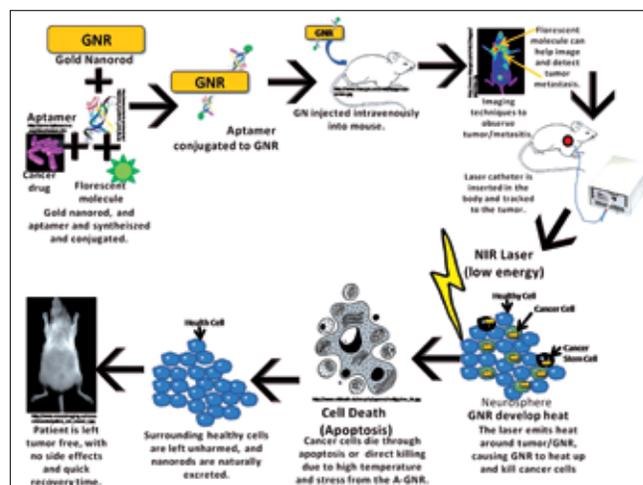


Figure 12: The complete nanothermal radiation treatment option

Once optimal concentration, laser wavelength, laser time of exposure, etc. has been established, the next step would be testing this method *in vivo*. No matter how well an experiment works in cell culture, there is no way to stimulate the actual effect without using real animals.^[22] A GBM tumor would be transplanted into the side of a lab rat or mouse, and be allowed to grow for about a month. Then, using the optimal parameters, the treatment would be used to test its specificity and efficacy in a *ex-vivo* live animal model. Hypothetically it should work the same, but the environments are extremely different, so it's hard to determine what the overall effect would be.

Final Thoughts

What's the point of this study? The reason that I am so dedicated to this project is that the real world application of this treatment method would really enhance, possibly revolutionize cancer treatment. More efficient, more personalized cancer treatments with fewer side effects are urgently needed, as is overcoming chemotherapy and radiation resistance of the tumors and reducing risk of metastasis by targeting cancer stem cells. This study has shown that nanothermal radiation is extremely successful in both killing the dangerous cells and causing virtually no side effects. Additionally, the combination of aptamer-targeted therapy can benefit all approaches of cancer treatment. My next goal is to prove this approach feasible with animal studies, further strengthen this approach in the near future.

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