Monoclonal Antibody Targeted Delivery of Boron Loaded Gold Nanoparticles to Pancreatic Cancer Cells for Boron Neutron Capture Therapy.

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Monoclonal Antibody Targeted Delivery of Boron Loaded Gold Nanoparticles to Pancreatic Cancer Cells for Boron Neutron Capture Therapy.

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

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Abstract

Pancreatic adenocarcinoma is an aggressive disease with an extremely high mortality rate. New therapies are needed to treat this deadly disease. Gold nanoparticles (GNP) are attractive vehicles for cancer therapeutics. The chemistry of gold allows for easy attachment of therapies. In addition GNP can also access tumors through the leaky vasculature. Boron Neutron Capture Therapy (BNCT) involves localizing non-toxic and non-radioactive Boron-10 to tumor cells. When a boron-10 atom is hit with low thermal energy neutrons it decays into destructive high-energy lithium ions and α-particles. By loading boron onto GNP, the therapeutic delivery to tumors can potentially be increased. The success of BNCT also relies on targeting the boron specifically to the tumor in order to localize damage to the tumor. Through attaching an antibody that recognizes a cell surface protein on pancreatic adenocarcinoma cells to the GNP the boron can be directed to the tumor. Here glycoprotein A33 is used as the target. Pancreatic adenocarcinoma cell line BxPC3 does not express A33, while another pancreatic cancer cell line CAPAN2 does. This model allows for the selectivity of boron loaded monoclonal antibody conjugated gold nanoparticles to be evaluated. In order to expose the cells to low thermal energy neutrons they must be transported to MIT Nuclear Reactor Lab, which involves cell culture in CO₂ independent conditions. Culture of cell in CO₂ independent conditions caused no significant morphological changes and importantly A33 expression did not change significantly in BxPC3 and CAPAN2 cell lines. An initial irradiation experiment was performed with monoclonal conjugated boron loaded gold nanoparticles constructs that provides a platform for additional investigation into nanoparticle targeted BNCT.
Acknowledgements

To my advisor, Drew Webb: Thank you for letting me join your lab my senior year. You’ve taught me a lot in a short period of time.

To my fellow lab members: Harini Natarajan, thank you for your support and nanoparticle advice. Karina Verma thank for helping split my cells while I was away.

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Introduction

Challenges of Treating Pancreatic Cancer

Pancreatic adenocarcinoma represents one of the largest challenges in cancer treatment today. In 2014 there will be an estimated 39,560 deaths due to pancreatic cancer, making pancreatic cancer the fourth most lethal form of cancer (Siegel et al., 2014). The 5 year survival rate of pancreatic cancer is less than 5% (Siegel et al., 2014).

Due to a lack of clinical symptoms, pancreatic cancer often goes undiagnosed until the cancer reaches advanced and often metastatic stages (Stathis and Moore, 2010). Upon diagnosis less than 20% of patients present with operable tumors (Vincent et al., 2011). Even with surgical resection most pancreatic cancer patients relapse due to the inaccessibility of the tumor in the pancreas (Neoptolemos, 2011). The most commonly used treatment for advanced pancreatic cancer is the nucleotide analog gemcitabine (Feig et al., 2012). However gemcitabine only modestly increased survival time from the former chemotherapeutic drug 5-Fluorouracil. However, it showed a significant improvement in the quality of life of the patients (Burris et al., 1997). Recent clinical trials have used gemcitabine combined with erlotinib, an epithelial growth receptor inhibitor. This combination therapy does come with some increase in toxicity and symptoms (Moore et al., 2007). However, it showed a slight but statistically significant increase in patient survival relative to treatment with just gemcitabine (Moore et al., 2007). In spite of minor advances using existing therapies, there is still an unmet need for novel therapies to combat advanced pancreatic cancer.

There are multiple factors that contribute to the chemotherapy resistance in pancreatic cancers. Pancreatic cancer metastases are highly genetically heterogeneous, which provides a means for selection for cells resistant to chemotherapy (Campbell et al., 2010; Yachida et al.,...
Pancreatic cancer stem cells have also been recently implicated in tumor growth and metastasis (Lee et al., 2008). Traditional chemotherapies are often inefficient at killing cancer stem cells, which provides a mechanism for resistance. New therapies are needed to treat this invasive disease in addition to traditional chemotherapies.

**Boron Neutron Capture Therapy**

Boron Neutron Capture Therapy (BNCT) seems to be a promising alternative to the current pancreatic cancer therapies. BNCT involves targeting a non-toxic, non-radioactive isotope of boron, boron-10, to tumor cells. When a boron-10 atom is hit with low thermal energy neutrons it decays into high-energy lithium ions and α-particles (Barth et al., 1990) (Fig 1). The high molecular weight of the species gives them a limited path length of about the radius of a cell. Localizing boron-10 within tumor cells limits cellular damage to within the tumor. The α-particles produced are not compromised by hypoxic environments, such as those found in the core of larger tumors (Barth et al., 1990). In contrast to most chemotherapies and radiation therapy, BNCT kills indiscriminately between rapidly proliferating cells and quiescent cells (Barth et al., 1990). This lack of specificity for proliferating cells indicates that BNCT could kill the non-neoplastic cells in the stroma that can influence tumor growth. Recently, BNCT has been shown to arrest and induce apoptosis in glioma stem cells (Sun et al., 2013). These advantages make BNCT an appealing alternative to traditional chemotherapies for treating pancreatic cancer.

Originally BNCT was developed as a treatment for glioblastoma multiforme (Hawthorne, 1993), partially because boron naturally localizes to brain tumors (Barth et al., 2005). The initial clinical trials of BNCT produced insignificant results (Barth et al., 1990). Later analysis revealed three key problems with this approach (Barth et al., 2005). One of the limitations of BNCT has been the difficulty of getting a high enough concentration of Boron-10 into the tumor
to inflict extensive damage. Secondly, there was enough residual $^{10}$B present in the blood at the time of irradiation to cause damage to the surrounding tissue. Finally low thermal energy neutrons did not have great enough penetrance to allow for sufficient radiation exposure for many tumors. Over the years various low molecular weight boron delivery agents including, BPA and BSH (Fig 2), have been used in BNCT clinical trials (Barth et al., 2005). More recent pre-clinical BNCT studies have placed an emphasis on using modern nanotechnology to facilitate boron delivery in order to overcome the challenges of BNCT, including a recent study that used liposomes to deliver $^{10}$B to xenografts in mice (Kueffer et al., 2013).

Figure 1: Schematic of the Mechanism of Boron Neutron Capture Therapy. When low thermal energy neutrons collide with boron-10 the collision produces destructive recoiling lithium ions and $\alpha$ particles. In order for BNCT to be effective Boron-10 is localized inside tumor cells, because the path-length of recoiling lithium ions and $\alpha$ particles is about the diameter of a mammalian cell.
Gold Nanoparticles

GNPs are attractive vehicles for therapeutics due to their very low toxicity in biological systems, easy linker chemistry due to stable dative linkages with thiols and high surface to volume ratio, which enables therapeutic and targeting moieties to be attached to a single particle (Conde et al., 2012). In addition, GNP can also access tumors through the leaky vasculature, which makes them ideal carriers for cancer therapeutics. We believe loading boron-10 on gold nanoparticles (GNP) would increase the boron concentration that could be introduced into the system.

Monoclonal Antibody Therapy

Due to the natural localization of boron to brain tumors, BNCT was primarily used for glioblastomas and cranial melanomas (Barth et al. 2005). However through a targeted delivery system, this limitation on BNCT might be eliminated. One characteristic of over 50% of pancreatic cancers is the expression of the cell surface protein glycoprotein A33 (Sakamoto et
Monoclonal antibodies targeted to A33 are rapidly endocytosed by cancer cells, which makes this protein an ideal target for therapeutic delivery (Mihaylova, 2006). The pancreatic adenocarcinoma cell line, CAPAN2, robustly produces glycoprotein A33 while another pancreatic adenocarcinoma cell line, BxPC3, has no detectible levels of A33 protein expression. This provides a useful model system for investigating targeted delivery.

**Boron-Loaded Monoclonal-Antibody-Conjugated Gold Nanoparticles**

In addition to the short penetrance of low thermal energy neutrons, there are two main issues with BNCT, insufficient boron load in the tumor to achieve maximal effect and the tendency of BSH and other boron compounds stay in the blood. We suggest a model where GNP particles are loaded with boron-10, to allow for high cellular cytotoxicity, and are conjugated to mAb in order to target the constructs specifically to pancreatic cancer cells overexpressing A33. The anti-A33 antibody (As33) is directionally conjugated to the gold with the variable region facing outward using a dithiol-PEG-hydrazine linker (Kumar et al., 2008). The hydrazine reacts with an oxidized glycosylation site on the Fc region of As33, while the dithiol enables an attachment to the gold (Kumar et al., 2008). The As33 antibodies remain stably attached to the GNP at about 15 antibodies per particle for approximately a month in solution before decreasing (Fig 4, Natarajan unpublished results). This indicates that the antibody conjugation will remain intact over the short time course of a cell based irradiation experiment.

Since BSH has a thiol group (Fig 2), BSH was directly attached to the gold nanoparticles through a dative linkage. The particles were backfilled with polyethylene glycol (PEG), which is a flexible molecule and increases biocompatibility by increasing the hydrodynamic radius, preventing it from getting flushed out by the reticuloendothelial system.
Figure 3: Schematic of Boron loaded monoclonal antibody conjugated gold nanoparticles.

Figure 4: As33 Antibody Loading and Stability on Gold Nanoparticles. As33 levels on gold nanoparticles were measured by ELISA each week for 8 weeks. The nanoparticle constructs (GNP-As33) were captured using protein A and assayed for antibody content with a goat anti-mouse Fab (Courtesy of Harini Natarajan).
This study aimed to evaluate the targeted delivery of monoclonal conjugated boron loaded gold nanoparticles to pancreatic cancer cells, CAPAN2 (A33+) and BxPC3 (A33-). In order to complete these experiments at the MIT nuclear reactor, the cells were conditioned to CO₂ independent conditions that did not drastically alter cell physiology, particularly A33 expression. An initial irradiation experiment was performed with monoclonal conjugated boron loaded gold nanoparticles constructs, this experiment provides a platform for additional investigation into nanoparticle targeted BNCT.
Materials and Methods

Cell lines and culture

BxPC-3 and CAPAN2, human pancreatic adenocarcinoma cell lines, were purchased from American Type Culture Collection (ATCC). Both cell lines were carried in RPMI media (Sigma #R6504) supplemented with 0.2% sodium bicarbonate (Sigma #S8761), antibiotic solution (100 units/ml penicillin, 0.1 mg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B; Sigma #A5955), and 10% fetal bovine serum (BioWest #S162H). The cells were passaged at 70-80% confluency at ATCC recommended dilution ratios 1:4 and 1:6 CAPAN2 and BxPC3 respectively.

Conditioning Cells to an Unregulated CO₂ Environment

BxPC3 and CAPAN2 cell lines were directly adapted to CO₂ Independent Media (GIBCO, Life Technologies # 18045) supplemented with 4 mM L-glutamine (GIBCO, Life Technologies #25030-081), 10% fetal bovine serum (BioWest #S162H) and antibiotic solution (100 units/ml penicillin, 0.1 mg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B; Sigma #A5955). The cells were seeded into CO₂ Independent Media and placed a 37°C incubator with 0% CO₂. According to the manufacturer’s instructions for the first passage, the cells were cultured in a closed flask while subsequent passages were cultured in an open flask. BxPC3 and CAPAN2 cell lines went through three passages in CO₂ Independent Media before use in experiments to assess viability under a variety of conditions.
Viability Assay to Compare Normal Conditions and CO\textsubscript{2} Independent Conditions

BxPC3 and CAPAN2 cell lines were seeded in triplicate at 5,000 cells per well in two separate 96 well plates. One plate was kept at 37°C in 5\% CO\textsubscript{2}, while another plate was maintained at 37°C in 0\% CO\textsubscript{2}. Under 0\% CO\textsubscript{2}, cells were either grown in RPMI or CO\textsubscript{2} Independent Media. The cells grown in CO\textsubscript{2} Independent media had been conditioned in CO\textsubscript{2} independent conditions for three passages (see above). Under 5\% CO\textsubscript{2}, the cells were grown in complete RPMI (see above). After 24 hours fresh media was added. As a positive control for cell death, the topoisomerase inhibitor etoposide (Sigma #E1383) was added to cells grown in normal conditions at a final concentration of 1 mM and incubated for 1 h. After 1 hour, the cell viability reagent WST-8 (Cell Counting Kit 8 CCK8; Dojindo #CK04) was added to all wells to reach a final concentration of 10\%. In addition a blank (media and 10\% WST-8) was added to each plate for both types of media. Following a three-hour incubation the reactions were read at 450 nm in a Molecular Devices M3 plate reader. Cell viability was determined as a percentage of the cells grown under normal conditions. Blank-corrected absorbance values of the treatment condition were divided by the blank-corrected absorbance values of the cells grow under normal conditions (RPMI, 5\% CO\textsubscript{2}, 37°C).

Protein Extraction and Quantification

Cells were pelleted in PBS pH 7.4 and frozen “dry” at -80°C. The cells were resuspended in mammalian Protein Extraction Reagent (mPER, Thermo #78501) containing 1X HALT protease inhibitor cocktail (AEBSF 1mM, Aprotinin 800 nM, Bestatin 50 µM, E64 15 µM, Leupeptin 20µM, Pepstatin A 10 µM; Thermo Scientific #87786) in an approximate ratio of 1 mL of mPER / 100 mg of pellet. The cell suspension was shaken at room temperature for 10
minutes. The supernatant lysate was carefully recovered after removal of cellular debris by centrifuging at 14,000 x g for 15 minutes.

The Pierce BCA Protein Assay Kit (Thermo #23235) was used according to manufacturer’s instructions to estimate total protein levels in the cell lysates. The BCA standard was diluted in a serial ½ dilution from a starting concentration of 2000 µg. The protein lysates were diluted 1:2 and 1:5. In duplicates the sample and protein standards were added in 1:8 ratio with the working reagent (25:24:1 Reagent A, Reagent B, Reagent C respectively) in a 96 well plate. After shaking to mix, the samples were incubated for 30 minutes at 37°C. The absorbance was measured at 562 nm in a Molecular Devices M3 plate reader. A blank-corrected BCA standard curve was created. From the equation generated by the standard curve the concentration of the protein samples were calculated.

**Western Blot**

In order to denature proteins, the protein lysates were heated for 5 min at 90°C in 1X Lane Marker Non-Reducing Sample Buffer (Thermo Scientific #39001). Samples (10 µg of protein) and 7 uL of Pre Prestained SDS-PAGE Standards (BioRad #161-0309) were separated by SDS PAGE using a 10% polyacrylamide Precise Protein Gel (Thermo Scientific #25221) in TRIS-HEPES-SDS buffer (Thermo Scientific #28398) at 45V for 30 minutes and then 150V, until the loading dye was near the bottom of the gel.

Upon removal, the gel was quickly washed in Nanopure water to remove excess buffer and detergent before gel and nitrocellulose membrane were equilibrated in 1 X Transfer Buffer (25 mM Tris Base, 192 mM glycine, and 20% methanol) for 20 minutes. The proteins were transferred to nitrocellulose membrane at 40V for 90 minutes in 1 X Transfer Buffer.
transfer the nitrocellulose membrane was washed three times with 1 X TBS-T (Tris-buffered saline containing 0.02% Triton X-100). The membrane was blocked in 5% Biorad blocker (Biorad #170-6404) in TBS-T for one hour at room temperature, followed by 3x washes with TBS-T. In order to probe for Glycoprotein A33 (GPA33) expression, the membrane was incubated overnight at 4°C in polyclonal Rabbit Anti-GPA33 antibody (Sino Biological #11277-RP02) at 1:10,000 in 5% Biorad blocker in TBS-T. After the primary antibody incubation, the membrane was washed three times with TBS-T, before incubation in Goat anti-Rabbit-IgG HRP conjugate (Thermo #31460) at 1:5,000 in 5% Biorad blocker in TBS-T for 1 hour at room temperature. All washing and antibody incubations were performed on a rotary shaker. After washing three times with TBS-T the membrane was sprayed with luminol chemiluminescent substrate (Denville Scientific #E2400). After 1 min, excess luminol reagent was removed with a Kimwipe. The blot was imaged using a BioRad GelDoc XR imager. Equivalent protein loading in lanes was evaluated by reprobing the blot for GAPDH. The same membrane was washed with TBS-T and incubated with rabbit anti GAPDH (Rockland #600-401) at 1:1,000 dilution in blocking solution overnight at 4°C. The same secondary antibody and imaging procedure was used as above.

**Gold nanoparticle (GNP) construction**

Conjugation of mAb to GNP was performed essential as described by Kumar et al (2008) and modified by Althari (2013). Briefly, the glycosylation site of As33 was oxidized using sodium periodate. The presence of aldehydes was confirmed using the Purpald assay. Then, the PEG-hydrazine-dithiol linker was added to the oxidized antibody. The hydrazine on the linker formed a stable hydrozone linkage with the oxidized As33. The linker-modified antibody was purified through molecular weight cut off column to remove excess linker and periodate. 100 uL
of the linkered antibody was incubated with 1 mL of 20 nm citrate stabilized gold colloid (Ted Pella #15705, manufactured by BBInternational, UK) for 20 minutes at room temperature with shaking. Methoxy-PEG-SH was added to the antibody-conjugated nanoparticles at 500 ug/mL. The nanoparticles were then spun at 4,000 x g for 30 minutes at 4˚C. The supernatant was discarded and the resulting pellet was resuspended in 2% PEG in PBS. The conjugated GNPs were resuspended (at 10^{12} particles/mL) in RPMI without bovine serum but containing 0.2% BSA (American Bioanalytical #AB0440). GNP concentration were calculated based on the published (Ted Pella & BBI) extinction coefficient data, which for these 20nm GNP is 7 x 10^{11} particles/mL colloid suspension with an “absorption” at 520 nm of 1.0 OD. The gold nanoparticles constructs were stored in the dark at 4˚C.

Figure 5: Periodate oxidation of glycosylation site of As33 and addition of PEG-hydrazine-dithiol linker.
Irradiation Experiments

Cells, that had been grown in CO\textsubscript{2} Independent Conditions for three passages, were seeded at 10,000 cells/well in duplicate 96 well plates (irradiated and non-irradiated). After 24 hours of growth in CO\textsubscript{2} Independent media in a 37°C incubator with 0% CO\textsubscript{2}, the media was replaced with CO\textsubscript{2} Independent media mixed with the appropriate treatment. Each condition was done in triplicate. The treatments are summarized in Table 1. Untreated cells were merely supplemented with fresh CO\textsubscript{2} Independent media. The treatments are as follows, 100 nM and 1\mu M of sodium mercaptododecaborate >99.5% enriched (Katchem, Czech Republic) with \textsuperscript{10}B (BSH), \textsuperscript{10}7/mL and \textsuperscript{10}8/mL GNP conjugated to mAb As33 (GNP-As33), \textsuperscript{10}7/mL and \textsuperscript{10}8/mL of GNP conjugated to mAb As33 and loaded with BSH (GNP-As33-BSH) (Table 1). The samples were incubated in a portable humidified 37°C incubator for 3 hours while transferred to MIT Nuclear Reactor Lab (NRL).

In order to expose the cells to low thermal energy neutrons, the samples were irradiated for 2 min at a beam power of 5 MW. Thermal neutron and boron doses were determined for us by Kent Riley using activation of gold foils placed in wells plate (containing complete RPMI medium) spaced around the plate as described in Althari (2013). Thirty minutes after the irradiation, the media was removed and replaced with fresh CO\textsubscript{2} Independent Media with 10% of the WST-8 reagent. This delay was due to safety protocols that involved determining the remaining radiation of the samples before it could leave the reactor core. Following a 3 hour incubation in a portable humidified incubator at 37°C, the plate was read at 490 instead of 450 due to available filters in the BioTek Elx800 plate reader located at NRL. A second reading was obtained 18.5 hours later. Cell viability was determined as a percentage of the untreated cells. Blank-corrected absorbance values of the treatment conditions were divided by the blank-
corrected absorbance values of the untreated cells. This process is summarized as a timeline in Figure 6.

Table 1: Summary of treatments for irradiation experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSH (Na₂B₁₂H₁₁SH)</td>
<td>100nM 1 µM</td>
</tr>
<tr>
<td>GNP-As33</td>
<td>10⁷ particles/mL 10⁸ particles/mL</td>
</tr>
<tr>
<td>GNP-As33-BSH</td>
<td>10⁷ particles/mL 10⁸ particles/mL</td>
</tr>
</tbody>
</table>

Figure 6: Timeline of irradiation experiment.
Results

Cell Growth in CO₂ Independent Environment

In order to carry out the irradiation experiments, the cells must be transported to the MIT Nuclear Reactor Lab. This involves the cells being transported and maintained in a non-CO₂ regulated humidified portable incubator at 37°C. Traditional cell culture medium (e.g. RPMI) is buffered by sodium bicarbonate, which relies on high CO₂ for its buffering capacity. Growing cells in traditional cell culture media without regulated CO₂ results in a pH imbalance and cell death. In order to carry out experiments outside of a CO₂ regulated environment, the cells were adapted to CO₂ Independent Medium (GIBCO) buffered with sodium phosphate and β-glycerophosphate and capable of maintaining long-term pH stability under atmospheric CO₂ conditions.

Significant changes in cellular function and viability could prevent adequate results from being obtained by future experiments. Since the cells are normally cultured in 5% CO₂ in RPMI media, we evaluated the change in cell morphology and cell viability when the cells were switched to CO₂ Independent Conditions (0% CO₂, CO₂ Independent Media).

As shown in Figure 7, cells adapted to the CO₂ Independent media did not have significantly altered cell morphology from cells grown in normal conditions. To further characterize cell growth in CO₂ independent conditions, the cell viability of cells grown in CO₂ independent conditions relative to normal conditions was determined using the Cell Proliferation Kit 8 (CCK-8, Dojindo) (Fig 8). CCK-8 uses an active reagent WST-8 that when reduced by live cells changes color. Using the CCK-8 assay, the culture of cells in the CO₂ Independent Media dramatically improves viability relative to RPMI under 0% CO₂ (Fig 8). In fact, cells grown for 24 h or 48h in RPMI in 0% CO₂ resulted in more cytotoxicity than cells grown under normal
conditions (RPMI, 5% CO₂) when treated with a high concentration (1 mM) of the topoisomerase inhibitor cancer drug etoposide (VP-16) (Fig 7). From this experiment it appears CAPAN2 is more viable that BxPC3 in CO₂ Independent Conditions (Fig 7.) Although since the CCK-8 cell viability assay is based on the extracellular reduction of the reagent (WST-8) by mitochondrial NADH, it is possible that CCK-8 could be influenced by changes in cellular metabolism as well as cell number and viability. The fact that WST-8 is extracellular makes it less cytotoxic to cells and allows for culture in the reagent for long incubation periods.

**Figure 7: Cell morphology in normal and CO₂ independent conditions.** Cells were grown in either normal conditions (RPMI, 5% CO₂) or CO₂ independent conditions (CO₂ Independent Media, Gibco and 0% CO₂). Cells were seeded at an equal number for normal and CO₂ independent conditions. Images were taken with 10X objective on the Nikon Eclipse TS100.
Figure 8: Cell viability in a non-CO$_2$ atmosphere. Prior to the experiment both BxPC3 (A) and CAPAN2 (B) cells were directly adapted to CO$_2$ Independent Media, by culture in the CO$_2$ independent media for three passages. Cells were grown in 96 well plates (5,000 cells/well) in a humidified but non-CO$_2$ atmosphere at 37°C, in either CO$_2$ Independent Media or RPMI Media (sodium bicarbonate buffer). Treatments were done in triplicate. After 24 and 48 hours in a non-CO$_2$ atmosphere, the viability was measured with the CCK-8 kit (Dojindo). Viability is represented as the percent of the control (cells grown under normal conditions; RPMI, 5%CO$_2$, and 37°C). Etoposide (VP-16; 1 mM) was used as a control for cell death. Cells treated with VP-16 were grown under normal conditions (RPMI, 5%CO$_2$, and 37°C). Bars represent averages of triplicates.
CO₂ Independent Conditions Do Not Alter A33 Expression

The main purpose of this experiment is to assess the targeted therapy through a monoclonal antibody designated As33, which is directed against A33. Under regular growth conditions BxPC3 cells do not express detectible levels of A33 while CAPAN2 cells express copious amounts of the antigen A33. The biggest concern, for these experiments, is that altering the growth conditions cells could change the expression of A33. As shown in Figure 9, A33 expression might be slightly decreased in CO₂ independent conditions however the overall pattern remains the same; BxPC3 cells lack detectible A33 expression, while CAPAN2 cells robustly express A33 (Fig 9).

Figure 9: A33 expression in BxPC3 and CAPAN2 under normal and CO₂ independent conditions. A33 expression was evaluated by a western blot. The upper arrow indicates membrane A33 and the lower arrow indicates the cytosolic form of A33. GAPDH was run as a loading control. 1) BxPC3 grown under 5% CO₂ in sodium bicarbonate buffered media (RMIPI, Sigma) 2) BxPC3 grown under 0% CO₂ in CO₂ Independent media (GIBCO). 3) CAPAN2 grown under 5% CO₂ in sodium bicarbonate buffered media 4) CAPAN2 grown under 0% CO₂ in CO₂ Independent media.
Irradiation Experiments

In order to test the toxicity of BNCT delivery through monoclonal antibody conjugated gold nanoparticles (GNP), pancreatic adenocarcinoma cells expressing A33, CAPAN2, and not expressing A33, BxPC3, were treated with gold nanoparticles conjugated to the monoclonal antibody As33 directed against the glycoprotein A33 and loaded with boron-10 (GNP-AsA33-BSH). After treatment the cells were exposed to low thermal energy neutrons, and the cytotoxicity was observed at two time points using the CCK-8 assay. The triplicates for each treatment were highly variable and clear outliers were discarded (see Appendix for raw data and neutron dose distribution). This experiment must be repeated with less variability for the following observations to be validated. GNP-As33-BSH did not have much of an effect on non-irradiated cells, but resulted in a large decrease in cell viability after irradiation for both GNP concentrations of $10^7$/mL and $10^8$/mL (Fig 10). There appeared to be no selectivity of GNP-As33-BSH due to A33 targeting because BxPC3 and CAPAN2 had very similar responses to GNP-As33-BSH (Fig 10).

The cells were also treated with GNP just conjugated to mAb As33 alone (GNP-AsA33) to determine if the BSH conferred additional cytotoxicity after irradiation. In irradiated BxPC3 cells, GNP-As33 reduced cell viability by approximately 60% and 80%, for GNP treatments of $10^7$/mL and $10^8$/mL respectively. Irradiated CAPAN2 cells pretreated with $10^7$/mL GNP-As33 had approximately an 85% decrease in cell viability. However when irradiated CAPAN2 cells were pretreated with $10^8$/mL GNP-As33 there was not much of decrease in cell viability. This inconsistence makes it hard to interpret the effect of GNP-As33. Additional experiments are needed to determine if this is experimental error or not. The cells were also treated with BSH alone to determine the effect of BSH alone before and after irradiation. This produced an
unexpected result that BSH reduced viability more in the non-irradiated cells than cells that had been exposed to low thermal energy neutrons (Fig 10).

The second reading, the following morning after irradiation, produced similar results except there appeared to be cell recovery in most irradiation treatments (Fig 10). Importantly the least recovery was seen in the GNP-As33-BSH irradiated cells (Fig 10). It appears that irradiation increases the toxicity of both GNP-As33-BSH and GNP-As33. From this experiment there does not seem to be a detectible level of selectivity based on A33 expression. However additional experiments, with less variability, will be needed to validate and expand these observations.
Figure 10: Cytotoxicity of Pancreatic adenocarcinoma cells, BxPC3 and CAPAN2, exposed to BSH and gold nanoparticle constructs after exposure to low thermal energy neutrons. The cells were grown in CO₂ independent conditions for 3 passages. Both BxPC3 and CAPAN2 were seeded at 10,000 cells per well. After 24 hours the cells were treated with the following conditions: 100 nM and 1µM of BSM, 10⁷ particles/mL and 10⁸ particles/mL GNP conjugated to mAb As33 (GNP-As33), 10⁷ particles/mL and 10⁸ particles/mL of GNP conjugated to mAb As33 and loaded with BSH (GNP-As33-BSH). After 3 hours incubation, the cells were exposed to low thermal energy neutrons (2 min at a beam power of 5MW). Thirty minutes after irradiation, 10% WST reagent in CO₂ independent media was added. Read 1 is 3.5 hours after irradiation and read 2 is 22 hours after irradiation. Bars represent averages of 2 or 3 values (See Appendix A Table 1 and Table 2). Cell viability was determined as the percent of the control (cells treated just with media).
Discussion

Future Directions

A. Repetition and reducing variability in BNCT irradiation experiments

A preliminary irradiation experiment was performed to examine cytotoxicity of nanoparticles in cells (Fig 10). There was a high level of variability within the replicate samples (Appendix, Table 1 and Table 2) that could be attributed to uneven distribution of the radiation across the wells of the plate. Following irradiation of the cells, the level of neutron flux was determined through analysis of gold foils by our collaborator Kent Riley. It was determined that the wells on the corners of the plate received a lower dose of thermal energy neutrons compared to wells closer to the center of the plate (Appendix, Fig 1). This could be corrected for by seeding the cells to a more limited area in the center of the plate.

It is also possible that despite allowing cells to adhere to the wells for twenty-four hours prior to the experiment, the use of RPMI without serum and supplemented with 0.2% BSA was disruptive to maintenance of maximal adhesion. Cells that did not adhere fully to the plate could have been disrupted during cell transport and handling. Future experiments should be performed to evaluate cell adhesion under these conditions to minimize well-to-well variability. Given the observed difference in cell growth between CAPAN2 and BxPC3, it will be important to determine specific cell numbers per well at the time of GNP treatment to equalize the GNP/cell dose of exposure.

B. Determining Targeting Specificity and Internalization of Nanoparticle Constructs

No significant selectivity correlated to A33 expression was observed with either the GNP-As33 and GNP-As33-BSH treatments prior to or following irradiation of the cells (Fig 9). This result was disappointing since prior work by our group (Pollack 2013) has demonstrated specific targeting of As33-conjugated GNP to CAPAN2 xenografts grown on the chick CAM in
vivo model. Future experiments will use bare GNP as a control to determine corresponding cytotoxicity (if any) in both BxPC3 and CAPAN2 cell lines. The observed lack of selectivity could be the result of nonspecific internalization or association with the membrane of A33 negative BxPC3 cells. Additionally, this lack of specificity could be caused by inadequate removal of media containing the functionalized GNPs, which might have had a cytotoxic effect on nearby cells after irradiation. This eventuality is of concern especially since there was an apparent BNCT effect in samples in this study where BSH was dissolved in the culture media (Fig 10). Future experiments will look at internalization of GNP-As33-BSH in both CAPAN2 and BxPC3 to look for A33 receptor-mediated endocytosis and compare results when the media is changed prior to irradiation so that the only nanoparticles present in the system are the GNP constructs internalized by the cells.

C. Increasing boron concentration on GNP through pEK peptide.

The apparent lack of BNCT-effect could also be due to suboptimal concentrations of $^{10}$B being taken up by cells on GNPs. The method of directly adding BSH onto the GNP used in this study likely limit the amount of BSH that can be loaded. In order for BNCT to be effective, high amounts of $^{10}$B need to be localized to the tumor cells (Barth et al 2005). Conjugating a small peptide loaded with BSH would allow for higher concentrations of boron per nanoparticle than the direct conjugation of BSH to GNP. This in turn could potentially increase therapeutic levels of $^{10}$B. Nowinski et al. have developed a novel peptide, poly-EK (pEK) which is ideal for boron loading due to its high lysine content, hydrophilicity, and lack of bulky aromatic residues (Nowinski et al., 2014). pEK has the sequence of EKEKEKEKE-PPPPC where the C-terminal cysteine residue facilitates a thiol-gold interaction to directly connect pEK to GNP through a dative linkage. The homo-proline stretch adds rigidity to allow for tighter packing enabling a
larger quantity of peptides to assemble on GNP. Additionally pEK is advantageous for boron loading because boron can be attached to the lysines, through the hetero-functional sulfo-MBS cross-linker (Fig 11). The NHS-ester group of sulfo-MBS reacts with primary amine groups, found on the lysines of pEK, and forms an amide bond. On the other end of sulfo-MBS is a maleimide group. The maleimide reacts with the thiol on BSH, creating a very stable thioether bond.

Furthermore, pEK was shown to perform similar in vivo functions as PEG without the biological toxicity; namely forming “stealth, non-fouling” GNP with high stability and low agglomeration characteristics in serum (Nowinski et al., 2014). Using boronated pEK would prevent the need to backfill the nanoparticles with PEG. PEG has been shown to cause liver toxicity in mice (Rudmann et al., 2013). Switching to boronated pEK would serve dual functions by increasing the boron load and eliminating the toxicity concern of PEG.
Figure 11: Boronation of lysines through sulfo-MBS. The amine of the lysine attacks the ester bond forming an amide bond. Then the malimide reacts with the thiol on BSH forming a stable thiolether bond.
Implications

A. Future targets: Pancreatic cancer stem cells

Recent work has identified stem cell-like pancreatic cells responsible for the highly metastatic nature of pancreatic cancer. In 2007 a study identified a population of pancreatic cells expressing the cell surface markers CD44, CD24, and ESA. When a small number of these cells were injected into nude mice, they all formed heterogeneous tumors, a common characteristic of cancer stem cells (CSC) (Li et al., 2007).

Another group found that CD133+ cells isolated from pancreatic cancer patients formed tumors in nude mice at a much higher rate than CD133- cells (Hermann et al., 2007). In addition Hermann et al. showed that gemcitabine, commonly used to treat pancreatic cancer, significantly enriched the CD133+ positive cells in a pancreatic cancer cell line. Additionally this study highlighted that pancreatic CSC were heterogeneous. Hermann et al. further identified a subpopulation, CD133+CXCR4+ that were highly invasive. They showed that treatment with anti-CXCR4 monoclonal antibody (mAb CXCR4) decreased in vitro cell migration of a pancreatic adenocarcinoma cell line (L3.6 pl).

Together these two studies highlight the complexity and importance of pancreatic CSC in pancreatic cancer metastasis and further indicate the inefficiency of chemotherapy to target these CSC. The question still remains whether there are two distinct pancreatic cancer stem cell populations, or if the makeup of CSC varies between patients. Despite the complexities these studies also provide some hope, because they point to surface markers that could hopefully be exploited for targeting moieties in the future to directly kill pancreatic CSC implicated in metastasis. The inhibitory effect of mAb CXCR4 on in vitro pancreatic cancer migration is particularly promising.
Recent work on cancer stem cells indicates that it may be time to rethink traditional chemotherapy methods, which might actually enrich for CSC in the tumor cell population. BNCT could provide an alternative to traditional chemotherapy and radiation. In fact BNCT has been shown to be effective in arresting and killing glioma cancer stem cells and progenitor cells (Sun et al., 2013). As the research narrows in on identifying surface markers for pancreatic CSCs, it is tempting to hypothesize that targeting GNP conjugated with boron-10 enriched compounds to pancreatic cancer stem cells using surface markers could help improve long term outcome of patients treated with BNCT.

Conclusions

Medical nanotechnology holds a lot of promise for the therapy of cancer and other severe conditions. At present, there is an unmet medical need for targeted cancer therapeutics. More broad-spectrum approaches could prove harmful to the patient and come with a variety of severe side effects. However, with a more targeted approach, the therapeutic could be delivered only to cells overexpressing a particular cell surface receptor through the use of highly specific monoclonal antibodies. Particularly in the case of pancreatic adenocarcinoma, there are very few options, surgical or otherwise, that can be used to treat the cancer, leading to an incredibly low survival rate. However, the use of gold nanoparticles allows for a high surface-to-volume ratio, which allows for the attachment of both therapeutic and targeting moieties. Additionally, gold is highly biocompatible as it is a noble metal and does not oxidize easily, as other metals do.

Nanotechnology may be able to revive boron neutron capture therapy (BNCT). Using monoclonal antibody-conjugated gold nanoparticles as a targeting mechanism for BNCT could potentially resolve two main problems with the clinical application of BNCT, high levels of boron left circulating in the blood and low levels in target tissues. Targeting of this therapy using
mAbs could potentially decrease levels of $^{10}$B in other tissues at the time of irradiation, while concentrating potentially several hundred BSH molecules on a single GNP could potentially help increase the therapeutic load. This thesis developed an experimental set up to evaluate the cytotoxicity of gold nanoparticles following irradiation of $^{10}$B-loaded and monoclonal antibody conjugated gold nanoparticles in an attempt to expand the applications of BNCT.
References


### Appendix A

**Table 1: Raw Data From Irradiation Experiment Initial Reading (3.5 h after irradiation).**
Highlighted values were omitted from analysis.

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**Table 2: Raw Data From Irradiation Experiment Second Reading (22.5 h after irradiation)**
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Figure 1: Thermal Flux (109 neutrons cm\(^{-2}\) s\(^{-1}\)) is greater in wells in the center than on the corners of the 96 well plate. (Data calculated courtesy of Kent Riley). Wells D7 and B4 contain gold foil covered with media, while wells E6 and G10 just contain gold foils.