CHARACTERIZATION OF HUMAN BLADDER CANCER CELL LINES FOR TARGETED ALPHA-PARTICLE THERAPY

By

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ABSTRACT

Bladder cancer ranked as the 12th most common cancer in the world in 2018 and was the 7th most prominent cancer in men. Current treatment options for non-muscle invasive bladder cancer do not include radiation therapy, as the bladder is a mobile organ and adjacent to many sensitive tissues. Standard of care treatment (BCG) is effective in only 60-70% of the patient population and the nonresponsive patients eventually require more radical treatment options. Given that 30-40% of the bladder cancer patients are not effectively treated, together with the high cost of more radical approaches, there is a huge need for new bladder cancer treatments. One option could be adding targeted radiation therapy delivered inside the bladder with standard of care BCG could improve the response rate for this cancer type, while lowering cost and possible complications. Thus, our hypothesis was that bladder cancer could be safely and effectively treated using alpha-particle therapy with Pb-214/Bi-214-labeled trastuzumab/cetuximab. A supportive prior pilot study demonstrated that alpha particle therapy was safe and effective in muscle-invasive bladder cancer patients, using intravesical Bi-213-cetuximab antibody. We first determined the EGFR1 and EGFR2 levels for 6 human bladder cancer cell lines in vitro and binding affinities of Tc-99m-[Trastuzumab] and Tc-99m-[Cetuximab] to the human bladder cancer cells. The purity of the radionuclide bound antibodies was determined using instant Thin Layer Chromatography, and specific binding of the radiolabeled antibodies to target receptors was measured. Next, we attached Pb-214/Bi-214, the novel radioactive agent emitting alpha particles, to the FDA-approved antibodies (Cetuximab and Trastuzumab) that target the EGFR1 and EGFR2 receptors expressed on bladder cancer cells. Then, Pb-214/Bi-214-labeled Trastuzumab and Pb-214/Bi-214-Cetuximab was demonstrated to provide efficient killing of the bladder cancer cells. Addition of increasing concentrations of radiolabeled antibodies resulted in 5-20% reduction in cell numbers after 24 hours. These effects were often statistically significant, but the overall effects were modest at 24 hours. However, cell viability was significantly reduced at 72-hour post treatment. Overall, the results at 72 hours after treatment suggest that higher doses of Pb-214/Bi-214-trastuzumab/cetuximab were effective in killing EGFR1/EGFR2-expressing human bladder cancer cells. This research demonstrated a new targeted radiation treatment for bladder cancer is was effective and cost-efficient. This work developed methods for moving the new bladder cancer treatment strategy forward to a phase I clinical trial.

This thesis is dedicated to my grandparents, for I know you all would be proud to see who I have become since I left your side; to my parents, Erhan and Aynur Erder, for giving up their dreams to believe in mine and moving to this unknown place with me so I would never feel lonely; and to my sister Ela Erder, for always encouraging me to be the book-smart, medicine-cored, immigrant dream girl of the family as she can't even stand the sight of blood and wants to be the high heels and high ceilings business girl of the family. I believe in you more than you ever will comprehend.

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This body of work could not have been written without the help of my family, countless mentors, colleagues, and friends. I will name some of them here.

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1. INTRODUCTION

1.1. Background

1.1.1. Bladder Cancer

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States with more than 600,000 deaths in 2020 [1]. Bladder cancer ranked 12th among cancers in the world in 2018 and was the 7th most predominant cancer in men [2]. In 2021, it ranked 4th most predominant cancer in men in the United States [3]. Estimated new cases for bladder cancer is 81,180 for both sexes (76% male patients), with estimated 17,100 deaths in both sexes (71% will be male patients) in 2022 [4]. The COVID-19 pandemic has reduced access to medical care impacted diagnosis and treatment of cancer. It is reasonable to expect a short-term drop in cancer incidence, followed with an increased diagnosis of advanced-stage disease and, ultimately, increased cancer mortality in the near future [5].

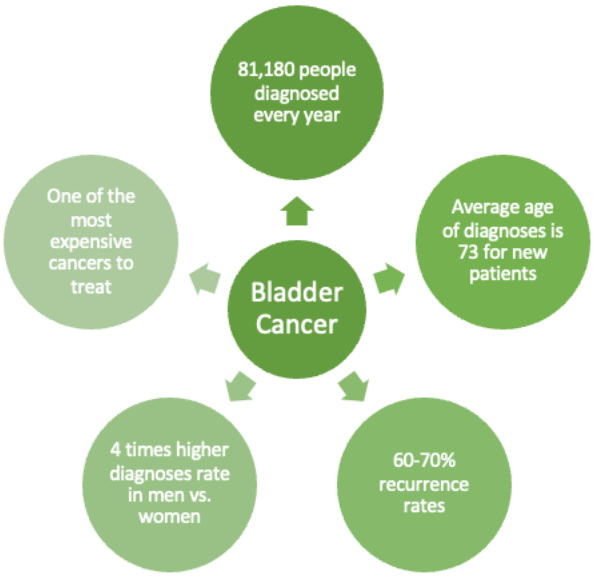

Figure 1.1. Bladder Cancer Facts.

1.1.2. Epidemiology

Of diagnosed patients, urothelial carcinoma accounts for more than 90% of all bladder cancers, which also accounts for 10-15% of kidney cancers diagnosed in adults [6]. Of all newly diagnosed cases of bladder cancer, about 80% is non-muscle invasive bladder cancer (NMIBC), also known

as superficial bladder cancer [6]. This is a heterogenous group of patients that includes three pathological stages: Ta, T1 and carcinoma in situ (CIS). The first pathological stage Ta describes confinement to the epithelium or the mucosa. T1 describes the invasion of subepithelial connective tissue or lamina propria. Lastly, carcinoma in situ (CIS) sometimes called Tis, describes the flat, high-grade, non-papillary carcinomas confined to the urothelium [7, 8]. Approximately 70% of NMIBC cases will be stage Ta, 20% will be T1, and 10% will be CIS [8]. Of those diagnosed with NMIBC, approximately 50-70% will recur and roughly 10-20% will progress to muscle invasive bladder cancer which is more likely to metastasize [8].

The high prevalence of non-invasive cancers makes them a significant financial and emotional burden for society, with the most detrimental burden being that non-muscle invasive bladder cancer (NMIBC) has a significant risk of recurrence [5]. Even with high initial treatment response rates for NMIBC, the majority of cases will recur [9]. The probability of recurrence at one year is anywhere from 15% to 61%, while at five years, it is anywhere from 31% to 78% [8].

It is also important to consider the effects of bladder cancer in the older population, the average age of diagnosis being 71 for women and 69 for men [10]. While the association between cancer and age is exemplified in almost all types of cancer, bladder cancer act as a reminder that with advancing age, clinical presentation and cancer outcomes are usually worse. Medical care for elderly patients requires specific knowledge of many key geriatric clinical issues to guide optimal cancer treatment plans [11]. Incorporation of a true functional assessment is a required component, since comorbidities, disabilities and functional status is important to predict cancer treatment outcomes. Advanced age is associated with worse outcomes, but stage and grade at diagnosis are key determinants of cancer prognosis. Previous studies have shown that advanced age increases the risk of advanced stage bladder cancer and high-grade disease if cancer is superficial [12]. High grade and muscle invasive tumors are much more likely to progress and metastasize compared to low grade and low stage cancers [12, 13], and even then, the management of the disease is costly and challenging, with a high chance of recurring. Radical cancer treatments are associated with increased therapeutic effects and decreased chances of recurrence; however, such treatments are not favorable with advanced patient age. One radical treatment is complete radical cystectomy with concurrent chemotherapy for muscle invasive bladder cancer patients [14] and is associated with many possible side effects and decreased quality of life [15].

Bladder cancer (BC) treatment was one of the more expensive when compared to other cancers for lifetime treatment costs per patient in 1996, and rates have only increased overtime [16]. In 2010, BC was the 9th most costly cancer to treat and was responsible for $3.98 billion in direct medical costs [17]. Under ideal circumstances, the lifetime cost for management of a single patient with NMIBC is $120,000, ranging from $96,000 to $230,000 in other estimates [18, 19]. This variability of the average cost is due to heterogeneity of the patient population, as well as stages and grades of the tumor and treatment planning [20]. Indirect cost of cancer is not quantifiable, from emotional burden to economic burden for patients, their caregivers and so forth. Because of the above discussed points, not only is it important to identify a BC treatment that is effective in eradicating cancerous cells and decreasing the chances of recurrence, but also minimizes the financial and health consequences of treatment for the patient population.

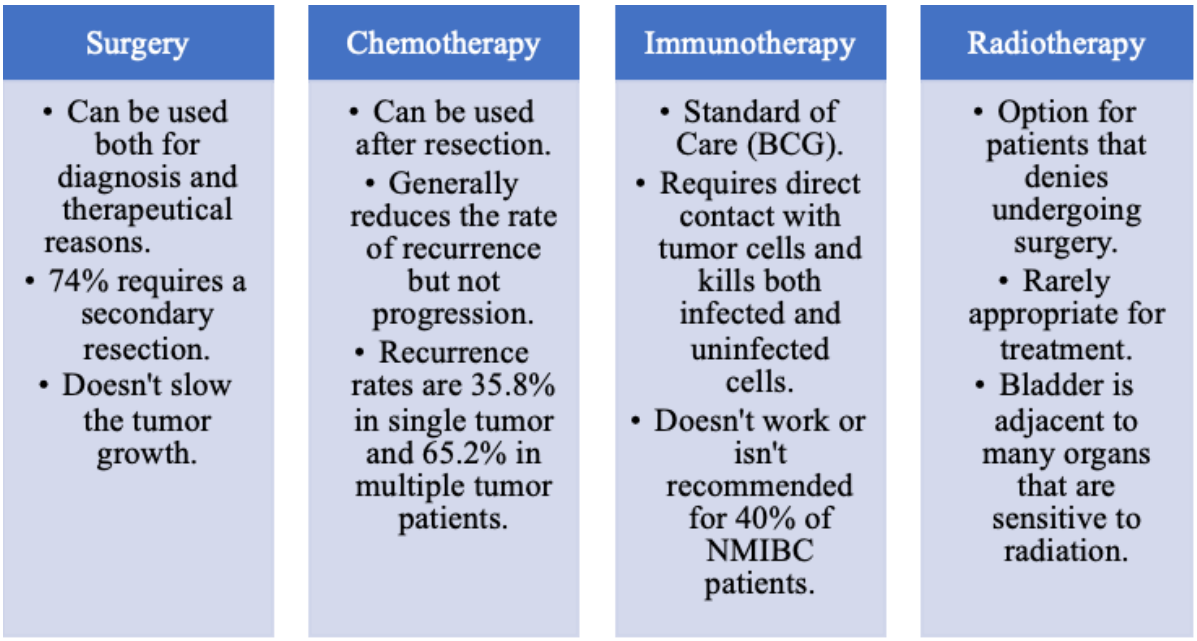

Figure 1.2. Current Treatment Options for NMIBC Patients.

1.1.3. Overall Summary

The current standard of care for NMIBC patients is transurethral resection (TUR), where a resectoscope is used to access patient's bladder and either (1) collect samples to analyze or (2) completely remove small tumors [21]. TUR is usually followed by intravesical immunotherapy, the gold standard of care being instillation of the bacterium Bacillus Calmette–Guerin (BCG) [22]. If NMIBC is diagnosed at an advanced stage or is recurrent, TUR is commonly repeated and

intravesical BCG immunotherapy is given to the patient [23]. Intravesical immunotherapy with BCG performs more effective than intravesical chemotherapy for high grade bladder cancers. If the cancer was not fully removed at TUR, or BCG is not effective on recurrence, then partial or radical cystectomy are recommended.

Timely and effective treatment for NMIBC can achieve good outcomes, potentially avoiding increase in recurrence rates and progression to MIBC. However, to optimize good outcomes, unnecessary medical interventions should be minimized, and cost effectiveness of the treatments should be prioritized. Currently, management of NMIBC and the clinical course of treatment options are varied and complicated, and inconsistencies between treatment plans are present [24]. This is due to response rates and the heterogenous nature of the cancer at this stage [24]. A novel treatment with higher response rate can help minimize inconsistencies between clinical treatment plans and lower the financial burden related to the treatment.

1.1.4. Surgical Intervention

First line for diagnosis and treatment of new bladder tumors is transurethral resection of bladder tumor (TURBT). This is a biopsy procedure where the doctor takes a tissue sample from the area where cancer may exist, and during the procedure doctor also will try to completely remove the cancerous growth. Once tumor is removed, the doctor may attempt to destroy any remaining cancer cells by fulguration or cauterization. Intravesical chemotherapy is generally applied into the bladder after TURBT for maintenance [25, 26].

One important aspect of TURBT is quality of resection. Result from MSKCC in 1312 patients with NMIBC referred by an outside urologist found residual tumor in 74% of patients who underwent a second TURBT [27]. In patients with high grade lesions, half the Ta tumors had residual disease and 15% were upstaged, for T1 tumors, 48% had persistent NMIBC and 30% were upstaged to muscle invasion [27].

1.1.5. Intravesical Therapy

Intravesical therapy describes instillation of a drug directly into the bladder via a catheter. Both chemotherapy and immunotherapy are intravesical therapies.

Chemotherapy is when the drug actively kills the growing cancer cells without having to use the body's immune system. It is generally administered right after TURBT as adjuvant therapy and can include drugs such as mitomycin, doxorubicin and epirubicin [28, 29]. These drugs generally

reduce the rate of recurrence but not the rate of disease progression [25]. A meta-analysis showed that TURBT and immediate perioperative dose of chemotherapy was better than TURBT alone (39% reduction in recurrence for single tumor, 56% reduction for multiple tumors), however recurrence was 35.8% in single tumor patients and 65.2% in multiple tumor patients, showing that one instillation alone is not sufficient for treatment [30]. Overall, intravesical chemotherapy reduces early recurrence percentage, and is the most effective treatment strategy for patients at low risk, but has no long term benefit as it does not prevent disease progression [31].

Immunotherapy is when treatment triggers immune activation to stimulate the immune system to fight the cancer. The immunotherapy used in the treatment of NMIBC is Bacillus Calmette- Guérin (BCG) [32]. BCG is a weakened strain of bacterium Mycobacterium bovis, originally developed as a vaccine for tuberculosis. BCG must come in direct contact with cancer cells, and the infection of these cells with BCG activates the immune system leading to killing of both infected and uninfected cancer cells [33]. For high grade and T1 tumors, use of intravesical BCG after TURBT is recommended, since the procedure alone isn't adequate [26]. BCG is instilled weekly for a total 6 treatments after the procedure and maintained for up to 36 months [22]. A meta-analysis done by AUA suggests 24% and 31% reduction in recurrence rates for BCG induction and maintenance [34]. BCG treatment eventually fails in up to 50% of patients that were treated, and half of this group is failed within the 6 month evaluation period [35]. Adding cytokine therapy to BCG as a way to enhance its effects, have initially showed good response rates, however long term studies showed 64% of the patients relapsing at 5 years with 20% having disease progression [36].

For patients that are unresponsive to BCG or if they elected not to undergo cystectomy, pembrolizumab and valrubicin are given in place of BCG for maintenance. Valrubicin, the only FDA-approved drug for BCG-refractory CIS is only effective in <10% of the patients at 2 years and none with coincident stage T1 disease [37]. Overall, BCG decreased cancer recurrence and progression for many but not all. However, other treatment options have increased risks of toxicity, treatment failure, and a delay to recovery [31].

1.1.6. Radiotherapy

As discussed above, cystectomy is the standard treatment option for recurrent BCG refractory, high grade tumors. However, many patients prefer to not undergo surgery, possibly due to older age, comorbidities, and desire to avoid a large operation [38]. Radiation therapy offers a

nonsurgical option for BCG refractory patient group. Generally, external beam radiation therapy is offered, where an outer source focuses radiation to the region of interest.

An earlier study showed that radiotherapy alone is not a better treatment option compared with more traditional treatments and it did not show any difference of cancer recurrence rates between treatments [39]. Another study showed that combined radiotherapy after maximum TURBT for high risk NMIBC patients resulted in complete response at 88% of the patients, where 80% survivors preserved their bladder and up to 70% were satisfied with their urinary function [40].

The 2015 NCCN bladder cancer guideline suggested that external beam radiotherapy was rarely appropriate for patients with stage Ta, T1 or Tis, due to many side effects from radiation damage to adjacent healthy tissues [41].

1.1.7. Novel Therapies

Currently several strategies are being explored to enhance the BCG response in NMIBC patients. The goal of new investigational treatment agents is to bypass the BCG refractory response.

Some of the current studies included are the immune checkpoint inhibitors like anti-CTLA-4 and anti-PD-1 antibodies that can enhance the T cell response in patients with BCG failure [42] or enhancing the BCG response by trapping it to exogenous fibrinogen or inhibiting IAP proteins by SMAC mimetics [43].

1.2. Project

1.2.1. Scientific Basis of the Study

Bladder cancer, being a very common diagnosis in the older population, needs novel therapies that will fight the incomplete responses from above-described options, while not causing significant financial or physical burden on the patients. One of the many options that could show significant advantages but is currently considered high risk to benefit ratio is radiotherapy. As discussed above many limitations are present such as side effects including damage to normal tissue when it comes to external beam therapy. However, as prior studies suggested, local instillation of radioisotopes can minimize the off-target side effects and damage to normal tissue, while causing substantial advantages for therapy when combined with other pharmaceuticals and therapies [44].

To make sure that the radioisotopes will be delivered in close proximity to the cancer cells, biomarkers that are significantly expressed on cancer cells can be used for targeting. Epidermal growth factor receptors 1 and 2 are known to be receptor biomarkers that are important drivers of

invasion and progression of many cancer cells and are heterogenous in their expression at the early stages of NMIBC [45-48]. In healthy body, their function is to regulate epidermal tissue development and overall homeostasis. EGFR is expressed all over the body due to above-described function and is known to be the prototype of the receptor tyrosine kinases, which itself belongs to the ErbB family of RTKs[49]. In pathological conditions, they become drivers of tumorigenesis, due to amplification and overexpression of proteins in cancer, their normal trafficking becomes abnormal, causing increased signaling in tumor development[50, 51]. Targeting both receptors with their respective antibodies labeled with a radioisotope that emits alpha particles causing cancer cell death with minimal effect on healthy tissues may be an effective and safe treatment option for many patients. Targeting EGFR1 with Bi-213-labeled cetuximab was previously reported as effective and without toxicity [44].

Currently above-described targeted radiation therapy (TRT) is popular among many investigators that focus on cancer treatments, where alpha and beta particles are of great interest. One of the earliest studies that demonstrated effective TRT was for neuroendocrine cancer patients treated with Lutathera, a beta particle emitter (Lu-177-DOTATATE). The Lutathera treatments increased progression free survival at 20 months to 65.2% compared to 10.8% at SOC group [52], it was approved by FDA in 2018 for somatostatin-receptor positive neuroendocrine cancer. Radium-223 chloride is another FDA approved radioisotope, and this alpha particle emitter was approved for castrate resistant prostate cancer with bone metastases [53]. Its bone mimetic properties reduced skeletal event risks and the treatment improved mean survival by 3.6 months, while being well tolerated [54].

Above shared studies and many others resulted in TRT becoming a popular area of investigation. Our current study extends the 2018 pilot study that labeled cetuximab with Bi-213, an alpha emitter, with the Bi-213-cetuximab instilled one time in the patient's bladder. Therapeutic efficacy was reported without adverse effects [55]. Of 12 patients treated, 2 patients showed no sign of CIS 44 and 30 months, respectively, after treatment, while another patient that was treated a second time showed eradication of CIS at 3 months. For rest of the cohort, the only benefit was increased time to cystectomy [55]. Their findings concluded that a higher dose and/or repeated instillations might improve the response rates and therapeutic efficacy. Taking these recommendations into consideration, and others from previous studies such as incorporating multiple antibodies for

increased efficacy in reaching cancer cells, we hope to demonstrate a novel option for safe and effective treatment of bladder cancer using two antibodies for targeting bladder cancer.

1.2.2. Advantages of Pb-214 from a Generator System For Radiation Therapy

This study is the first ever to utilize a novel generator system that was invented by Niowave, a Lansing-based supplier of radioisotopes. The Niowave system uses Rn-222 (3.8 d half-life) as the parent that generates Pb-214 (half-life=26.8 min), a short-lived radionuclide that decays with beta emission to Bi-214 (half-life=19.7 min), which subsequently decays by beta emission to Po-214 (0.16 msec) that decays with alpha-particle emission. The decay of Pb-214 results in the emission of two beta particles and one alpha particle. The Pb-214 obtained from this generator is highly pure with an extremely low lead concentration, thus allowing for effective radiation therapy without pharmacological effects and/or toxicity. The α-particle-emitting therapeutic agents, like Pb-214/Bi-214-labeled antibodies, are particularly well-suited for local radiation therapy, as they deliver 1000X more radiation locally than beta (β-) particles, while minimizing damage to normal tissue when compared with other standard techniques, such as external beam radiation due to their considerable bigger radius compared to beta particles. Alpha particles cause many double stranded breaks in DNA when in close proximation to cells and result in death of the cancer cells and absence of resistance to this mechanism.

1.2.3. Order of Studies

The research proposed in this project will provide preliminary in vitro data for a human phase I study that will evaluate Pb-214/Bi-214-labeled cetuximab/trastuzumab (radiotherapy) in combination with BCG treatment (immunotherapy) for bladder cancer. By demonstrating a novel option for the safe and effective treatment of bladder cancer, the field of oncology may be able to adopt this as standard of care for bladder cancer after additional phase II studies. The first aim of the research project focused on validating the expression of the biomarkers (EGFR1 and EGFR2) in seven relevant bladder cancer cell lines that are known to express these receptors in varying quantities. The quantification of the number of receptors per cell in each of the 7 cell lines will provide us with a basis for the second aim. The second aim is focused on in vitro killing assays using these cell lines and treatment with various concentrations of Pb-214/Bi-214 labeled trastuzumab and cetuximab antibodies and compared to non-labeled antibodies as well as common chemotherapy agent (doxorubicin). The results from this research project will be part of the

Investigative New Drug application for the future phase I clinical trial for this novel treatment option.

2. MATERIALS AND METHODS

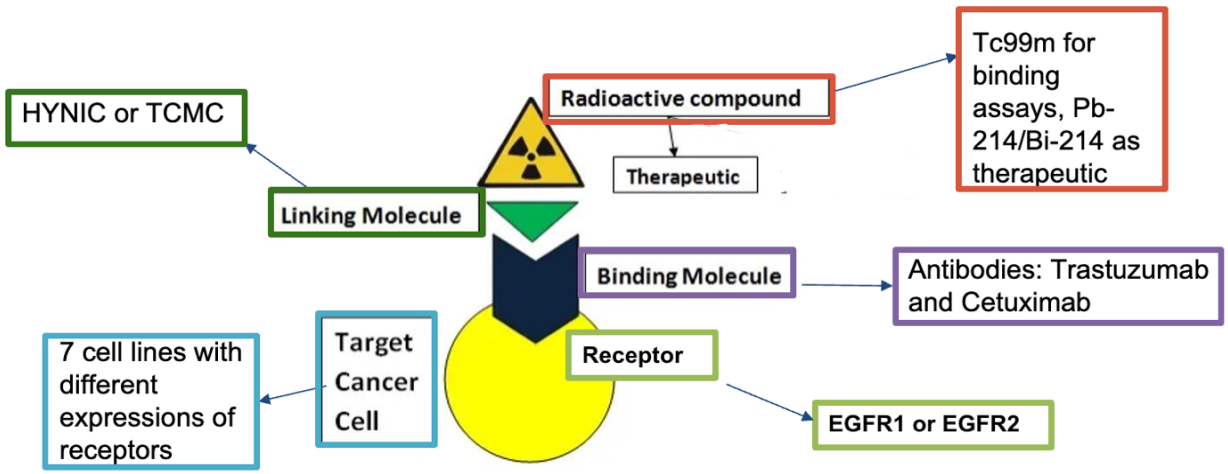

Figure 2.1. Summary of Reagents of Use for Radiopharmaceutical Ligand and Receptor Dynamic.

2.1. Cell Lines

RT4, T24, SW780, SCaBER, 5637, TCCSUP and 3T3/NIH cell lines were purchased from ATCC (Manassas, VA, USA). These cell lines have been studied before to examine the levels of EGR1 and EGFR2 receptors [45]. Siddiqui et al suggested that RT4 had overexpression of EGFR2, SCaBER and 5637 had overexpression of EGFR1, SW780 had overexpression of both receptors, and T24 and TCCSUP had low expression of both receptors. The 3T3/NIH cell line was included as the negative control since it did not express either EGFR1 or EGRR2.

RT4 and T24 cells were grown in McCoy's Media + 10% Fetal Bovine Serum (FBS) + 1% Penicillin G-Streptomycin (Pen-Strep)+ 1% L-Glutamine, SW780 and 3T3/NIH cells were grown in Dulbecco's Modified Eagle Media + 10% FBS + 1% Pen-Strep + 1% L-Glutamine, SCaBER and TCCSUP cells were grown in Eagle's Minimum Essential Media + 10% FBS + 1% Pen-Strep + 1% L-Glutamine, and 5637 cells were grown in Roswell Park Memorial Institute 1640 Media+ 10% FBS + 1% Pen-Strep + 1% L-Glutamine. All cells were grown at 37 °C with 5% CO_2.

2.2. Antibodies

Kanjinti (Trastuzumab-anns) was purchased from Amgen (https://www.pi.amgen.com/~/media/amgen/repositorysites/piamgen.com/kanjinti/kanjinti_pi.ashx) and is a FDA-approved therapeutic antibody and biosimilar of trastuzumab. It is a recombinant DNA-derived humanized monoclonal immunoglobulin G1 kappa antibody, with binding to

EGFR2. Erbitux (cetuximab), a chimeric (mouse/human) monoclonal antibody, was purchased from Lilly (https://uspl.lilly.com/erbitux/erbitux.html#pi). It is an FDA-approved therapeutic antibody with binding for EGFR1. Human IgG1 Kappa-UNLB was purchased from Southern Biotech (Birmingham, AL). This antibody was isotype matched with Kanjinti and Erbitux, and does not bind EGFR1 or EGFR2, and served as a non-binding control in the killing assays.

2.3. Radioactive Materials

Tc-99m was purchased from Cardinal's Flint Pharmacy and delivered to MSU's radiopharmacy. Each shipment was 50 mCi in 0.5 mL isotonic saline at the time of receipt. Pb-214 was provided from Niowave (Lansing, MI), a supplier of medical radioisotopes. HYNIC and TCMC chelators were conjugated to the antibodies at 6:1 molar ratios (chelator:Ab) and were used to bind Tc-99m and Pb-214/Bi-214, respectively, to the antibodies. The HYNIC and TCMC were available for conjugation using prepared vials (kits) containing the appropriate weights of the chelators that had been vacuum dried by Benchtop Freeze Dryer/Lyophilizer (SP Scientific Virtis Advantage EL; Warminster, Pennsylvania), and stored at -20 °C in vials capped under vacuum. Tricine and tin chloride ($SnCl_2$) kits were prepared similarly and had 36.7 mg of Tricine and 51 mg of $SnCl_2$ per kit. The tricine solution (100 mL of ddH20 with 3.67 g tricine) was adjusted to be a pH of 7.0 and degassed (vacuum and ultrasonic bath) before addition of the $SnCl_2$ (in ethanol). A total of 1.0 mL was added to each vial followed by freeze drying.

2.4. Overall Order of Experiments

2.4.1. Specific Binding assays

A selected cell line was plated with four replicates for each of the two antibodies with 8 dilutions and incubated overnight. Antibodies were conjugated with HYNIC (6:1 molar ratio) and dialyzed in PBS overnight for removal of unconjugated chelators. The radioactivity dose was received the following day, and injected into the Tricine/SnCl2 kit vial, with 15-minute incubation, and then transferred and incubated with each HYNIC-conjugated antibody for 1 hour to allow for Tc-99m binding. Purification to remove unbound radioactivity was performed using G25 column chromatography, and the highest activity vials in the first peak from the column was tested for purity by instant thin layer chromatography (iTLC) and protein concentration using Bradford (absorbance=595 nm). After confirming purity (high Tc-99m binding), the selected fraction was then used to prepare a dilution plate with previously decided highest concentration (10-30 nM)

and 1:2 serially diluted along the column with four replicates per antibody concentration. The 96-well plate containing the cells added 24 hours earlier was then removed from incubator and washed twice with 150 mL of 1X PBS. Blocked conditions (single replicates for each concentration) received 20X higher concentration of unlabeled antibodies as compared with highest concentration of Tc-99m-labeled antibody to be tested while unblocked triplicates received media. Following 15 minutes of incubation, the Tc-99m-labeled antibodies from the previously prepared dilution plate were added. After 45 minutes of incubation, wells were aspirated and washed twice with 200 mL of 1X PBS. Wells then received 100mL of the respective media and ATPlite protocol was then followed to measure total cell number per well and lysates were collected in Polymerase Chain Reaction (PCR) vials for measuring the radioactivity. Data were then analyzed to quantify specific binding affinity (Kd) and receptor numbers per cell.

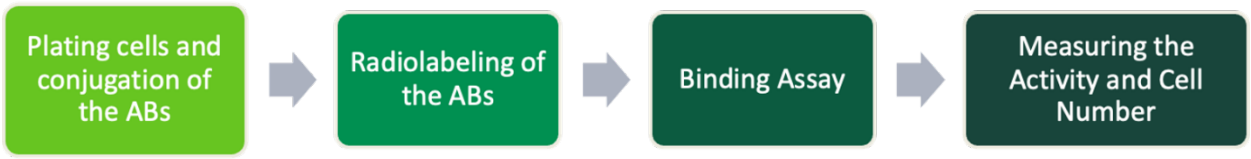

Figure 2.2. Order of Experiments for Validating Expressing of EGFR 1 and 2, and Determining SB and Kd values for respective antibodies.

2.4.2. Antibody Assays as Controls Without Pb-214

The day prior to the assay each cell line was plated in 96-well plates for six triplicate treatment conditions on the following day as follows: 1. Trastuzumab antibody alone, 2. Cetuximab antibody alone, 3. Trastuzumab and cetuximab combination treatment, 4. One negative control (no treatment), 5. Isotype-match IgG1, and 6. one positive control with doxorubicin. Cells were incubated overnight. Dilution plate for each condition was prepared the next day with a 1:2 dilution ratio, for 8 total dilutions of each treatment. After incubation was completed, cells receive their respected treatment from the dilution plate, negative controls received only fresh media, and positive control received the dilution series of doxorubicin. After 24 hours of incubation, ATPlite protocol was followed to measure total cells per well and data were analyzed to quantify % of surviving cells for each treatment condition.

2.4.3. Pb-214/Bi-214 Killing Assays

The day prior to the assay each cell line was plated in 96-well plates for five triplicates treatment conditions on the following day as follows: 1. Pb-214/Bi-214-TCMC-Trastuzumab antibody alone, 2. Pb-214/Bi-214-TCMC-Cetuximab antibody alone, 3. Pb-214/Bi-214-TCMC-Trastuzumab and Pb-214/Bi-214-TCMC-cetuximab combination treatment, 4. One negative control (no treatment), 5. Pb-214/Bi-214-TCMC-Isotype-match IgG1, Antibodies were conjugated with TCMC at a molar ratio of 6:1 (TCMC:Ab) and dialyzed overnight to remove unconjugated TCMC. The Pb-214/Bi-214 dose was received the next day and used to radiolabel antibodies. A dilution plate for each condition was prepared with a 1:2 dilution ratio. Cells receive their respected treatment from the dilution plate, the negative control did not receive any treatment but fresh media, and after all treatment additions the plate was incubated for 24 hours. At the end of incubation, the ATPlite protocol was followed to measure cell numbers, and data were analyzed to quantify % of surviving cells for each treatment condition.

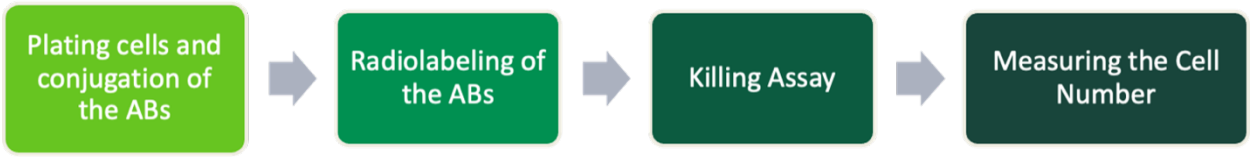

Figure 2.3. Order of Experiments for Demonstration of Killing of BC cancer cells with PB-214/Bi-214 Labeled antibodies.

2.5. Detailed Protocols

2.5.1. Specific Binding Assay

2.5.1.1. Plating cells

Cells were washed with 1X PBS (ThermoScientific; Waltham, MA, USA) and were detached from the cell culture plates using 1X TrypLE Express (gibco; Waltham, MA, USA). Cell counts were measured using an Eve Automated Cell Counter (NanoEnTek; Seoul, South Korea) and cells were plated at 30,000 cells/well with 100 mL of their respective complete media into 96-well clear-bottom, black plates and incubated overnight at 37 °C and 5% CO_2. For experiments to test specific binding, two antibodies per cell line were used. The unblocked condition was done in triplicate with eight 1:2 serial dilutions of radiolabeled antibody. A single replicate of blocked condition with eight 1:2 serial dilutions of radiolabeled antibody, but first at least 20 molar fold of unlabeled

antibody (relative to highest radiolabeled antibody condition) was added before the radiolabeled antibody dilutions. For each cell line, a total of 1.92 million cells was needed for binding experiments, and 650,000 cells for the ATPlite standard curve.

2.5.1.2. *Conjugation of antibodies with hydrazino nicotinate (HYNIC)*

Previously prepared and frozen HYNIC kits were removed from -20 ºC freezer and allowed to warm to room temperature. Lyophilized Na_2HPO_4 buffer (pH=7.4) was resuspended by the addition of ddH_2O and added to the HYNIC kit as 2 mL of buffer per 1 mg of antibody. After dissolving the freeze-dried HYNIC in the vial, the appropriate amount of antibody was added, and the solution was mixed by pipetting. The vial was then covered in aluminum foil to keep in the dark during incubation at room temperature for 1 hour with gentle mixing on a rocker. The sample was then dialyzed overnight against 1 L PBS (1X pH=7.4) buffer.

2.5.1.3. *Overnight Dialysis*

A Slide-A-Lyzer Dialysis Cassette for 10,000 Da molecular weight cutoff (MWCO), 0.5-3mL capacity (Thermo-Fischer; Waltham, MA, USA) was soaked in 1 liter PBS (pH 7.4) for 5 minutes. The cassette was removed, and a corner was marked for injecting the solution. A 3mL capacity syringe with 18G needle was used to collect previously prepared conjugated antibody which was injected into the cassette slowly. The remaining air between the membranes was removed and cassette was placed back into the 1L PBS beaker, with float to remain at top of PBS. After 4 hours, the PBS was replaced, and cassette was then placed in the beaker overnight for additional dialysis. Cassette was then removed from the beaker, and another 3mL syringe with 18G needle was used to push air into the cassette from the opposite of previously marked corner and the dialyzed sample was collected and place in a glass vial and stored at 4 °C until needed.

2.5.1.4. *Column Preparation*

A total of 25 grams of Sephadex^{TM} G-25 Superfine resin (GE Healthcare; Chicago, IL, USA) was mixed in 100mL of ddH2O in 300mL glass beaker and allowed to swell. Washing with ddH_2O was completed twice when the separation of filled resin (bottom) and ddH20 (top) was achieved. ddH_2O was poured off to get rid of fine particles and refreshed for washing. Following the two washes, ddH_2O were removed and exchanged with PBS (1X) and were washed 2 more times and left to swell overnight at 4 °C. The swelled G25 resin was added to disposable 10mL polypropylene columns (Thermo-Scientific; Waltham, MA, USA) and left to settle overnight.

2.5.1.5. *Tc-99m Radiolabeling*

Approximately 50 mCi of radioactivity in 0.5mL saline was ordered and delivered from Cardinal Health. After receipt the exact mCi of radioactivity was measured using a dose calibrator (Model #CRC-25R, Capintec, Florham Park, NJ) and recorded in the lab notebook. The total Tc-99m received was then added to the previously prepared Tricine/$SnCl_2$ Kits and incubated for 15 minutes at room temperature (light-protected). Following the incubation, the Tc-99m/tricine/$SnCl_2$ was added to previously prepared HYNIC-conjugated antibodies and incubated for 45 minutes at room temperature (light-protected).

2.5.1.6. *Purification*

During the Tc-99m incubation period, the previously prepared polypropylene columns containing G25 resin were resuspended and equilibrated with PBS. Purification of the radiolabeled antibody was performed by adding a total of 1 mL of the sample containing the Tc-99m that was mixed with HYNIC-conjugated antibody, and collecting 1.0 mL fractions in 1.5 mL vials, with 1.0 mL PBS added each time a fraction was collected. Approximately 9 vials were collected until an initial protein peak activity and a second free Tc-99m peak was measured. Every fraction was measured for activity and time of measurement on the dose calibrator. The fractions with high activity vials following the column purification were set aside for further analyses by iTLC.

2.5.1.7. *iTLC (instant Thin Layer Chromatography)*

Solvents selected were PBS and 2-butanone (MEK), with 300 mL of each solvent added to glass developing vials. 2 mL of sample were placed at the bottom of the strip cut from iTLC-SG-Glass microfiber chromatography paper impregnated with silica gel, (Cat. No SGI0001, Agilent Technologies, Folsom, CA), just above solvent height, and strips were placed into the glass vials until the solvent front was run to the end of the strip. Strips were cut into two pieces and each piece was measured on the gamma counter (Wizard2 Gamma Counter, Model#2480-0010, Perkin Elmer; Singapore) for quantification of purity. A purity above 95% was acceptable using 'bound/(bound+free)x100%', or '(bottom/total strip)x100%'.

2.5.1.8. *Bradford Protein Assay*

The fractions from G25 column chromatograph with >95% purity were measured for protein concentration using Bradford Protein Concentration Assay, which allowed calculation of specific

activity of the Tc-99m-labeled antibody (mCi/mg). The selected fraction was stored at 4 °C until preparation of dilution series.

2.5.1.9. *Dilution Plate Preparation*

Dilution series were prepared at 1:2 ratio, starting at 40 nM as the highest condition. Stock was prepared after Bradford analysis of the radiolabeled antibody. Dilutions were prepared by adding 150 mL of the intended stock to the wells containing the highest concentration and adding 75 mL of media (differed according to cell line) to the lower wells. 75 mL from the top well was transferred to the next lower dilution, mixed, then repeated to prepare the 1:2 dilution plate with 8 different dilutions for a total of four replicates.

2.5.1.10. *Cell Plate Dilution Addition*

The media from cells incubated overnight in the 96-well plate was aspirated and cells were washed twice using 200 mL of 1X PBS. After washing was completed, unblocked wells received 50 mL of their respective media and blocked condition received 50 mL of unlabeled antibody stock that was at a 20 times higher concentration than that of the highest Tc-99m-labeled antibody dilution. The plate was placed on a rocker in 5% CO_2, 37 °C incubator for 15 minutes. After incubation, the plate was removed from the incubator and wells received 50 mL of the Tc-99m-labeled antibody from the dilution plate and the plate was placed back onto the rocker in the incubator for 45 minutes. After incubation period, wells were aspirated and washed twice with 200 mL of 1X PBS, which was then aspirated again, and wells received 100 mL of their respective media.

2.5.1.11. *ATPlite Assay*

All wells received 50 mL of the mammalian cell lysis solution and the plate was shaken at 700rpm for 5 minutes. This was followed by the addition of 50 mL of the ATPlite buffer and lyophilized substrate solution mixture and was shaken at 700rpm for 5 minutes. 96-well plate was then dark adapted for 10 minutes and read for luminescence using IVIS Lumina LT Series III machine with Living Image Software 4.7.2 (PerkinElmer; Waltham, MA, USA). The output was later analyzed to create an equation from a standard curve that correlates the photon flux (photon/sec) to the cell number by Microsoft Excel.

2.5.1.12. *Measurement of radioactivity with the gamma counter*

After completion of ATPlite, each well contained a total volume of 200 mL and were collected into PCR vials for radioactivity measurement in the Gamma Counter (CPM) for 1 minute per

sample or until 10,000 total counts were collected (1% statistical error), whichever was first. Decay corrections on data and conversions from CPM to mCi were done manually in Excel. The absolute efficiency of the gamma counter was 1.42 x 10^6 CPM/mCi based on prior calibrations.

2.5.1.13. *Analysis of Data*

Dose calibrator measurement and Bradford protein value for the selected fraction was used to quantify the specific activity at the time for initial purification. ATPlite data was analyzed by the same-day standards for cell count and was converted into total cell count from photon/sec values on Excel by linear fit analysis. Triplicates were utilized for standard error calculations. Specific binding values were calculated using GraphPad Prism (Dotmatics; Boston, MA, USA) where a baseline analysis was computed with the following equation: Specific Binding=Total Binding (unblocked triplicate conditions) – NonSpecific Binding (blocked single replicate conditions). Specific binding value (Bmax) was then converted into number of receptors with the following equation: Specific Binding (in moles) *Avogadro ($6.023*10^{23}$). The number of total receptors for the experiment was then divided by the average total cell number in the wells that was quantified by Living Image Software from bioluminescence data, to obtain the number of total receptors (EGFR1 or EGFR2)/cell values.

2.5.2. *Pb-214 Killing Assay*

2.5.2.1. *Control Experiments*

2.5.2.1.1. Plate cells

For each cell line in the killing assay experiments, three antibodies were used for control studies with four different treatments, together with untreated cells for normalization. For antibody control experiments, the treatments include Trastuzumab alone, Cetuximab alone, Trastuzumab+Cetuximab combined, and IgG Isotype control antibody alone (no EGFR1 or EGFR2 binding). For all treatments the cells were plated as triplicates at 30K cells/well in 100 mL of media for the day before treatments were added. All treatments with antibodies included 8 serial dilutions.

Positive control for the killing assay was performed with Doxorubicin (Dox), where two triplicates of cells were plated per cell line at 30K cells/well in 100 mL of media. The first set of triplicates received Dox dilution at 1:2, starting from 0.8 mg/well, and the second set of triplicates didn't receive any treatment and used as negative control.

2.5.2.1.2. Concentrating Antibodies

Antibodies were concentrated using Protein Concentrator Polyethersulfone (PES; Pierce™; Waltham, MA, USA, 10K MWCO, 2-6 mL) and the final goal was 3000 mg/mL antibody concentration. Trastuzumab, received in powder form, was weighed, and resuspended in ddH_2O. Cetuximab and IgG were received in liquid form; the reported concentrations were used as a guide for initial addition of the antibodies to the concentrators. The antibodies were concentrated by centrifuging at 3260g for 10 minutes, 3 times. ddH_2O was added to bring the total volume to 2 mL after each centrifuge round. The final product was stored in a 1.5 mL vial at 4 °C until dialysis.

2.5.2.1.3. Overnight Dialysis of Antibodies

A 1L beaker was filled with PBS (1X, pH=7.4, 4 °C) and a Slide-A-Lyzer Dialysis Cassette (10,000 MWCO, 0.5-3mL capacity; Thermo-Fischer; Waltham, MA, USA) was soaked in the PBS for 5 minutes. The cassette was then taken out of the solution and a corner was marked for the syringe. A 3 mL capacity syringe with 18G needle was used to collect previously prepared antibodies which were injected into the cassette slowly. Remaining air between the membranes were removed and the cassette was placed back into the 1L PBS beaker. After 4 hours, the PBS was changed to fresh cold PBS, and the cassette was then placed in the beaker overnight in the refrigerator for improved dialysis. The cassette was then removed from the beaker and another 3 mL syringe with 18G needle was used to push air into the cassette from the opposite of previously marked corner. The dialyzed sample was collected into a glass vial and stored at 4 °C until needed.

2.5.2.1.4. Dilution Plate Preparation for unlabeled antibody experiments

The dilution series were prepared as 1:2 serial dilutions starting at 4952, 4944 and 3203 nM per well at the highest condition for Trastuzumab, Cetuximab and IgG Isotype antibodies, respectively. The stock solutions were prepared based on Bradford assays after the antibody samples were concentrated. Dilutions were prepared by adding 150 mL of the highest dilution to the starting wells and adding 75mL of respective media for chosen cell line for the lower wells. Next, 75 mL from the top well were transferred to the lower wells and this step was repeated until the lowest well (8^{th} dilution) for a total of 3 columns (triplicates) for each treatment condition.

2.5.2.1.5. Dilution Plate to Cell Plate addition

The cell plate that was incubated overnight at 37°C and 5% CO_2 was removed from the incubator and wells were washed twice with 200 mL of 1X PBS. Trastuzumab, Cetuximab, and IgG

conditions received 50 mL of their respective media and 50 mL of the dilution series of their respective antibodies. Trastuzumab+Cetuximab condition received 50 mL of Trastuzumab and 50 mL of Cetuximab from the dilution plate. The negative control received 100 mL of fresh media and positive control (Dox) received 50 mL of fresh media and 50 mL of their dilution of Doxorubicin. Cell plate was then placed back in the incubator for 24 hours at 37 °C and 5% CO_2.

2.5.2.1.6. ATPlite Assay after treatments

After overnight incubation with unlabeled antibody or Pb-214/Bi-214-labeled antibody treatments, the cell plate was removed from the incubator. To minimize the loss of cells from wash and aspiration, wells were not washed. All wells received 50 mL of the mammalian cell lysis solution followed by the addition of 50 mL of the ATPlite buffer and lyophilized substrate solution mixture and was shaken at 700rpm for 5 minutes each time. The 96-well plate was then dark adapted for 10 minutes and read for the luminescence in IVIS Lumina LT Series III machine with Living Image Software 4.7.2. The output was later analyzed to create a linear equation that correlated the photon flux (photon/sec) to the cell numbers using Microsoft Excel.

2.5.2.1.7. GraphPad

Grubbs equation was used to analyze for outliers in negative control to minimize the irrelevant error. 5% was chosen as the significance factor. ATPlite data was analyzed by the same-day standards for cell count and was converted into cell numbers from photon/sec values in Excel by linear fit analysis. Triplicates were utilized for standard error calculations. The average of the triplicates for each condition was normalized with negative control to quantify the rate of killing. Significance was measured by ANOVA.

2.5.2.2. *Cell Killing Experiments*

2.5.2.2.1. Plate cells

For each cell line of the killing assay experiments, three antibodies were used, and each cell line has 4 conditions with a standard for normalization. For Pb-214/Bi-214 Killing Assay, conditions were Pb-214/Bi-214-[Trastuzumab], Pb-214/Bi-214-[Cetuximab], Pb-214/Bi-214-[Trastuzumab+Cetuximab], Pb-214/Bi-214-[IgG Isotype Control] and cells that were not treated (3 replicates in each row of the 96-well plate) for normalization. All treatments included triplicates with 30K cells/well in 100 mL of media and all treatments included 8 dilutions.

2.5.2.2.2. Concentrating Antibodies and Dialysis

Antibodies were concentrated and dialyzed as previously described (2.5.2.1.3.).

2.5.2.2.3. Buffer Exchange for TCMC chelator conjugation

Previously dialyzed antibodies are placed in the Pierce™ Protein Concentrator (PES, 10K or 30K MWCO, 2-6 mL) and centrifuged at 3260 g for 8 minutes until the volume was reduced to 90% of the initial volume. The remaining samples were diluted to original volume with Carbonate buffer (0.1 M NaHCO3 and 5 mM Na_2CO_3 in ddH2O; pH 8.6) and centrifuged 3 more times or until the desired buffer exchange was achieved. The Bradford assay was then used to measure the protein concentration of the samples, and vials are stored in 4 °C until radiolabeling.

2.5.2.2.4. TCMC conjugation

Previously prepared and frozen TCMC kits were removed from -20°C freezer and allowed to warm to room temperature. Kits were resuspended by the addition of 2 mL of buffer per 1 mg of antibody and were diluted further if needed for equivalent volume of the stocks after addition of antibodies. The vials were then covered in foil and incubated in room temperature for 2 hours on a rocker.

2.5.2.2.5. Removal of Unconjugated TCMC Chelator

To remove the unconjugated chelator from the conjugated antibodies, the buffer was exchanged to 0.15M NH_4OAc buffer, pH 7.0, using zeba desalting columns (40K MWCO, 2mL) by centrifugation at 1000g. Final stocks were stored at 4 °C until radiolabeling.

2.5.2.2.6. Stock Pb-214/Bi-214 pH Balancing

Pb-214 (0.1M HCl) was received from Niowave and measured for dose, time, and volume. A total of 200 mL of 5 M NH_4OAc (pH 7) was added to the vial containing the Pb-214/Bi-214 chloride and it was vortexed for 3 seconds to achieve pH 6.5.

2.5.2.2.7. Radiolabeling of the three Antibodies (TCMC-conjugated trastuzumab, TCMC-conjugated cetuximab, and TCMC-conjugated IgG control Ab)

TCMC-conjugated antibodies received 1/3rd of the total radioactivity each (final volume aimed to be at 1000 mL) and vortexed for 3 seconds and incubated in an Eppendorf heating block with 350 rpm, for 15 mins at 37 °C.

2.5.2.2.8. Purification of radiolabeled antibodies

A 40K MWCO, 2mL Zebra desalting column was centrifuged at 3260 g for 3 minutes to wash off the existing liquid. 1mL of 1X PBS was then added and the column was spun twice for 3 minutes

(with addition of 1 mL PBS) and one more time at 4 minutes. The radiolabeled antibodies were then placed in separated pre-equilibrated spin columns and were spun for 5 minutes. The purified Pb-214/Bi-214-labeled antibodies (liquid below spin columns after centrifugation) were used to prepare the dilution plates.

2.5.2.2.9. Dilution Plate Prep

Dilution plate was prepared to have 3 replicates each for Pb-214/Bi-214-labeled Trastuzumab, Pb-214/Bi-214-labeled Cetuximab, and the combined Pb-214/Bi-214-labeled Trastuzumab/Cetuximab and triplicates for Pb-214/Bi-214-labeled IgG. A total of 150 mL of the radiolabeled antibodies were placed in the initial wells and 75 mL of the sample from the upper well was transported to the lower wells with 75 mL of media to obtain a 1:2 dilution series.

2.5.2.2.10. Transfer from Dilution Plates to Cell Plates

The 96-well plates with cells seeded the prior day were removed from the incubator and washed twice with 200 mL 1X PBS. Single antibody treatments (in triplicate) received 50 mL of fresh media, while the combination antibody treatment (triplicates) received no addition and the untreated control cells received 100 mL. The solutions in the previously prepared dilution plate were then added to the 96-well plate containing the cells, 50 mL of Pb-214/Bi-214-Trastuzumab to Trastuzumab and Combo triplicates, 50 mL of Pb-214/Bi-214 -Cetuximab to Cetuximab and Combo triplicates, and 50 mL of Pb-214/Bi-214 -IgG to IgG Isotype Control triplicates. Plate was then placed back in the incubator for 24 hours at 37 °C and 5% CO_2.

2.5.2.2.11. iTLC and Bradford Assays

iTLC was conducted on the purified radiolabeled antibodies. 2 mL of the sample was placed at the bottom of the strips (TEC-Control Chromatography Strips for In-111/Y-90, Model# 150-772, BIODEX, Shirley, NY) and the strips were placed in a glass vial containing 300 mL of 5M NH_4OAc (pH=4) and a second vial with PBS for each antibody. After the solvent reached the top, the strip was cut in half and each half was counted in the Gamma Counter to measure radioactivity and calculate purity. Above 95% purity for 'bound/(bound+free)', or 'bottom/total strip' was considered acceptable. Bradford Protein Assay was also completed on purified labeled antibodies to quantify finalized protein concentration.

2.5.2.2.12. ATPlite Assay and Graphpad Analyses

The ATPlite assay and Graphpad analyses were done as previously described (2.5.2.1.6. and 2.5.2.1.7.).

2.5.2.3. *Further Killing Analysis*

2.5.2.3.1. Plate cells

For SCaBER and SW780 cell lines, further analysis was completed. Each cell line has 3 conditions, as follows, Pb-214/Bi-214-[Trastuzumab+Cetuximab], Pb-214/Bi-214-[IgG Isotype Control] and cells that were not treated. All treatments included triplicates with 25% confluent wells, approximately 7K cells/well in 100 mL of media.

2.5.2.3.2. Concentrating Antibodies and dialysis

Antibodies were concentrated and dialyzed as previously described (2.5.2.1.3.).

2.5.2.3.3. Buffer Exchange for TCMC chelator conjugation

Previously dialyzed antibodies are placed in the Pierce™ Protein Concentrator (PES, 10K or 30K MWCO, 2-6 mL) and centrifuged at 3260 g for 8 minutes until the volume was reduced to 90% of the initial volume. The remaining samples were diluted to original volume with Carbonate buffer (0.1 M NaHCO3 and 5 mM Na_2CO_3 in ddH2O; pH 8.6) and centrifuged 3 more times or until the desired buffer exchange was achieved. The Bradford assay was then used to measure the protein concentration of the samples, and vials are stored in 4 °C until radiolabeling.

2.5.2.3.4. TCMC conjugation

Previously prepared and frozen TCMC kits were removed from -20°C freezer and allowed to warm to room temperature. Kits were resuspended by the addition of 2 mL of buffer per 1 mg of antibody and were diluted further if needed for equivalent volume of the stocks after addition of antibodies. The vials were then covered in foil and incubated in room temperature for 2 hours on a rocker.

2.5.2.3.5. Removal of Unconjugated TCMC Chelator

To remove the unconjugated chelator from the conjugated antibodies, the buffer was exchanged to 0.15M NH_4OAc buffer, pH 7.0, using zeba desalting columns (40K MWCO, 2mL) by centrifugation at 1000g. Final stocks were stored at 4 °C until radiolabeling.

2.5.2.3.6. Stock Pb-214/Bi-214 pH Balancing

Pb-214/Bi-214 (0.1M HCl) was received from Niowave and measured for dose, time, and volume. A total of 200 mL of 5 M NH_4OAc (pH 7) was added to the vial containing the Pb-214/Bi-214 chloride and it was vortexed for 3 seconds to achieve pH 6.5.

2.5.2.3.7. Radiolabeling of the Antibodies (TCMC-conjugated trastuzumab+cetuximab, and TCMC-conjugated IgG control Ab)

TCMC-conjugated antibodies for both conditions received 4.0 mCi of Pb-214 to 1 mL of total volume and vortexed for 3 seconds and incubated in an Eppendorf heating block with 350 rpm, for 15 mins at 37 °C.

2.5.2.3.8. Purification of radiolabeled antibodies

A 40K MWCO, 2mL Zebra desalting column was centrifuged at 3260 g for 3 minutes to wash off the existing liquid. 1mL of 1X PBS was then added and the column was spun twice for 3 minutes (with addition of 1 mL PBS) and one more time at 4 minutes. The radiolabeled antibodies were then placed in separated pre-equilibrated spin columns and were spun for 5 minutes. The purified Pb-214/Bi-214-labeled antibodies (liquid below spin columns after centrifugation) were used to prepare the dilution plates.

2.5.2.3.9. Addition of Radiolabeled Antibodies to Cell Plates

The 96-well plates with cells seeded the prior day were removed from the incubator and washed twice with 200 mL 1X PBS. Final samples for above conditions were then directly added to the cells. SCaBER and SW780 cells in 96 well plate were treated at 25% confluency with either nothing (untreated condition), Pb-214-IgG (68μCi, 5μCi/ug), or Pb-214-cAB (Trastuzumab+Cetuximab) (38 mCi, 1.8 μCi/μg). Plate was then placed back in the incubator for 72 hours at 37 °C and 5% CO_2.

2.5.2.3.10. iTLC and Bradford Assays

iTLC was conducted on the purified radiolabeled antibodies. 2 mL of the sample was placed at the bottom of the strips (TEC-Control Chromatography Strips for In-111/Y-90, Model# 150-772, BIODEX, Shirley, NY) and the strips were placed in a glass vial containing 300 mL of 5M NH4OAc (pH=4) and and a second vial with PBS for each antibody. After the solvent reached the top, the strip was cut in half and each half was counted in the Gamma Counter to measure radioactivity and calculate purity. Above 95% purity for 'bound/(bound+free)', or 'bottom/total

strip’ was considered acceptable. Bradford Protein Assay was also completed on purified labeled antibodies to quantify finalized protein concentration.

2.5.2.3.11. ATPlite Assay and Graphpad Analyses

After 72 hours of incubation, the cell plate was removed from the incubator. To minimize the loss of cells from wash and aspiration, wells were not washed. All wells received 50 mL of the mammalian cell lysis solution followed by the addition of 50 mL of the ATPlite buffer and lyophilized substrate solution mixture and was shaken at 700rpm for 5 minutes each time. The 96-well plate was then dark adapted for 10 minutes and read for the luminescence in IVIS Lumina LT Series III machine with Living Image Software 4.7.2. Graphpad analyses were done as previously described (2.5.2.1.7.).

3. RESULTS

3.1. Antibody Radiolabeling Results

The antibodies Trastuzumab, Cetuximab, and IgG were successfully radiolabeled with Tc-99m and Pb-214. The specific activity of Tc-99m-labeled antibody averaged 19.24 mCi/mg (range= 11.74-42.65) and specific activity of Pb-214-labeled antibody averaged 0.18 mCi/mg (range=0.12-0.27). The percentage of radioactivity bound to the antibody (purity) was measured by iTLC on the selected G-25 column fraction prior to using for experiments. The iTLC values >95% were accepted as sufficiently pure. The iTLC data are presented in Figure 3.1 and Table 1.1.1 for Tc-99m, and Figure 3.2. and Table 1.1.2 for Pb-214.

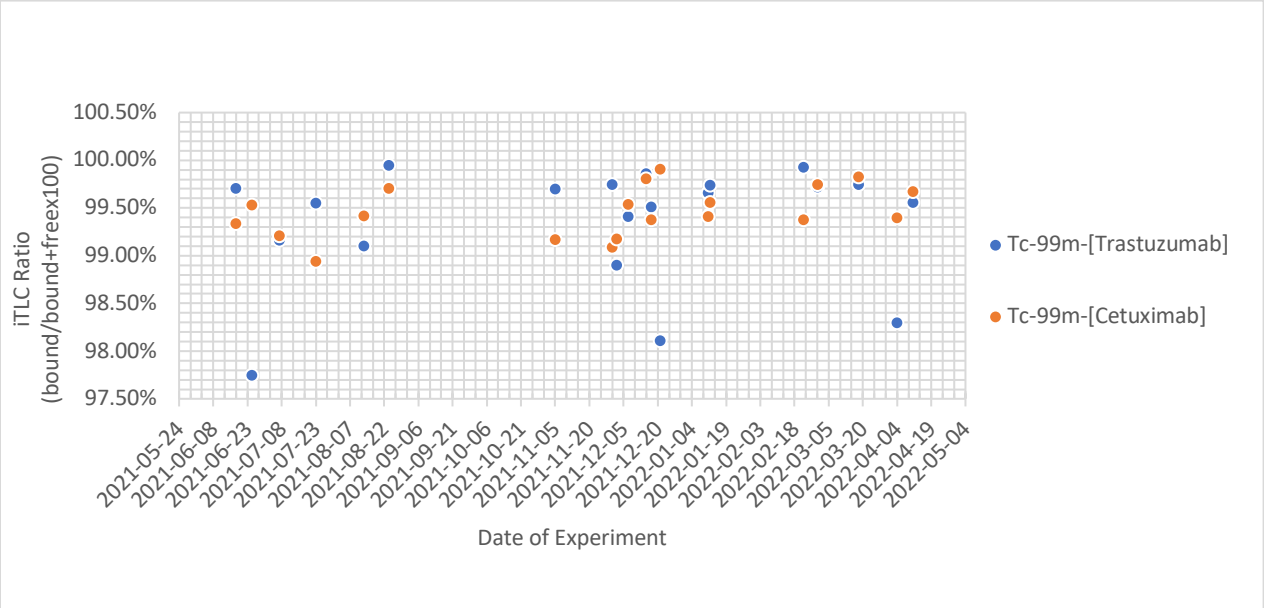

Figure 3.1 iTLC Results for Tc-99m radiolabeling of Antibodies. Data are presented as % values of Tc-99m bound to each antibody divided by total Tc-99m (bound+free) or (bound/bound+free) x 100%. A value of 95% was accepted as sufficiently pure to use in experiments, meaning that at least 95% of Tc-99m was bound to the antibody.

	Tc-99m-[Trastuzumab]	Tc-99m-[Cetuximab]
Mean	99%	99%
SD	0.01	0.00

Table 1.1.1. Summary of iTLC values for the Tc-99m-labeled antibodies.

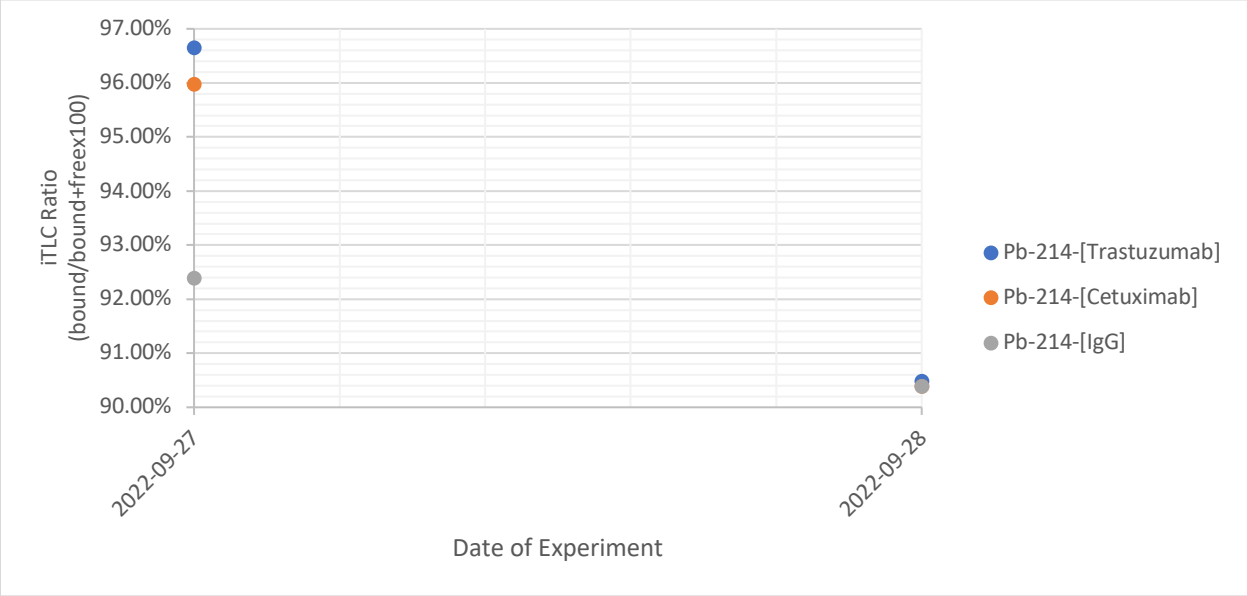

Figure 3.2 iTLC Results for Pb-214 radiolabeling of Antibodies. Data are presented as % values of Pb-214 bound to each antibody divided by total Pb-214 (bound+free) or (bound/bound+free) x 100%. A value of 90% was accepted as sufficiently pure to use in experiments, meaning that at least 90% of Pb-214 was bound to the antibody.

	Pb-214/Bi-214-[Trastuzumab]	Pb-214/Bi-214-[Cetuximab]	Pb-214/Bi-214-[IgG]
Mean	95%	94%	92%
SD	0.04	0.04	0.06

Table 1.1.2 Summary of iTLC values for the Pb-214/Bi-214-labeled antibodies.

3.2. Receptor Level Validation

3.2.1. Specific binding of antibodies to cells and determination of Kd (affinity)

Specific binding experiments with Tc-99m labeled antibodies were completed only if iTLC results were over 95% and Bradford Protein Concentration values were reasonable for the selected fraction used (>1/10th of the original conjugated antibody stock). After preparation of the dilution plate, addition of the dilutions to cell plate, incubation, washing, and cell count by ATPlite, the final collected samples were measured for radioactivity (CPM) with a gamma counter. The CPM values were converted into mCi using 1.42 million CPM/mCi as the conversion factor that was determined during calibration of the gamma counter. Further analyses were completed with GraphPad Prism as described in the methods section. The values are reported in Table 1.2.1.

Date of Experiment	Cell Line	Tc-99m-labeled-Antibody	Bmax (nM)	Kd	Fit R^2
11/21/21	3T3	Trastuzumab	3.94 E-09	Unstable	N/A
11/21/21	3T3	Cetuximab	-5.67 E+09	Unstable	N/A
01/12/22	5637	Trastuzumab	1.57 E-07	1.44	0.98
01/12/22	5637	Cetuximab	9.71 E-07	0.11	0.94
02/22/22	T24	Trastuzumab	2.55E-07	0.29	0.98
02/22/22	T24	Cetuximab	2.07E-07	0.08	0.97
03/18/22	RT4	Trastuzumab	4.28E-06	0.88	0.97
03/18/22	RT4	Cetuximab	8.00E-06	0.19	0.97
03/18/22	SW780	Trastuzumab	1.72E-06	0.72	0.99
03/18/22	SW780	Cetuximab	1.13E-05	0.23	0.95
04/04/22	SCaBER	Trastuzumab	1.89E-07	0.97	0.97
04/04/22	SCaBER	Cetuximab	6.79E-06	0.18	0.94
04/11/22	TCCSUP	Trastuzumab	3.41E-08	0.59	0.91
04/11/22	TCCSUP	Cetuximab	5.89E-07	0.01	0.97

Table 1.2.1 Bmax and Kd Values for Cell Lines with Their Respective Antibodies.

*R^2 values were computed by first analyzing the baseline corrected Specific Binding values to correct Total Binding and Non-Specific Binding values, and then using the One-Site Specific Binding Non-Linear Fit Analysis on GraphPad. They represented the quantitative fit of the values in a theoretical curve fit that was computed by Kd and Bmax values entered in to GraphPad.

The graphs that were computed by One-Site Specific Binding Non-Linear Fit Analysis by GraphPad in all cell lines are shown in Figure 3.3.1 to 3.3.12.

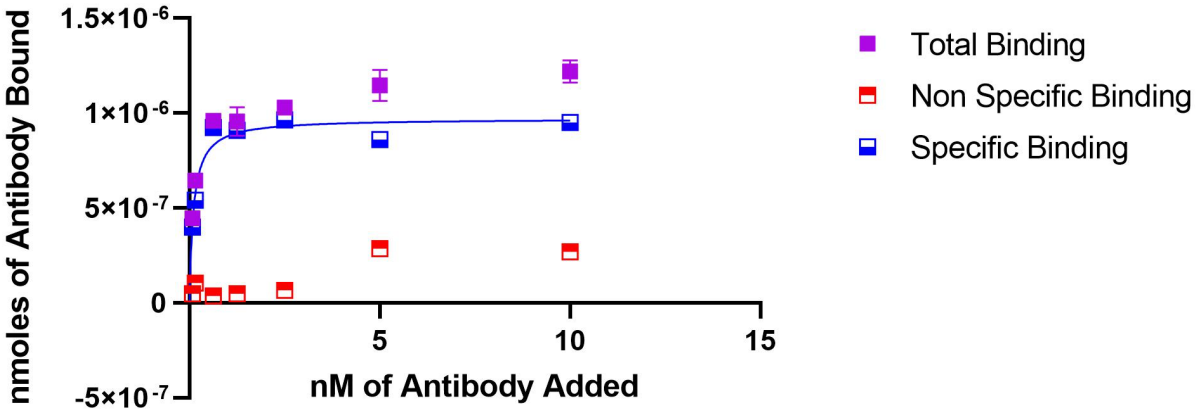

Figure 3.3.1 Tc-99m-Cetuximab binding results for the 5637 cell line.

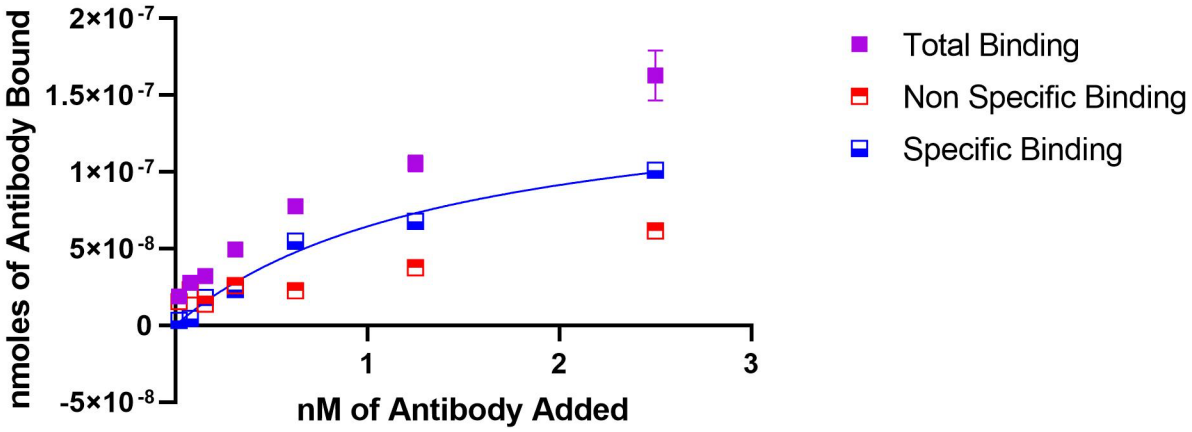

Figure 3.3.2 Tc-99m-Trastuzumab binding results for the 5637 cell line.

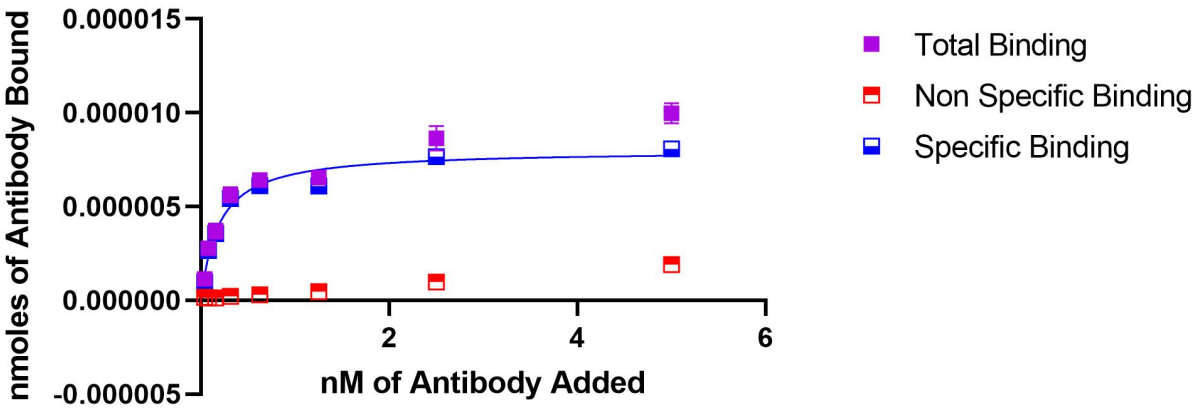

Figure 3.3.3 Tc-99m-Cetuximab binding results for the RT4 cell line.

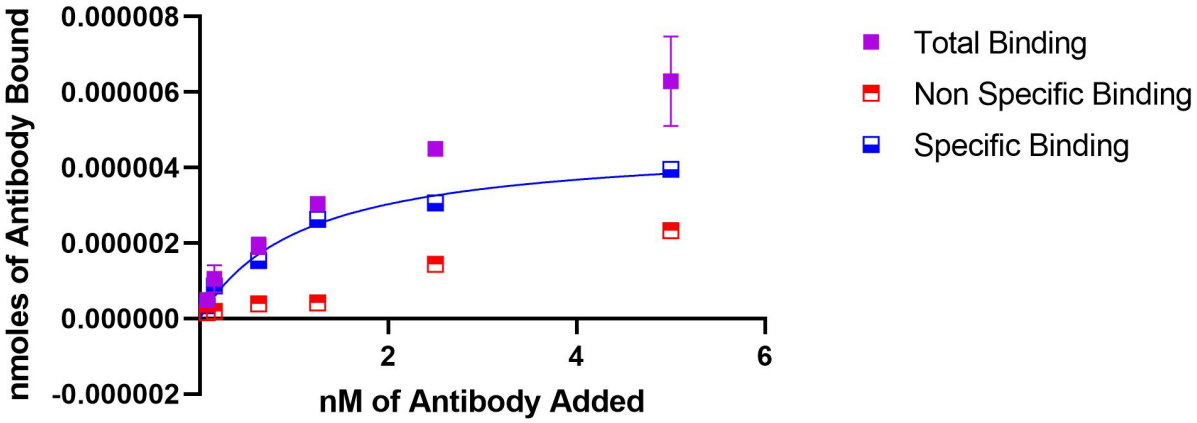

Figure 3.3.4 Tc-99m-Trastuzumab binding results for the RT4 cell line.

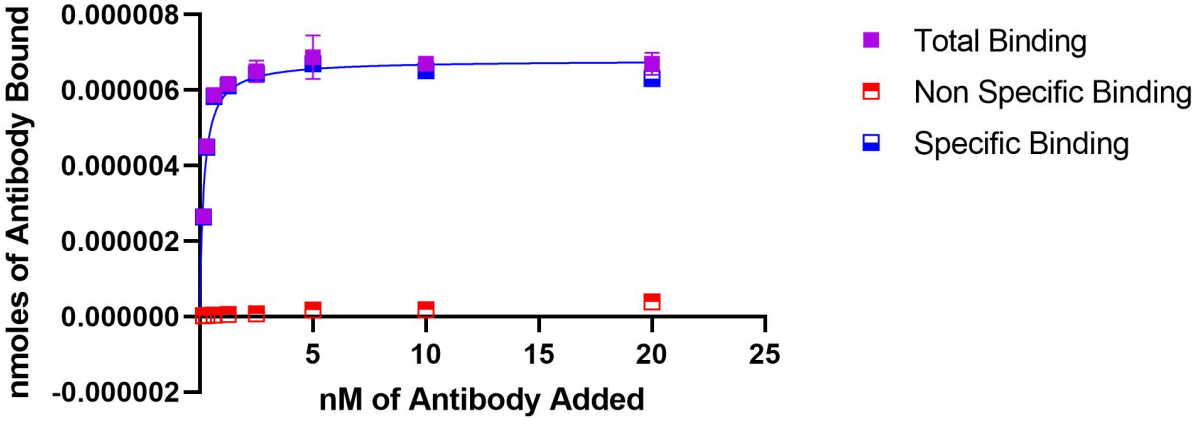

Figure 3.3.5 Tc-99m-Cetuximab binding results for the SCaBER cell line.

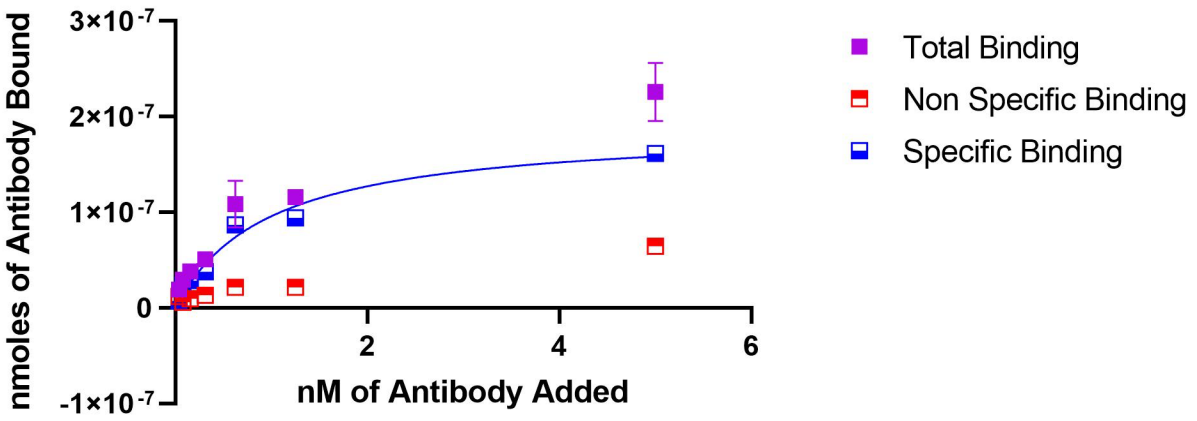

Figure 3.3.6 Tc-99m-Trastuzumab binding results for the SCaBER cell line.

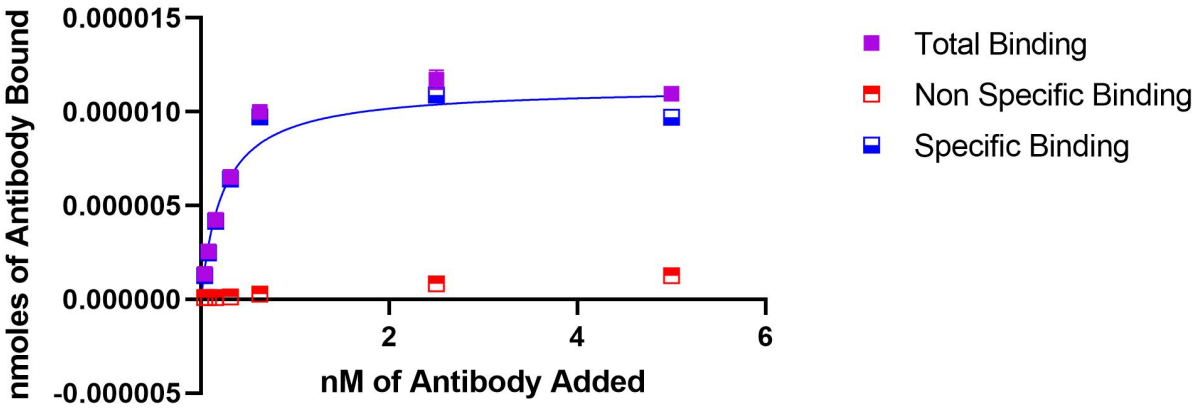

Figure 3.3.7 Tc-99m-Cetuximab binding results for the SW780 cell line.

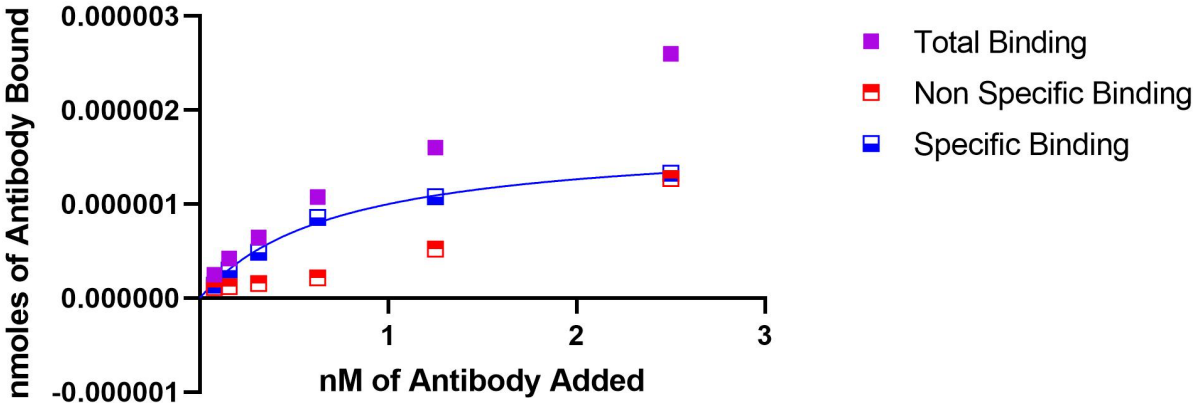

Figure 3.3.8 Tc-99m-Trastuzumab binding results for the SW780 cell line.

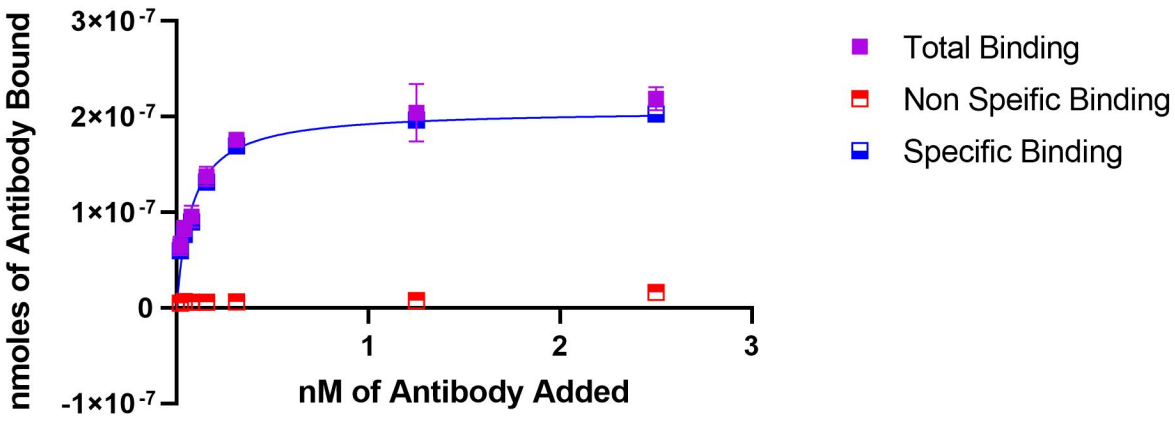

Figure 3.3.9 Tc-99m-Cetuximab binding results for the T24 cell line.

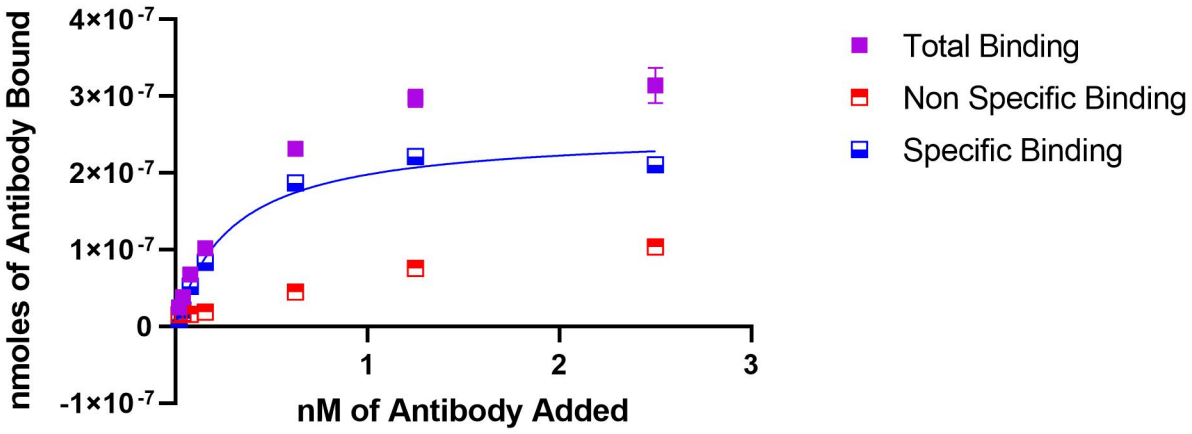

Figure 3.3.10 Tc-99m-Trastuzumab binding results for the T24 cell line.

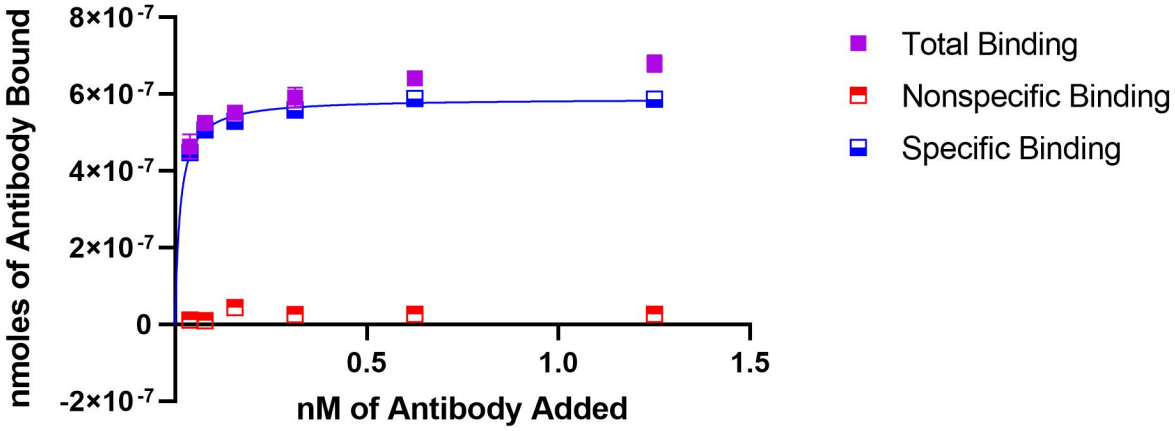

Figure 3.3.11 Tc-99m-Cetuximab binding results for the TCCSUP cell line.

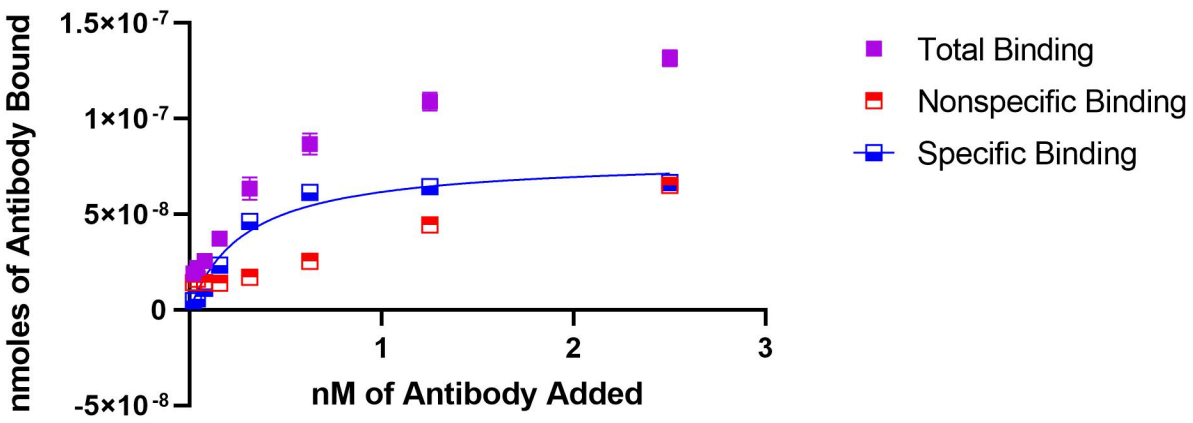

Figure 3.3.12 Tc-99m-Trastuzumab binding results for the TCCSUP cell line.

Analysis of binding data for the 3T3 cell line confirmed no expression of either EGFR1 or EGFR2, as there was no binding for the radiolabeled Trastuzumab or Cetuximab. The 3T3 was used as the negative control since it was known not to express EGFR1 or EGRF2.

3.2.2. Cell Numbers for Specific Binding Assays

Cell numbers in the binding assays were quantified with the ATPlite Assay after incubation of the radiolabeled antibodies and the washing steps. Living Image Software provided the photons/second values (luminescence), which were converted to cell numbers by a standard curve. This was a linear dilution of cells that were prepared prior followed by determination of a linear equation for photon flow/second and cell numbers in the wells. Values were accepted only if within the standard curve. The replicates typically had a relative standard error of less than 5%. Table 1.2.2 reports these data.

Date of Experiment	Cell Line	Antibody	Mean Cell Count	Standard Deviation	Relative Standard Deviation
11/21/21	3T3	Trastuzumab	2.13E+04	3.83E+01	0.18
11/21/21	3T3	Cetuximab	4.70E+04	2.12E+01	0.05
01/12/22	5637	Trastuzumab	1.44E+04	1.24E+01	0.09
01/12/22	5637	Cetuximab	1.43E+04	1.52E+01	0.11
02/22/22	T24	Trastuzumab	6.24E+03	2.25E+01	0.36
02/22/22	T24	Cetuximab	9.37E+03	1.35E+01	0.14
03/18/22	RT4	Trastuzumab	1.47E+04	1.73E+01	0.12
03/18/22	RT4	Cetuximab	1.97E+04	2.18E+01	0.11
03/18/22	SW780	Trastuzumab	1.79E+04	1.33E+01	0.07
03/18/22	SW780	Cetuximab	1.84E+04	1.29E+01	0.07
04/04/22	SCaBER	Trastuzumab	1.83E+04	6.09E+00	0.03
04/04/22	SCaBER	Cetuximab	1.93E+04	1.30E+01	0.07
04/11/22	TCCSUP	Trastuzumab	2.72E+04	1.21E+01	0.04
04/11/22	TCCSUP	Cetuximab	2.50E+04	6.16E+00	0.02

Table 1.2.2 Cell Number ATPlite analysis.

3.2.3. Receptor Numbers

Results from Table 1.2.1 and Table 1.2.2 were used to analyze cells and their expressions of the EGFR1 and EGFR2. The EGFR1 and EGFR2 receptors per cell for the studied cell lines are presented in Table 1.2.3.

Date of Experiment	Cell Line	Antibody	Receptor Type	Bmax (Σ#receptor)	Cell Count	# of Receptors
11/21/21	5637	Trastuzumab	EGFR2	9.46E+07	1.44E+04	6.58E+03
11/21/21	5637	Cetuximab	EGFR1	5.85E+08	1.43E+04	4.09E+04
01/12/22	T24	Trastuzumab	EGFR2	1.53E+08	6.24E+03	2.46E+04
01/12/22	T24	Cetuximab	EGFR1	1.25E+08	9.37E+03	1.33E+04
02/22/22	RT4	Trastuzumab	EGFR2	2.58E+09	1.47E+04	1.76E+05
02/22/22	RT4	Cetuximab	EGFR1	4.82E+09	1.97E+04	2.44E+05
03/18/22	SW780	Trastuzumab	EGFR2	1.03E+09	1.79E+04	5.77E+04
03/18/22	SW780	Cetuximab	EGFR1	6.82E+09	1.84E+04	3.71E+05
03/18/22	SCaBER	Trastuzumab	EGFR2	1.14E+08	1.83E+04	6.23E+03
03/18/22	SCaBER	Cetuximab	EGFR1	4.09E+09	1.93E+04	2.12E+05
04/04/22	TCCSUP	Trastuzumab	EGFR2	2.06E+07	2.72E+04	7.56E+02
04/04/22	TCCSUP	Cetuximab	EGFR1	3.55E+08	2.50E+04	1.42E+04

Table 1.2.3 Receptor Numbers per Cell Line per Antibody.

Receptor number values were compared to previous literature that reported a semiquantitative analyses by flow cytometry results for the cell lines that were used in this study. Comparison Graphs are shown in Figure 3.4.1. and 3.4.2.

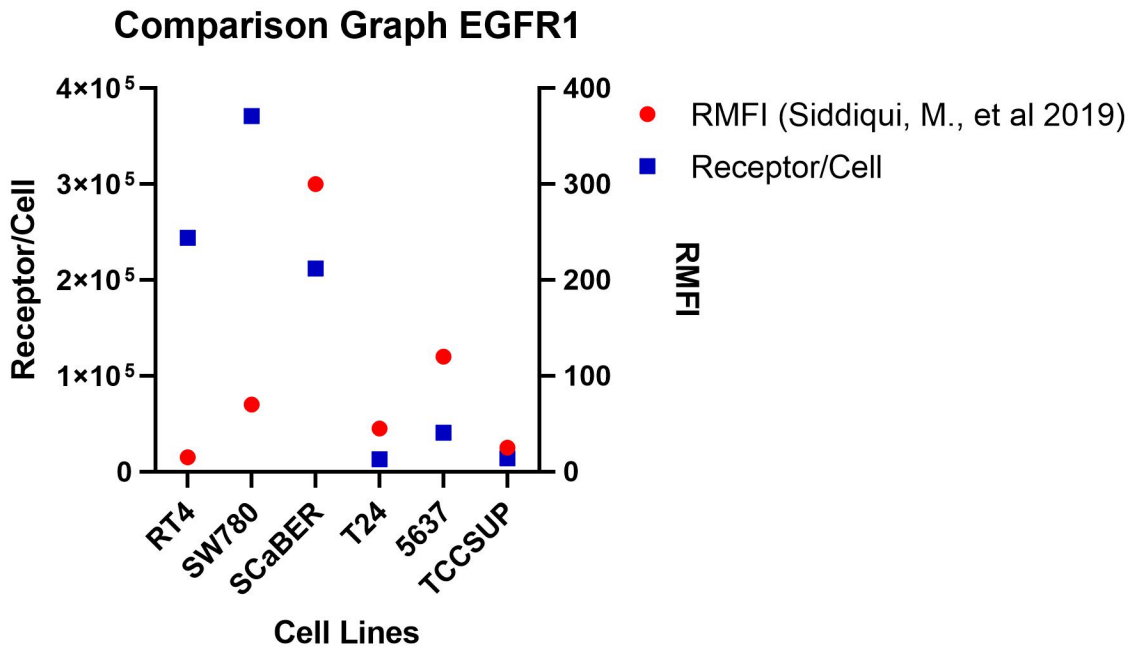

Figure 3.4.1. Comparison of above found EGFR1 number/cell for the discussed 6 cell lines to the RMFI values shared in previous literature by Siddiqui, M., et al 2019 [45].

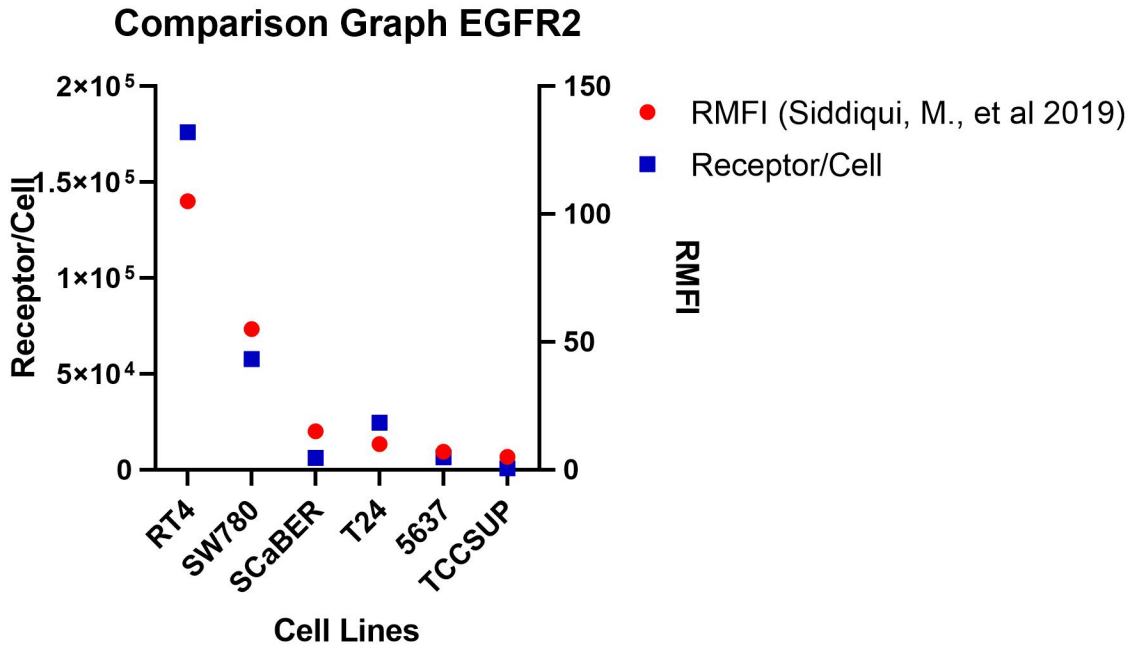

Figure 3.4.2. Comparison of above found EGFR2 number/cell for the discussed 6 cell lines to the RMFI values shared in previous literature by Siddiqui, M., et al 2019 [45].

3.3. Efficacy of Targeting and Killing with Pb-214

3.3.1. Control experiments for cells treated with antibodies without Pb-214

All cell lines were treated with each antibody alone, or in combination, with control replicates that were not treated. The treatment groups were compared using Two-Way ANOVA with GraphPad. Figures 3.5.1 to 3.5.14 present the comparisons for each cell line.

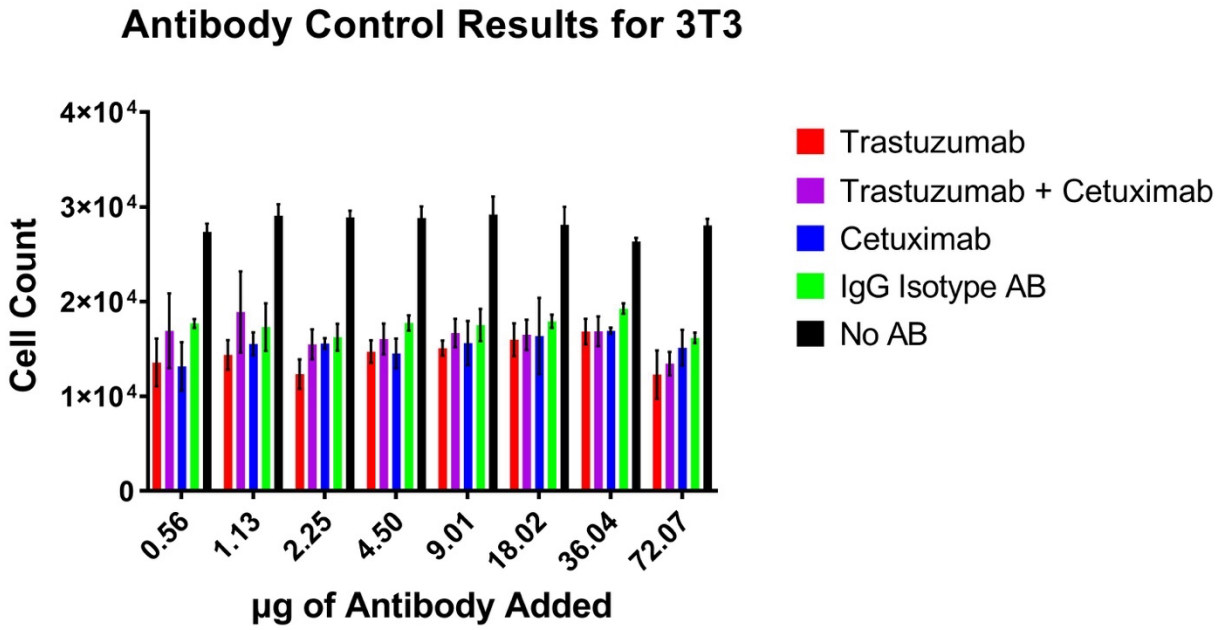

Figure 3.5.1. 3T3 cell line with antibody treatments.

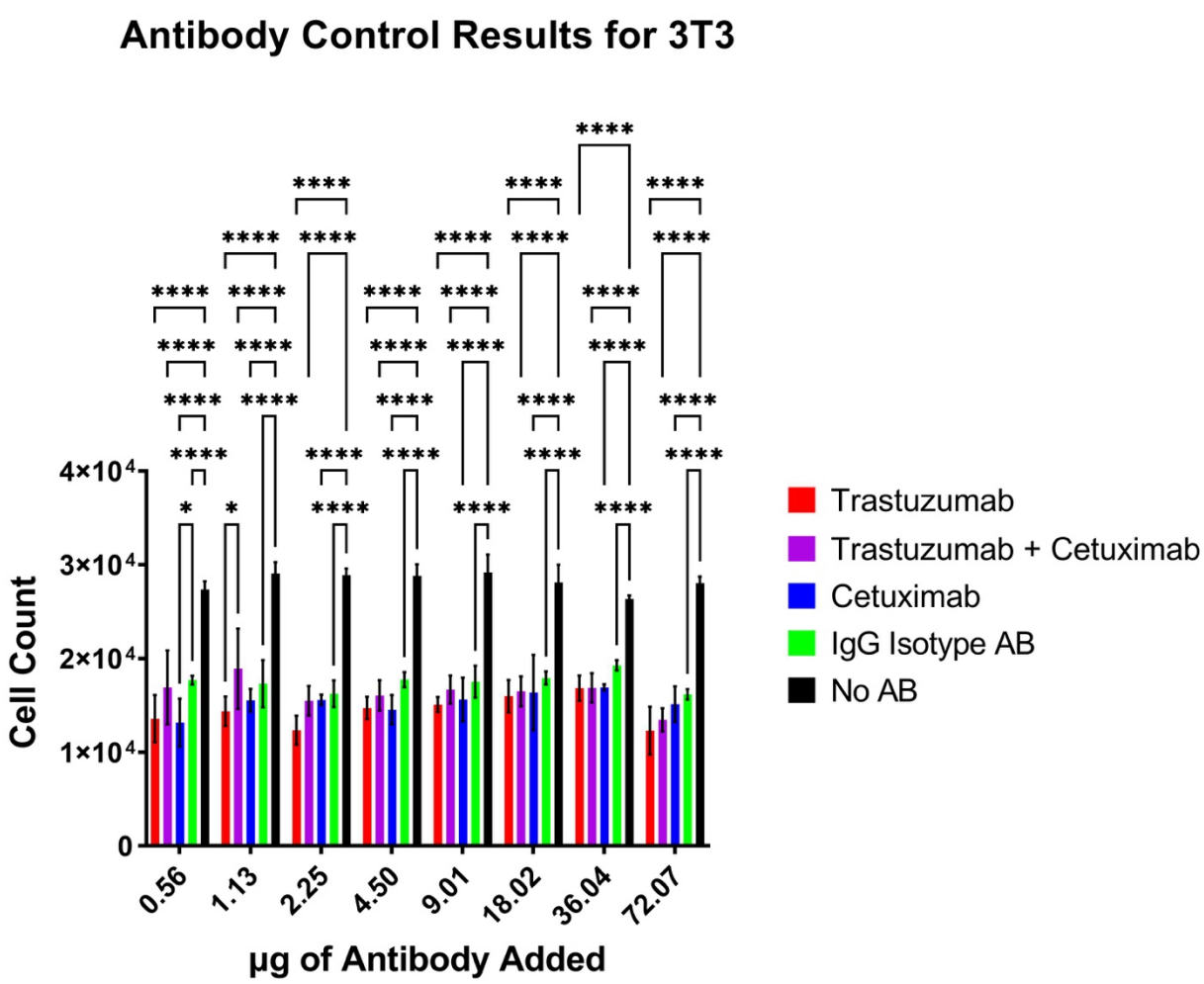

Figure 3.5.2. 3T3 cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002, ****: <0.0001).

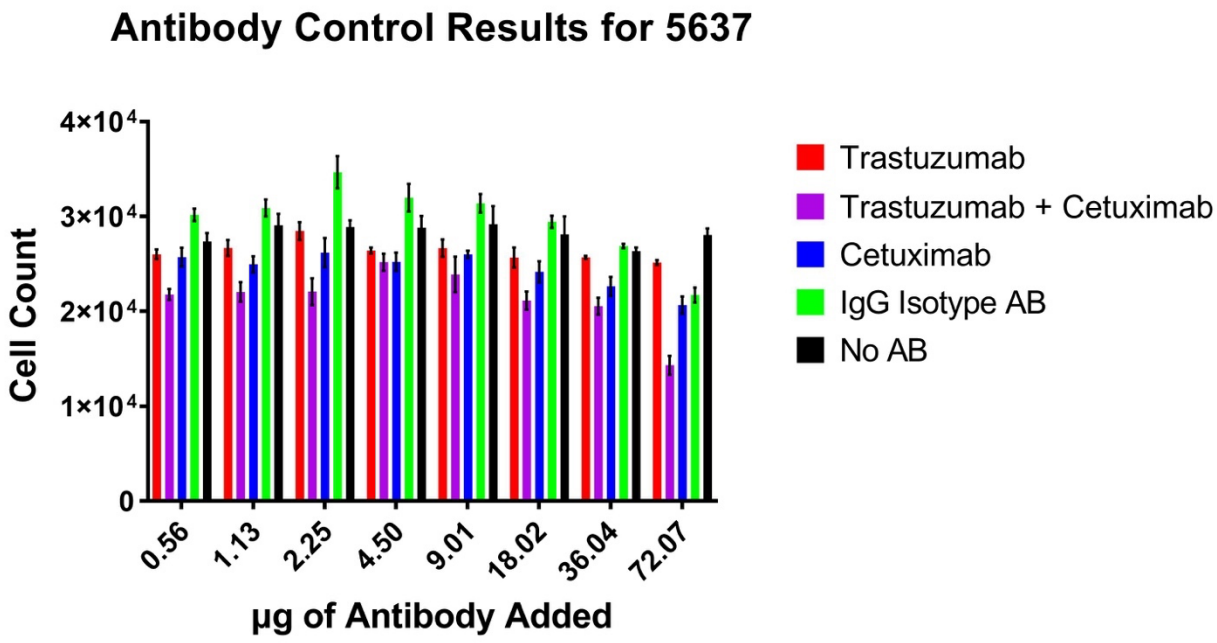

Figure 3.5.3. 5637 cell line with antibody treatments.

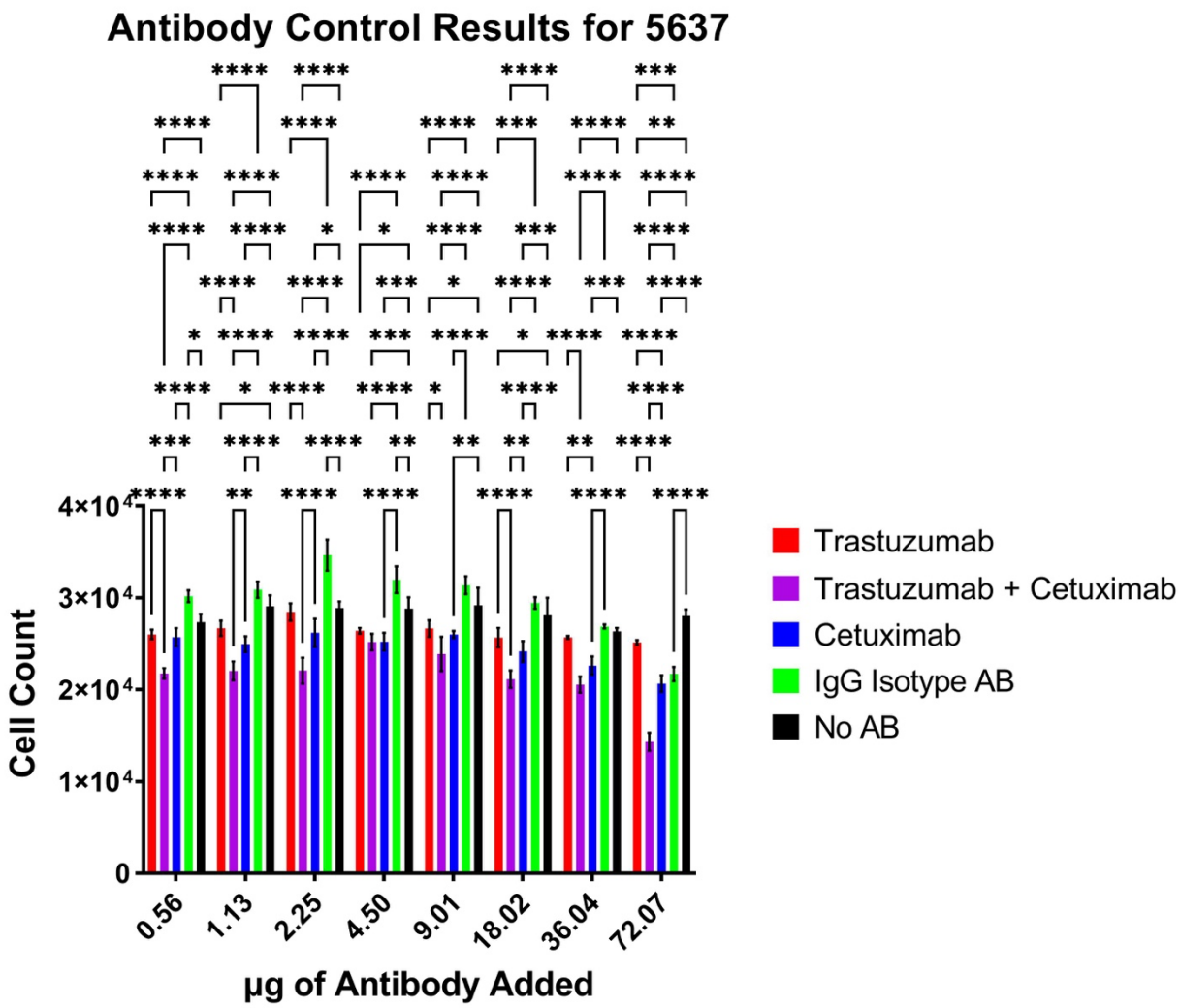

Figure 3.5.4. 5637 cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002, ****: <0.0001).

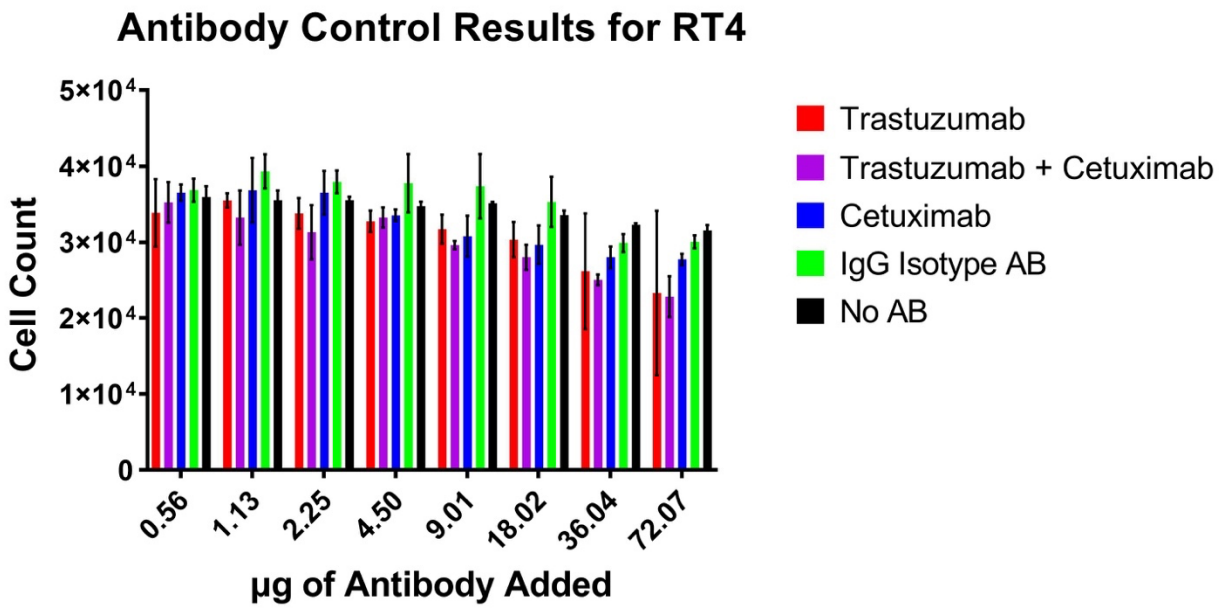

Figure 3.5.5 RT4 cell line with antibody treatments.

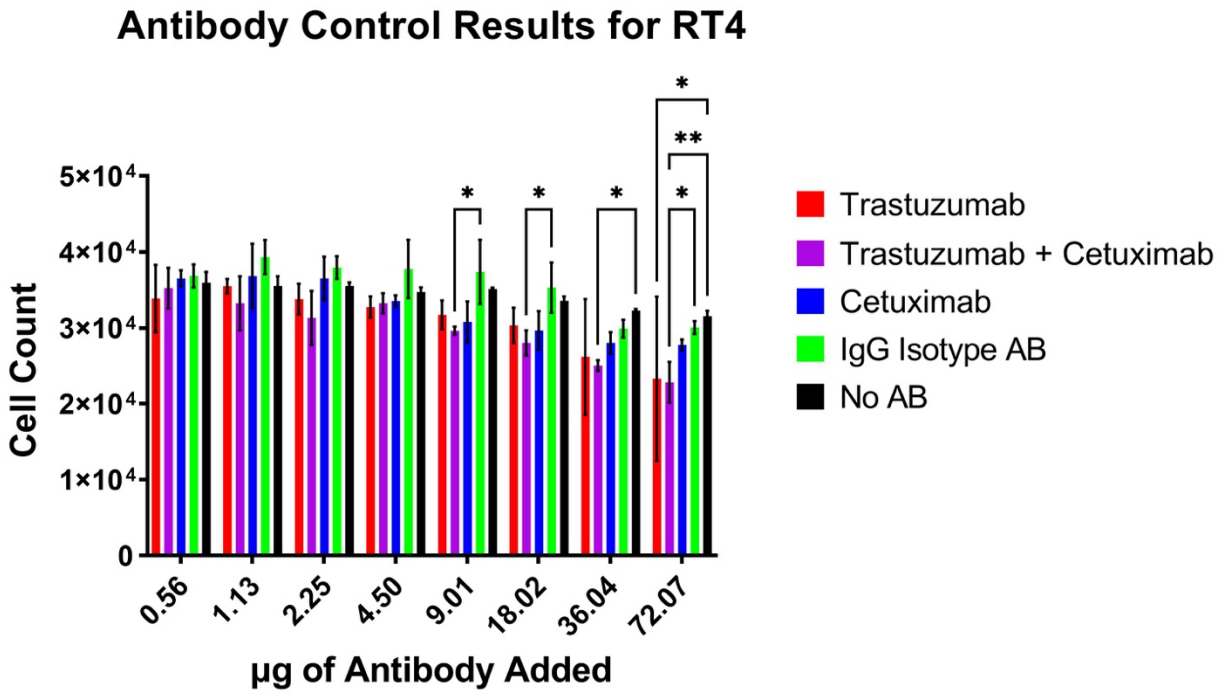

Figure 3.5.6. RT4 cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002).

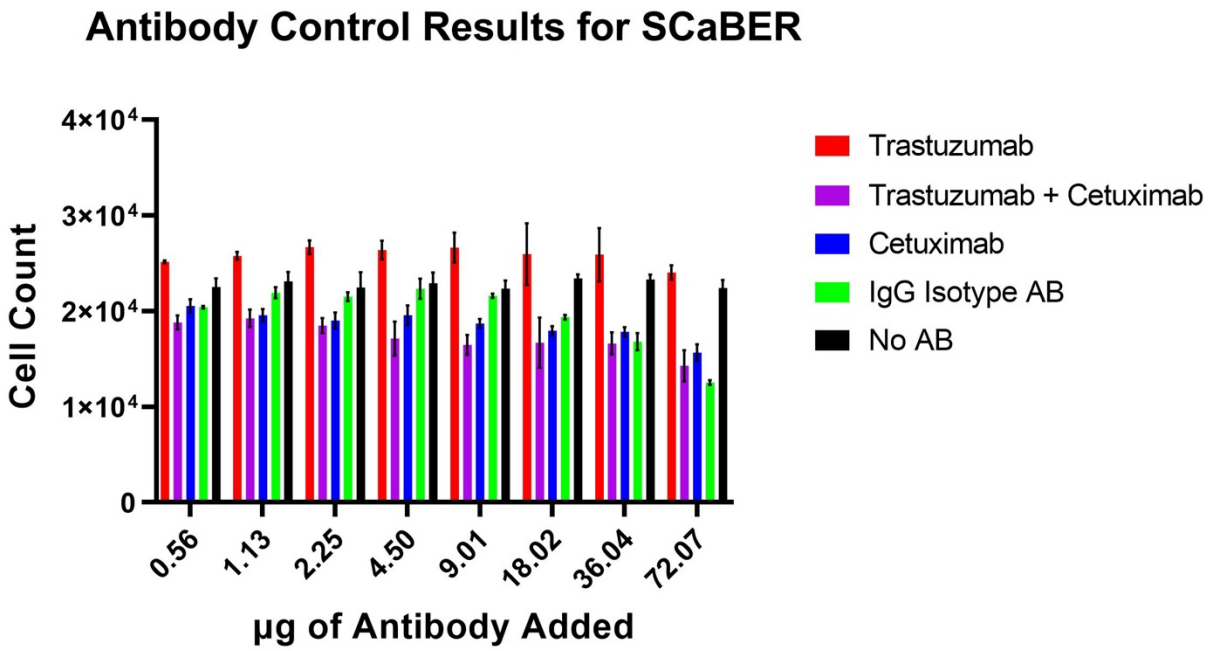

Figure 3.5.7 SCaBER cell line with antibody treatments.

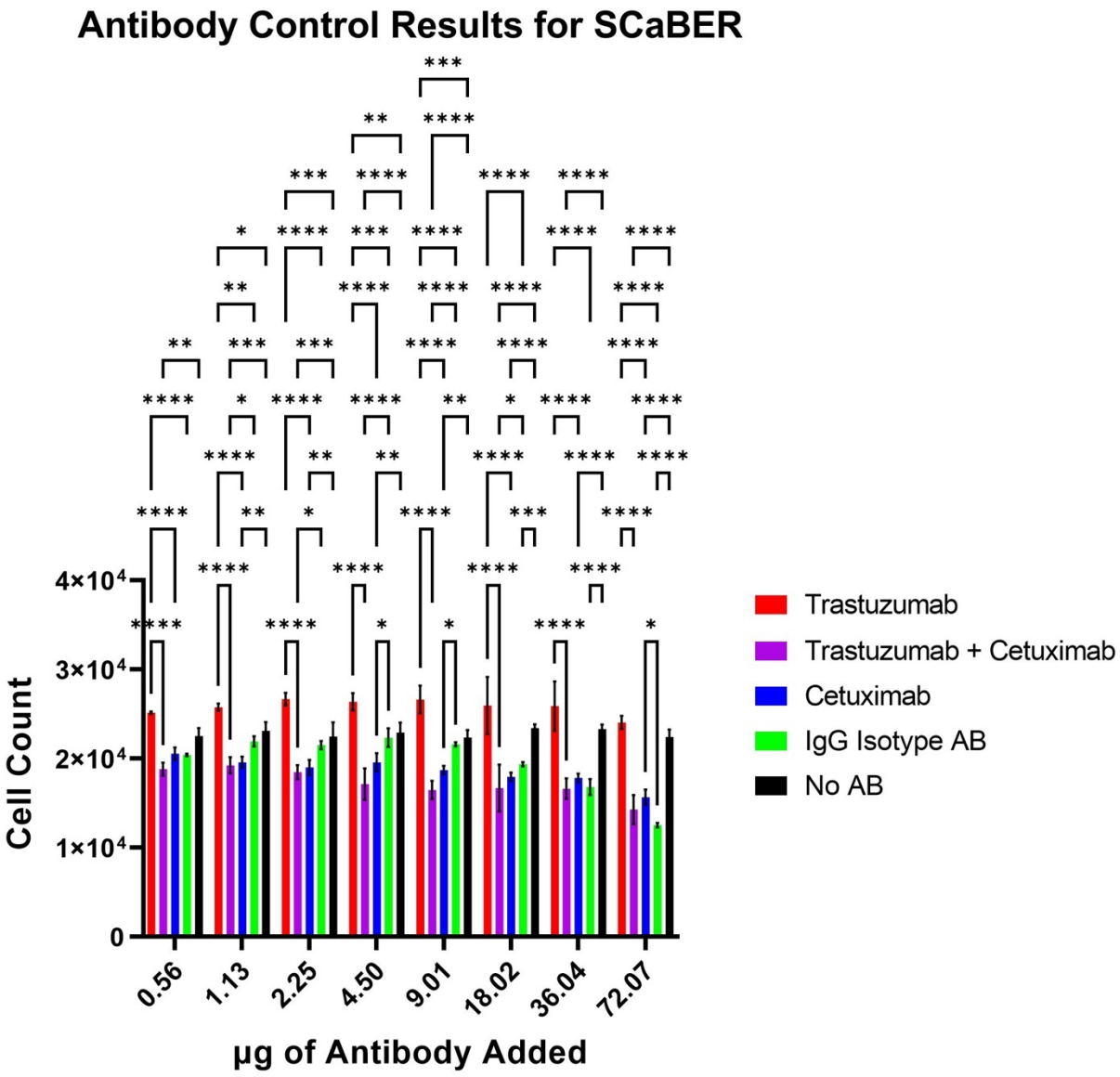

Figure 3.5.8. SCaBER cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002, ****: <0.0001).

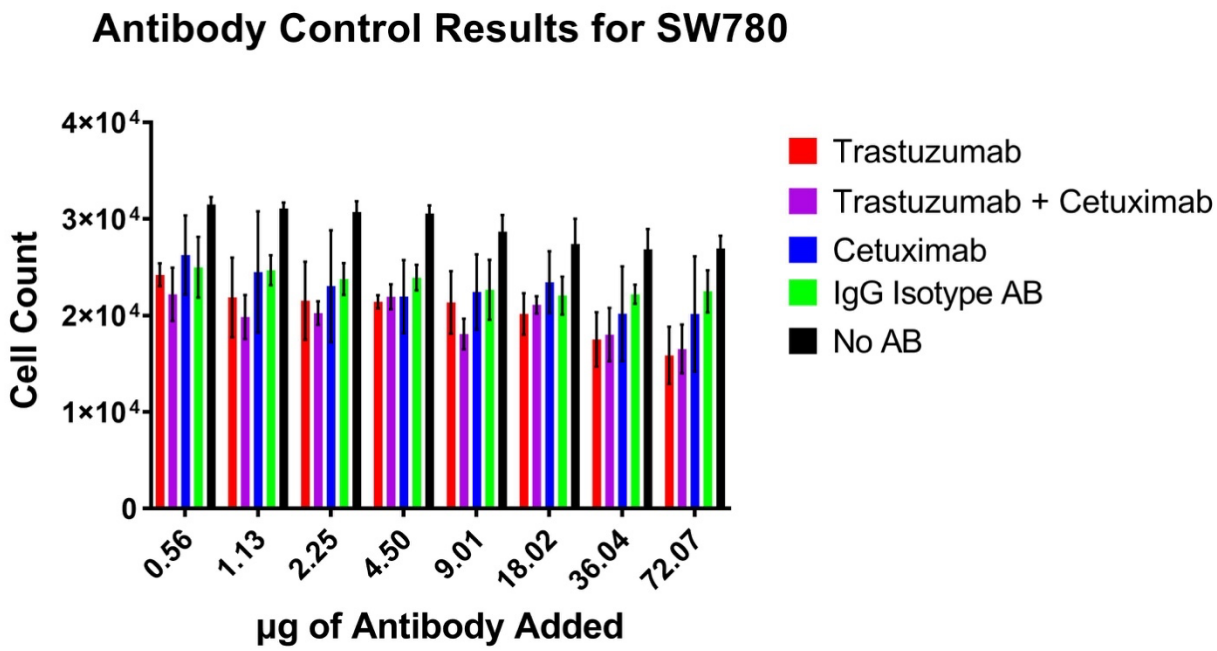

Figure 3.5.9. SW780 cell line with antibody treatments.

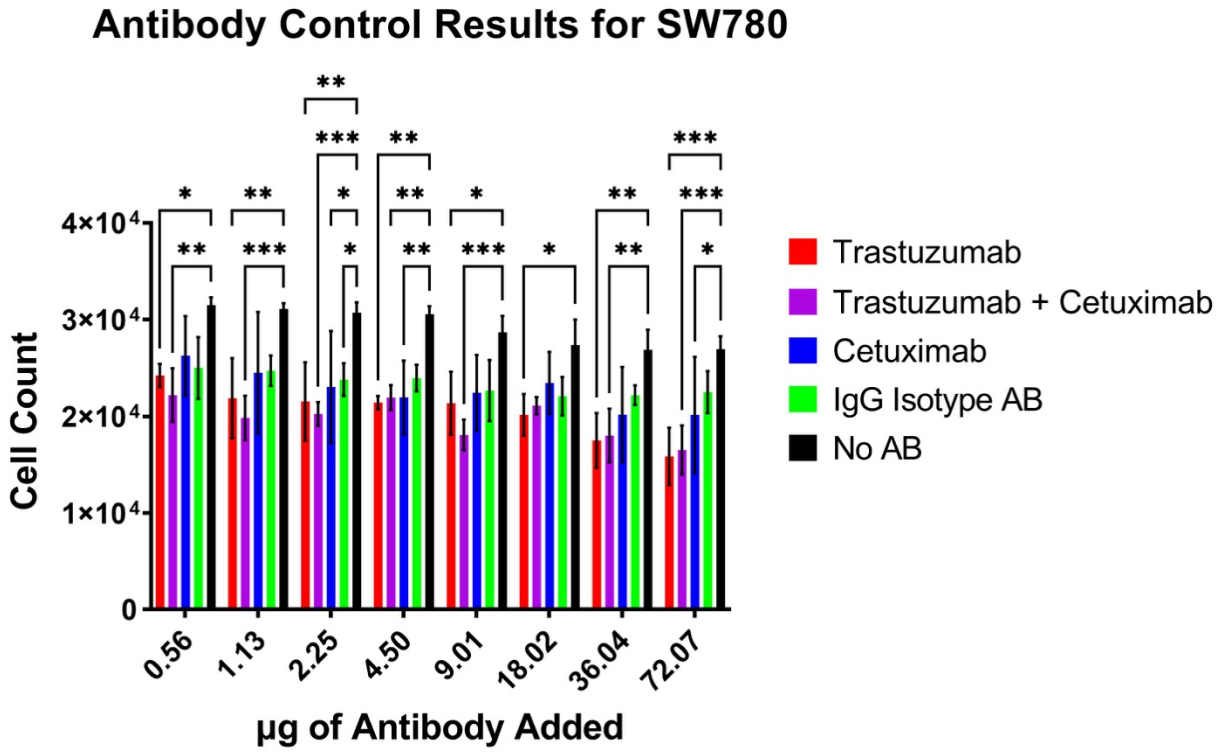

Figure 3.5.10. SW780 cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002).

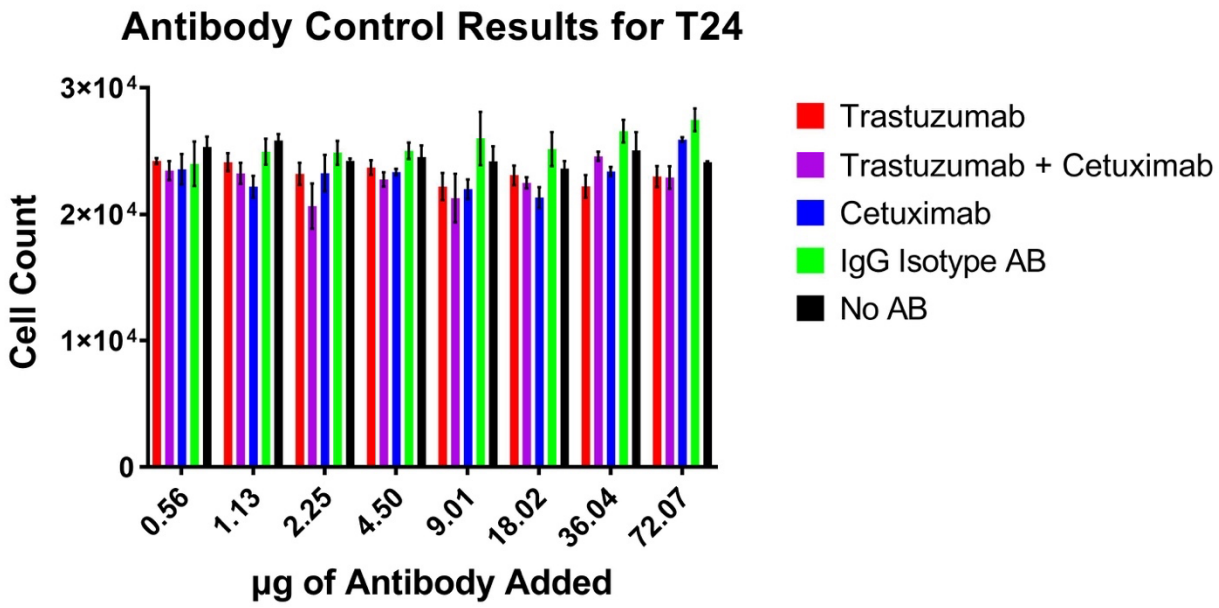

Figure 3.5.11. T24 cell line with antibody treatments.

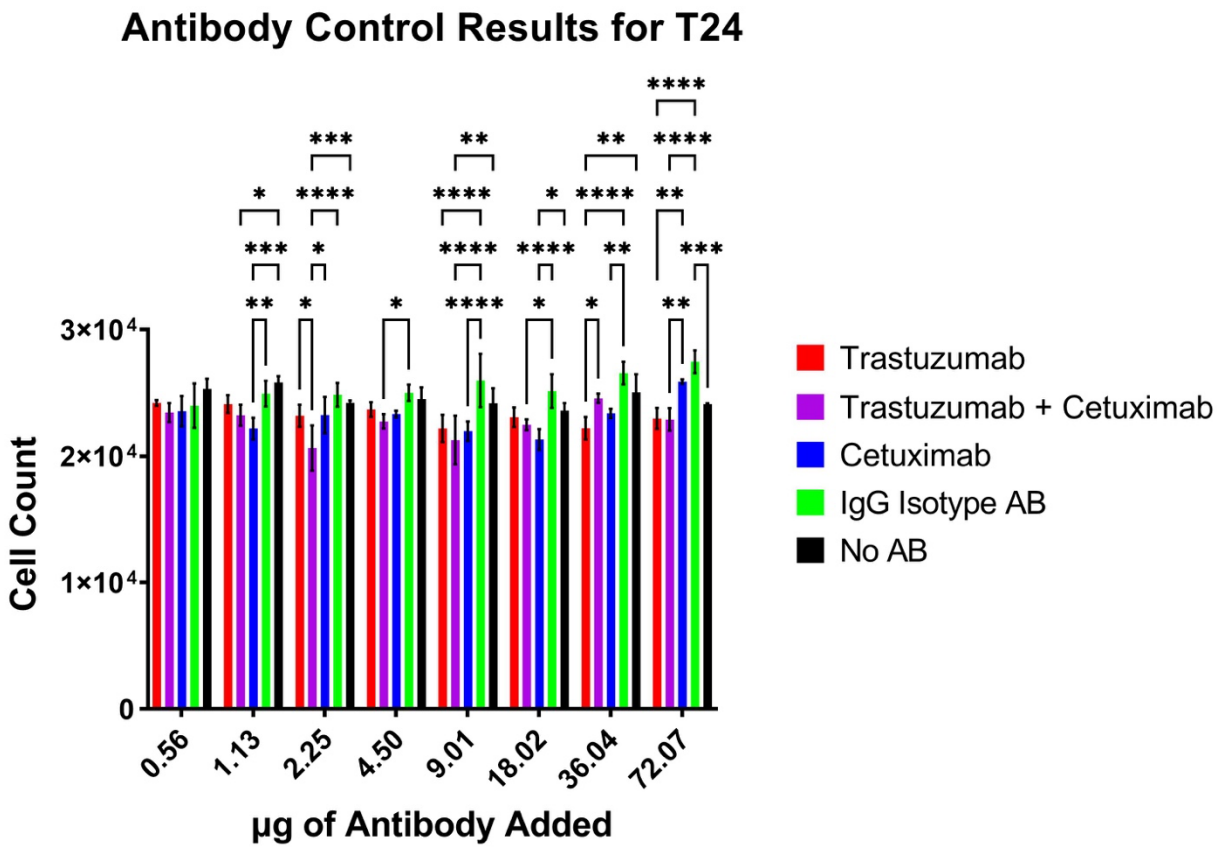

Figure 3.5.12. T24 cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002, ****: <0.0001).

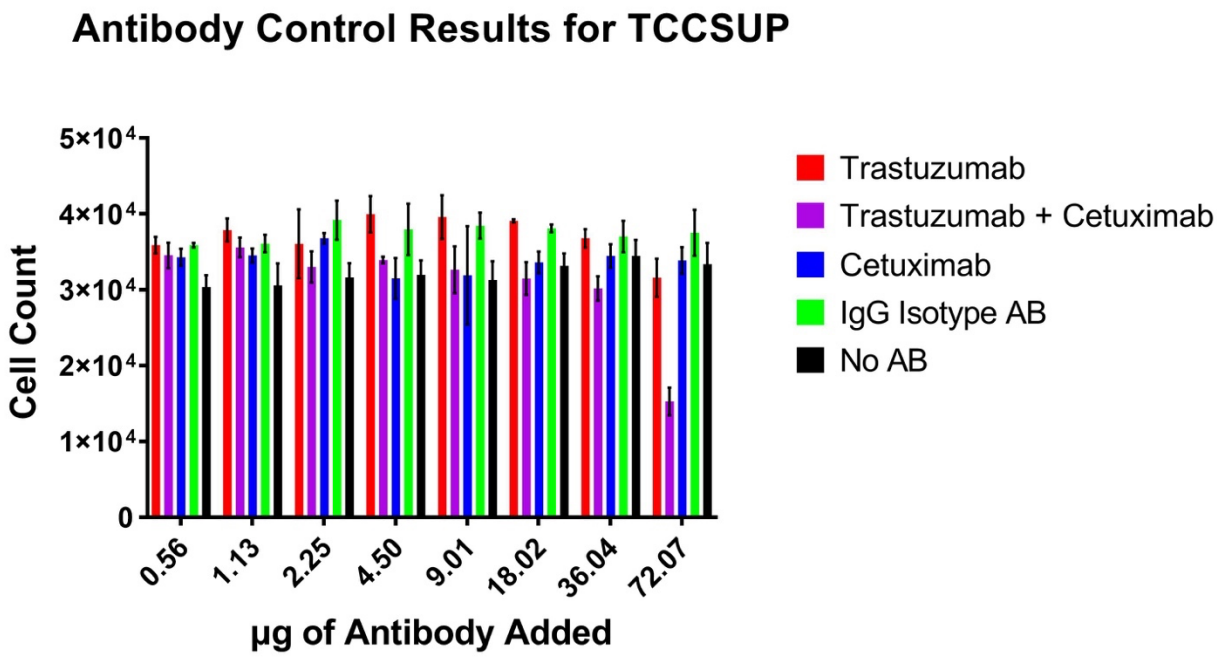

Figure 3.5.13 TCCSUP cell line with antibody treatments.

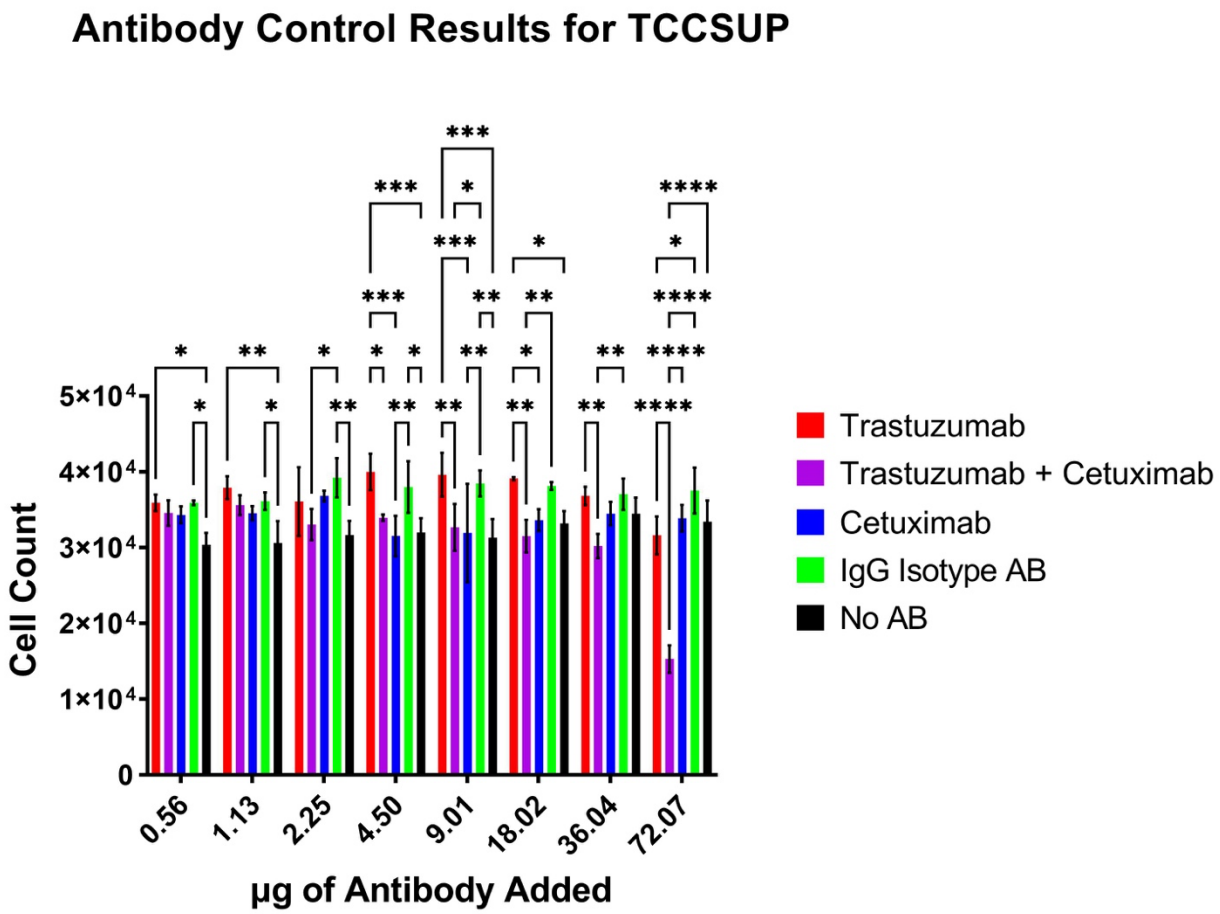

Figure 3.5.14. TCCSUP cell line with antibody treatments with statistical analysis (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002, ****: <0.0001).

3.3.2. Killing assays with Pb-214

3.3.2.1. Overnight Incubation Analysis

In this experiment the level of added radioactivity was different for the treatments when it should have been equivalent. Also, the specific activities of the radiolabeled antibodies were different. This was another variable that was not controlled in these preliminary experiments. Due to limited Pb-214/Bi214 shipments, there was not sufficient time to optimize the radiolabeling method. Figures 3.6.1 to 3.6.3 present this analysis for chosen cell lines.

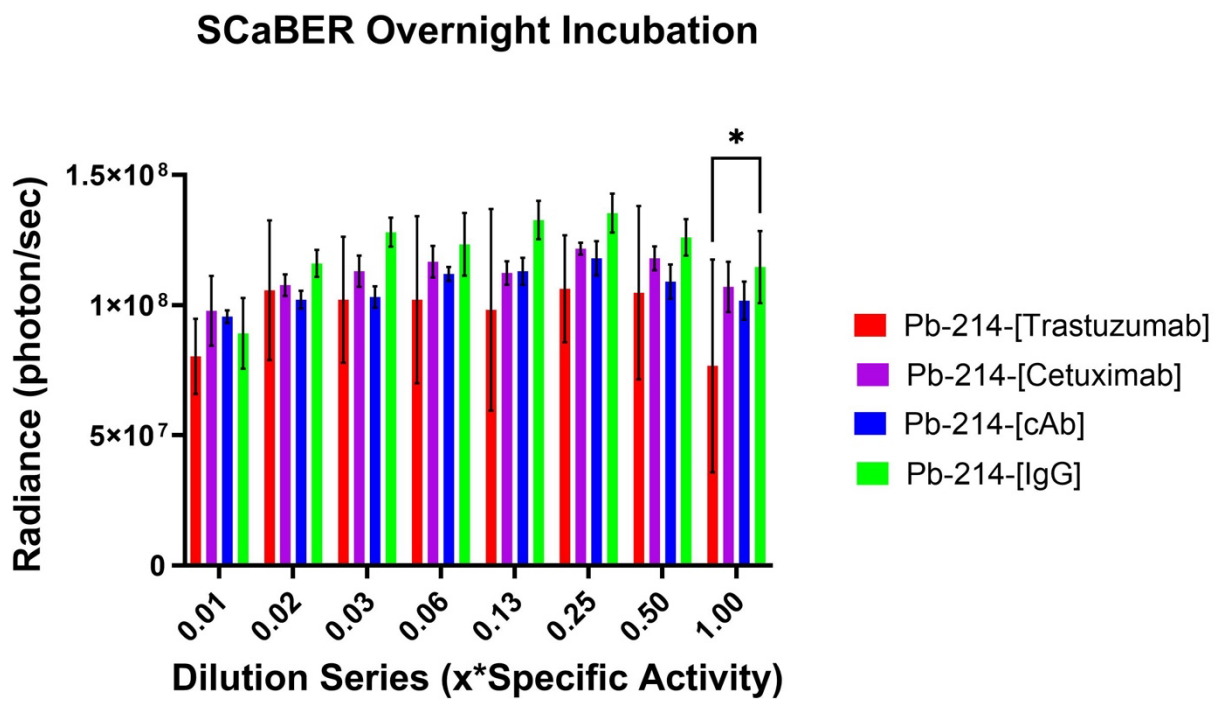

Figure 3.6.1. SCaBER cell line with Pb-214/Bi-214 treatment after overnight incubation. µCi/well at the 100% dose is as follows: Pb-214/Bi-214-[Trastuzumab]=9 /well, Pb-214/Bi-214-[Cetuximab]=6 µCi/well, Pb-214-[cAB]=7.5 µCi/well, Pb-214/Bi-214-[IgG]=6 µCi/well. (Asterisks Guide: *: <0.03).

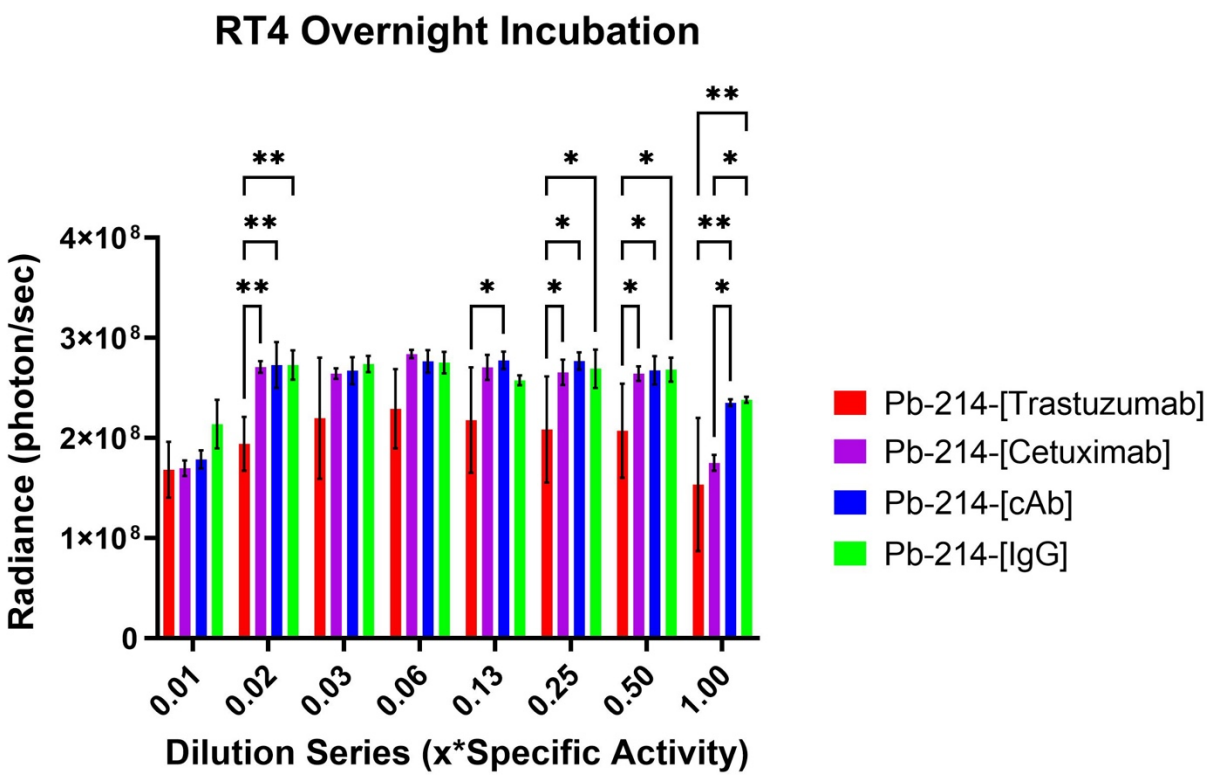

Figure 3.6.2. RT4 cell line with Pb-214/Bi-214 treatment after overnight incubation. μCi/well at the 100% dose is as follows: Pb-214/Bi-214-[Trastuzumab]=13 μCi/well, Pb-214/Bi-214-[Cetuximab]=18 μCi/well, Pb-214/Bi-214-[cAB]=15.5 μCi/well, Pb-214/Bi-214-[IgG]=15 μCi/well. (Asterisks Guide: *: <0.03, **: <0.002).

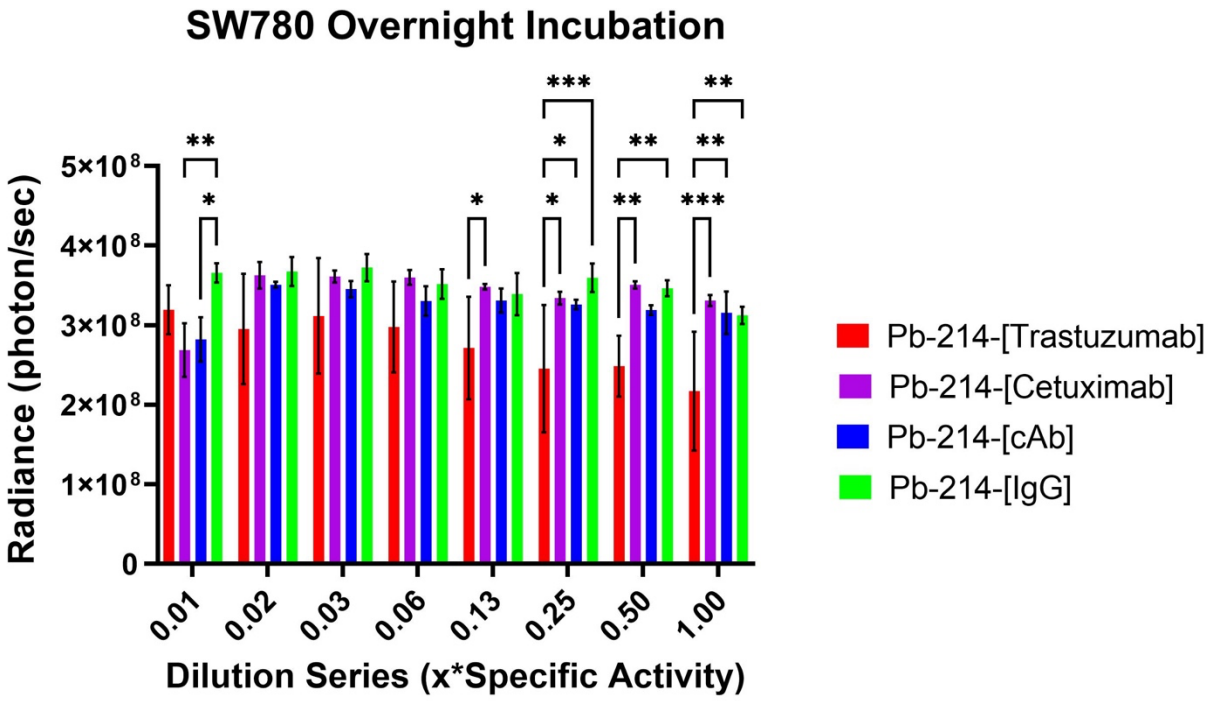

Figure 3.6.3. SW780 cell line with Pb-214/Bi-214 treatment after overnight incubation. µCi/well at the 100% dose is as follows: Pb-214/Bi-214-[Trastuzumab]=17µCi/well, Pb-214/Bi-214-[Cetuximab]=15µCi/well, Pb-214/Bi-214-[cAB]=16µCi/well, Pb-214/Bi-214-[IgG]=13µCi/well. (Asterisks Guide: *: <0.03, **: <0.002, ***: <0.0002).

3.3.2.2. *72-hours Incubation Analysis*

Two cell lines (SCaBER and SW780) were treated with either no treatment, combination antibody, or with IgG isotype AB, with no dilution. The treatment groups were compared using baseline correction on GraphPad and further analyzed by One-Way ANOVA for statistical significance, and results are graphed for radiance which previously proved to be in linear relationship with cell count. Figures 3.7.1 and 3.7.2 present the comparisons for each cell line.

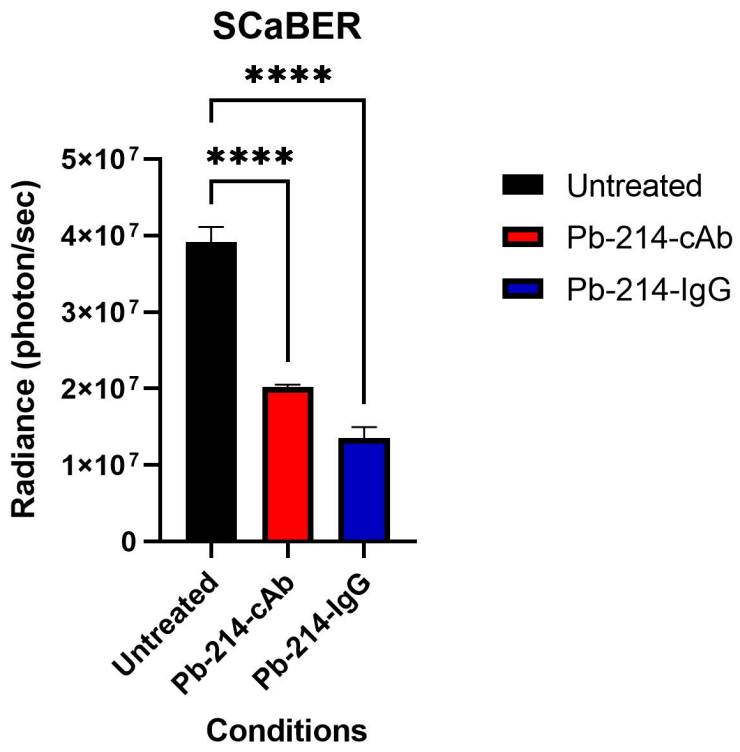

Figure 3.7.1. SCaBER cell line with Pb-214/Bi-214treatments at 72 hours post-incubation (Asterisks Guide: ****: <0.0001).

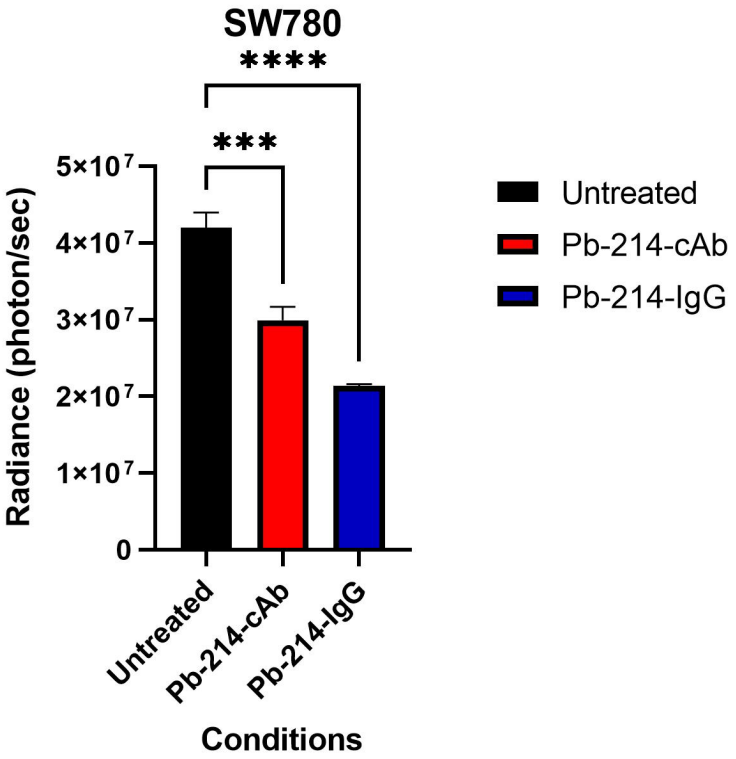

Figure 3.7.2. SW780 cell line with Pb-214/Bi-214 treatments at 72 hours post-incubation (Asterisks Guide: ***: <0.001, ****: <0.0001).

4. DISCUSSION

4.1. Radiolabeling antibodies and quantification of receptors

4.1.1. Radiolabeling with Tc-99m

Cetuximab and Trastuzumab are FDA-approved therapeutic antibodies and were utilized for binding studies to determine the levels of EGFR1 and EGFR2, respectively, in various bladder cancer cell lines. The binding of each antibody to its target receptor is known to block signaling pathways and thus reduce cancer growth. This aspect was also evaluated and will be discussed in a later section. The antibodies were radiolabeled with Tc-99m to measure specific binding to bladder cancer cells, determination the binding affinities as well as the EGRF1 and EGFR2 receptor numbers per cell. Prior to using the Tc-99m-labeled antibodies for binding experiments it was necessary to establish that the Tc-99m was tightly bound to each antibody. This was accomplished by iTLC, where the % of Tc-99m bound to antibody was determined. This was calculated as follows: (Tc-99m-labeled antibody) divided by the sum of [Tc-99m-labeled antibody + free Tc-99m (not attached to antibody)] x 100%. Above 95% was accepted to be pure enough for experimentation, and less than 95% was rejected. In over 30 experiments where antibodies were radiolabeled with Tc-99m, all results were with iTLC were greater than 95%. For the 20 different days that the antibodies were radiolabeled with Tc-99m for experiments conducted for this thesis, 99.4$\pm$0.6% and 99.5$\pm$0.3% were the mean$\pm$SD values for the % Tc-99m bound to Trastuzumab and Cetuximab, respectively. These results showed the low variability between the different days, suggesting that the radiolabeling was efficient and reproducible.

4.1.2 Radiolabeling with Pb-214/Bi-214

As discussed above Cetuximab and Trastuzumab are FDA-approved therapeutic antibodies and were utilized for binding studies to determine the levels of EGFR1 and EGFR2, respectively, in various bladder cancer cell lines. IgG was used as a control antibody, where previous data showed no binding to EGFR1 and EGFR2, and this control allowed measurement of the effect of radiolabeled antibody that was known to target cancer receptors versus radioactive material that weren't targeted to cancer receptors. The binding of each antibody to its target receptor is known to block signaling pathways and thus reduce cancer growth. This aspect was also evaluated and will be discussed in a later section. The antibodies were radiolabeled with Pb-214/Bi-214 to determine if the targeted radiation delivery to the bladder cancer cells would kill them, and to

determine if the effect was dependent on the level of radioactivity added. Prior to using the Pb-214/Bi-214-labeled antibodies for binding experiments it was necessary to establish that the Pb-214/Bi-214 was tightly bound to each antibody. This was accomplished by iTLC, where the % of Pb-214/Bi-214 bound to antibody was determined. This was calculated as follows: (Pb-214/Bi-214-labeled antibody) divided by the sum of [Pb-214/Bi-214-labeled antibody + free Pb-214/Bi-214 (not attached to antibody)] x 100%. Above 85% was accepted to be pure enough for experimentation, and less than 85% was rejected. The difference between purity values for Tc-99m and Pb-214 was due to Pb-214 being a novel radioactive material and still at the working phases for perfecting the conjugation. For the 2 different days that the antibodies were radiolabeled with Pb-214/Bi-214 for experiments conducted for this thesis, 94.4$\pm$4%, 94.1$\pm$4%, and 91.7$\pm$6% were the mean$\pm$SD values for the % Pb-214/Bi-214 bound to Trastuzumab, Cetuximab and IgG, respectively. These results showed low variability between the different days, suggesting that the radiolabeling was efficient and reproducible.

4.2. Expected Kd (Binding Affinity) vs Experimental Kd

The dissociation constant (Kd) represents the inverse of the affinity of the receptor for the ligand when at equilibrium. Kd is the ratio of antibody dissociation rate to antibody association rate, meaning the smaller the Kd, less antibody is required for the binding to be complete. The two antibodies used in this study had Kd values previously determined. Trastuzumab-anns (Kanjinti) was reported as 5.6 nM [56] while Cetuximab was reported as 0.38 nM [57]. The Kd values can be determined with various types of assays, one common being ELISA. For determination of Kd, environmental conditions are of utmost importance, experiments are typically done on ice and with a set period for each step. In our case however, we completed specific binding assays at 37 C^o to match how the treatment would be done in the clinical trial. Our protocol did allow us to determine the Kd and Bmax values quantitatively for each cell line. For all cell lines, the Kd (mean$\pm$SD) for Tc-99m-cetuximab binding to EGFR1 was 0.82 + 0.39 nM. Similarly, the Kd (mean$\pm$SD) for Tc-99m-trastuzumab binding to EGFR2 for all cell lines was 0.13+0.08 nM. Our data show high affinity, specific binding that was comparable to the literature values, with differences for the reasons noted.

4.3. Comparison of receptors to previous literature

Autenrieth et al. showed in their previous study with multiple bladder cancer cell lines that there were variable numbers of EGFR1 and EGFR2 as determined by flow cytometry analyses[55]. Their results were a guide for us to choose the cell lines with different levels of EGFR1 and EGFR2. Flow cytometry is an assay that allows detection of gated cell populations. While flow cytometry results can provide information on receptor binding characteristics, they are semi-quantitative at best and do not enable Kd determination or receptors per cell. Our goal was a more accurate and reliable quantitative analyses of receptor numbers. To accomplish, we opted for specific binding experiments with 8 concentrations of radiolabeled antibody and analyzed the data with GraphPad Prism to determine radiolabeled antibody saturation binding and that number was used to calculate the number of receptors per cell, for each cell line. Autenrieth et al. studies didn't include blocked conditions or different antibody concentrations in their protocol[55]. Our studies allowed us to determine non-specific binding and to find specific binding values by subtracting non-specific binding results from total binding results, as well as to determine the saturation binding. When our results are compared with Autenrieth et al. (Figure 3.4.1. and Figure 3.4.2.), there was generally good agreement, but certain differences were also demonstrated due to differences in assays [55]. The blocking condition of our assay resulted in more quantitative results for specific binding and receptor numbers that were calculated. For EGFR1 levels in the tested cell lines, our data were in a different order from highest to lowest when compared with results from Autenrieth et al. [55]. For 6 cell lines that were known to have expression of EGFR1, their cell line with the lower EGFR1 was RT4 while this cell line had the second highest levels of EGFR1 in our studies. Their two cell lines with highest EGFR1 levels were in the middle range for our results. For EGFR2 levels, in contrast, our data were in complete agreement with the Autenrieth et al. results [55]. While the differences between our study and Autenrieth with EGFR1 were significant, our results are expected to be more accurate and quantitative.

4.4. Choosing ATPlite for cell numbers in the assays

Cell numbers were needed for a quantitative and accurate determination of the EGFR1 and EGFR2 receptors per cell. Since experiments used 30,000 cells per well that were plated the night before, it was necessary to consider apoptosis or cell growth, as well as the effect of washing steps that might detach and remove cells during the assay. Multiple options were possible for cell counting.

Visual inspection was a method where human error was undeniable, but also it was a time-consuming option, considering the decay of radioactivity was also important during the visual inspection. Another trusted method was Bradford Protein assay, where the protein levels of the wells was related to cell numbers by a predetermined linear equation. While it did minimize the human error, it had the same disadvantage as visual inspection, it was time consuming.

ATPlite assay focused on ATP, which was a marker for cell viability due its presence in all metabolically active cells. It utilized the fact that ATP concentration declined rapidly when cells undergo necrosis or apoptosis, which makes monitoring for ATP a good indicator of cytocidal, cytostatic and proliferation effects. By adding D-Luciferin and luciferase to the wells, the ATP from the cells reacted and produced light, which then was quantified with the IVIS instrument. In our experiments, ATPlite assays were sensitive down to 500 cells in 100 μL, and it allowed for precise quantitative analyses of cell numbers. It also was accomplished in less time, since it was only 30 minutes to prepare and run the assay, and the instrument was also tolerant of the radioactivity, resulting in our preference for this protocol over the other methods that were discussed.

4.5. Using both Kanjinti and Erbitux for targeting EGFR1 and EGFR2

Most non-muscle invasive bladder cancers are heterogenous at the early stages where our proposed bladder cancer treatment with Pb-214-labeled antibodies would be used. EGFR1 and EGFR2 have been shown to be overexpressed in early stages and with one, or both receptors overexpressed in 85% of early bladder cancers. In the first part of this study, we were able to find receptor numbers for various bladder cancer cell lines that had different levels of EGFR1 and EGFR2. Having a quantitative result for the levels was important to determine if there was a difference in the treatment response for the Pb-214 labeled antibody treatment, which would later help with personalized treatment options.

4.6. Effect of Unlabeled Antibodies & Killing with Radiolabeled Antibodies

Unlabeled antibody control assays were undertaken to help determine if treatments with Pb-214/Bi-214 labeled antibody were from the radioactivity or could also be due to the biological effects of the antibodies, as the antibodies blocked signaling pathways important to cancer growth. Our goal was to determine if significant effects would be observed or not. In general, addition of increasing concentrations of cetuximab, trastuzumab, or both together resulted in 5-20% reduction

in cell numbers after 24 hours. While these modest effects were often statistically significant, especially at higher trastuzumab/cetuximab concentrations, the results were not highly impressive. Considering the tightness of data due to our treatment model, the overall effects were modest. What can be summarized for certain is that the additions of cetuximab, trastuzumab, or the combination did not increase the growth of bladder cancer cells rather, the antibodies had a slight negative effect on growth. Increased growth of cells due to IgG control antibody treatment (versus no addition) was an unexpected result. This unexpected finding requires further experimentation to understand the mechanism.

Pb-214/Bi-214 labeled antibodies were also utilized for overnight killing assays. Data was graphed to specify the dilution series, as 100% dose for antibodies in each treatment and experiment differed due to different doses of Pb-214/Bi-214 targeted delivery (2.5.2.2.8.). Our results showed statistically significant killing when Pb-214/Bi-214 was conjugated to different antibodies, but due to low delivery doses of Pb-214/Bi-214, a redo of these experiments with all cell lines will be beneficial and should be considered by future researchers. For our purposes, delivery of higher and pure doses of Pb-214/Bi-214 from Niowave didn't have a clear timeline, which resulted in our further analysis of killing which is discussed in 4.7.

4.7. Further Killing Analysis where cell count was measured 72 hours post-incubation

Additional experiments were conducted based on initial results. Triplicates of 3 treatment conditions were plated at 25% cellular confluency per well. For treatment conditions Pb-214/Bi-214-[cAb] and Pb-214/Bi-214-[IgG]), all of the dose was distributed to the wells at 100% without dilutions. The cells were then incubated for 72 hours and ATPlite analysis was completed to measure radiance (photons/sec), which had a linear relationship with cell count. Treatment triplicates were then compared to their control triplicates, where no treatment was given. Results showed significant killing for Pb-214/Bi-214-[cAb].

One point of significance was the effect of Pb-214/Bi-214-[IgG] vs Pb-214/Bi-214-[cAb] for killing cells. The expected outcome would be to have similar killing or better killing with Pb-214/Bi-214-[cAb] treatment due to binding to specific receptors compared to no specific binding as IgG is used as a control isotype Ab. The unexpected results of IgG condition having a better killing outcome compared to cAb conditions was likely due to the difference in specific activity. The treatment of Pb-214/Bi-214-[IgG] was 68 μCi (specific activity=5 μCi/mg) for the treatment

dose, while Pb-214-[cAb] was only 38 μCi (specific activity=1.8 μCi/μg) for treatment dose. This significant difference in the radiolabeling ratio is most likely due to the availability of the purified Pb-214 dose, as experimental steps are the same for both dose preparation as discussed in 2.5.2.3.1 to 2.5.2.3.8. This should further be evaluated in the future.

A second point of significance was the killing from the Pb-214/Bi-214-[Ab] condition vs no treatment condition. As shown in Figures 3.5.1 to 3.5.14, treatment with only antibodies didn't show any conclusive killing compared to no treatment conditions, and any statistical significance that were shown was discussed in 4.6. as not significant. In 72 hours post-incubation model, we showed not only statistically significant killing but also proof that once formulation of Pb-214/Bi-214-labeled targeting antibodies are perfected, it is likely to be highly effective for treatment of bladder cancer.

Future experimentation should also consider cellular changes such as apoptotic changes in DNA and cell membranes.

4.8. Short-lived Pb-214 vs longer-lived Pb-212 for bladder cancer therapy

FDA-approved radiopharmaceuticals for cancer therapy provide their radiation doses primarily by β– decay. The shorter path length and higher linear electron transfer rates of a-particles in clinical applications have yet to be fully explored, but initial results as discussed by Autenrieth et al. are encouraging. As Pb-212 was not easily accessible (available at MSU in 2023), Pb-214 was an attractive alternative and the shorter half-life (26.8 mins vs 10.6 hours) allows for the potential of fractionated dosing to improve anti-tumor immune responses [58]. With the Pb-214 generator of the correct size, one can treat a different patient every hour because the generator can be eluted every hour if needed. Due to the 27-minute half-life of Pb-214, the Pb-214 in the generator accumulates to 75% of maximum by 2 half-lives later, or in approximately 1 hour. This type of efficiency (Pb-214/Bi-214 available each hour) for patient studies is not possible with other radioisotopes proposed for a particle therapy. Short half-life is also important when considering the addition of Pb-214 labeled antibodies to the standard of care (BCG) that is directly instilled in the bladder. In just two half-lives (2 x 27 minutes), 75% of the dose instilled in the patients' bladder will decay to provide the radiation dose, in three half-lives this value goes up to 87.5% of the dose. To minimize the loss of the instillation by excretion from the bladder, shorter half-life would be

of utmost importance for the comfort of the patient. For Pb-214/Bi-214, 3 half-lives would be ~1.5 hours while for Pb-212, 3 half-lives would be 30 hours and thus not feasible for the patient.

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