A STUDY OF THE HUMANIZED MONOCLONAL ANTIBODY CT109 IN COMBINATION WITH FOLFIRI AND BORTEZOMIB AS A TREATMENT FOR COLORECTAL CANCER

by

Kelly Arias Cardenas

A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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A Study of the Humanized Monoclonal Antibody CT109 in Combination with FOLFIRI and Bortezomib as a Treatment for Colorectal Cancer

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Kelly Arias Cardenas
Bachelor of Science
George Mason University, 2015

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Fall Semester 2020
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to my loving family.

ACKNOWLEDGEMENTS

I would like to thank the many friends, relatives, and supporters who have made this happen. Special thanks to the team over at the Prince William County Science Accelerator.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS AND SYMBOLS

5-FU 5-Fluorouracil
ADCC ..... Antibody-Dependent Cellular Cytotoxicity
AKT1 ..... Protein Kinase B
Bcl2/Bcl-xl ..... B-cell Lymphoma 2/B-cell Lymphom-extra large
BTZ ..... Bortezomib
CEACAM5 ..... Carcinoembryonic antigen-related cell adhesion molecule 5
CEACAM6 ..... Carcinoembryonic antigen-related cell adhesion molecule 6
CDKN1A ..... Tumor suppressor 21
CTLA4 ..... Cytotoxic T-lymphocyte-associated protein 4
CT109 ..... Caerus Therapeutics 109
DNA ..... Deoxyribonucleic acid
dUMP ..... Deoxyuridine-5'-O-monophosphate
dTMP ..... Deoxythymidylate
FdUMP ..... Fluorodeoxyuridylate
FUTP ..... Fluouracil Triphosphate
H109 ..... Humanized 109 (CT109)
HIgG ..... Human immunoglobulin G
IKK ..... IKB Kinase
LAG-3 ..... Lymphocyte-activation gene 3
LV ..... Leucovorin
MMP2 ..... Matrix Metalloprotease 2
MoAbs ..... Mooclonal Antibodies
NFAT ..... Nuclear Factor of Activated T cell
NF-KB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells
NK ..... Natural Killer
PARP ..... Poly (ADP-ribose) polymerase
PBS ..... Phosphate Buffered Saline
PD1 ..... Programmed cell death protein 1
PDL1 ..... Programmed death-ligand 1
Pi ..... Π
TIGIT ..... T cell Ig and ITIM domain
TIM-3 ..... T-cell immunoglobulin and mucin-domain containing-3
TS ..... Thymidylate synthase
VEGF ..... Vascular endothelial growth factor
VISTA ..... V-domain Immunoglobulin suppressor of T cell activation

ABSTRACT

A STUDY OF THE HUMANIZED MONOCLONAL ANTIBODY CT109 IN COMBINATION WITH FOLFIRI AND BORTEZOMIB AS A TREATMENT FOR COLORECTAL CANCER

Kelly Arias Cardenas, M.S.

George Mason University, 2020

Thesis Director: Dr. Alessandra Luchini

Colorectal cancer ranks as the 2nd leading cause of cancer death in the United States. New drugs are urgently needed to increase the survival of patients with colorectal cancer. FOLFIRI is the most commonly used chemotherapy treatment in colorectal cancer, yet most patients develop side effects that severely affect quality of life. Bortezomib, an anti-cancer drug approved for multiple myeloma and lymphoma has been reported as an effective treatment in colorectal cancer that enhances apoptosis and attenuates tumor growth. Stromatis Pharma has developed a humanized monoclonal antibody, CT109, against CEACAM6 (new checkpoint inhibitor) that has demonstrated its promising antitumor activity against pancreatic and colorectal cancer in preclinical models. Monoclonal antibodies (moAbs) can be used in combination with drugs to treat cancer as adjunct therapy. MoAbs minimize treatment toxicity and are more effective at shrinking

tumors, inhibiting tumor growth, prolonging life, and/or improving quality of life. The purpose of this study was to evaluate the functional capabilities of CT109 either alone or in combination with chemotherapy treatments. We used antibody-dependent cellular cytotoxicity (ADCC) assays, immunoblotting and cell viability assays as an in-vitro assessment of the CT109 efficacy in combination with chemotherapy drugs. Results show that the combination of CT109 with Bortezomib enhanced poly ADP ribose polymerase (PARP) cleavage and increased apoptosis in HT29 cells. Further, the synergistic inhibition and/or reduction of tumor growth, in combination with FOLFIRI, were observed *in-vivo*, in a colorectal adenocarcinoma tumor xenograft model in nu/nu mice. We found that the humanized monoclonal antibody CT109 enhanced the anti-cancer effect of both treatments. These data support the use of a novel monoclonal antibody, CT109 as an adjunct therapy to treat this deadly malignancy.

CHAPTER ONE

Colorectal cancer and pancreatic cancer rank the 2nd and 3rd leading cause of cancer related deaths in the United States respectively. New drugs are urgently needed for those two cancer types. Incidence rate of colorectal cancer has been escalating over time. The 'rise' of colorectal cancer in developed countries can be attributed to the increasingly aging population, unfavorable modern dietary habits and an increase in risk factors such as smoking, low physical exercise and obesity(*1*). A key player in colorectal cancer is AKT1, also known as protein kinase B. AKT1, serine/threonine kinase, inhibits apoptosis and induces cell migration, adhesion and invasion by activating MMP2 (matrix metalloprotease 2) as seen in Figure 1. AKT1 also induces cell proliferation by inhibiting p-53 (tumor suppressor 53).

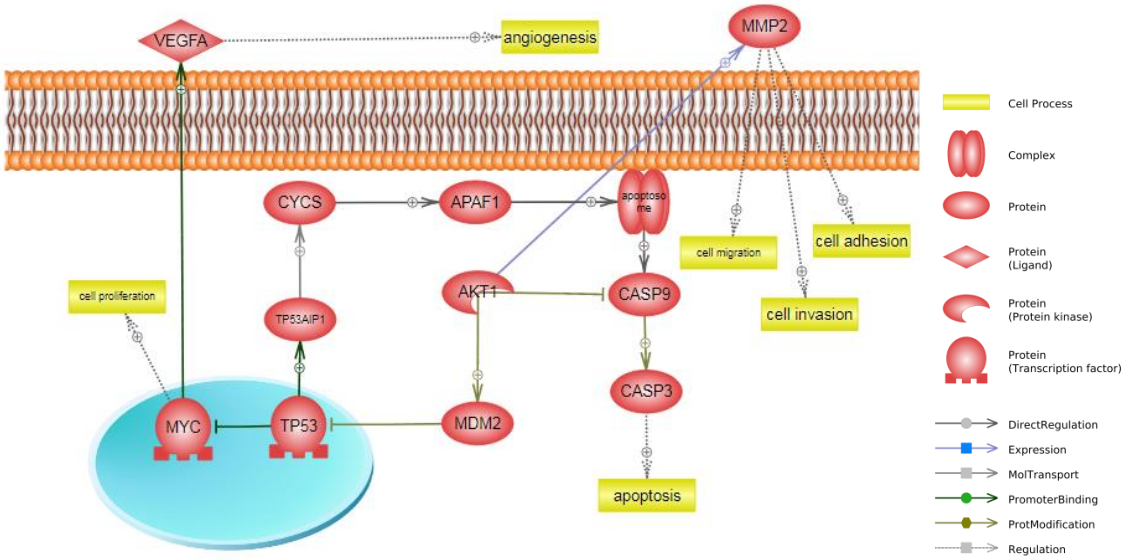


Figure 1: Colorectal Cancer Pathways of Interest.

The current standard-of-care chemotherapy for colorectal cancer is the combination regimen FOLFIRI, however additional targeted therapy may be recommended to those patients that would benefit from it. Target therapy targets the cancer's specific proteins, genes and tissue environment that helps it grow and spread. FOLFIRI refers to the combined treatment of 5-Fluoruracil (5-FU), Irinotecan and folinic acid. 5-FU inhibits thymidylate synthase and by doing so exerts an anti-proliferative effect. 5-FU is phosphorylated to FdUMP (deoxyuridine monophosphate). FdUMP then inhibits an important enzyme for nucleotide synthesis called thymidylate synthase. Thymidylate catalyzes the conversion of dUMP to dTMP (deoxythymidine monophosphate) which is required for DNA synthesis. Depletion of dTMP leads to DNA damage and ultimately apoptosis. 5-FU also gets converted to FUTP (fluorouridine triphosphate) which incorporates into RNA causing chain termination (*2*). Irinotecan, a

derivative of camptothothecin, inhibits topoisomerase I causing DNA breakage and eventually cell death. SN-38 produced in the body, starting at the liver by carboxylesterase, is the active metabolite of irinotecan. SN-38 appears to reversibly stabilize the topoisomerase I cleavable complex, resulting in single-strand DNA breaks and inhibition of DNA relegation. DNA synthesis is thus blocked in the presence of topoisomerase I inhibitors, leading to irreversible inhibition of DNA synthesis with double-strand DNA breaks. These events induce arrest of the cell cycle in the S-G2 phase and ultimately cause cell death (*3*). Lastly, folinic acid, also known as leucovorin calcium, although not a chemotherapeutic agent it is used to lessen the toxic side effects of 5-FU in healthy cells. Leucovorin Calcium also helps increase cytotoxicity by supplying tissues with reduced folate. In the absence of folate cofactors FdUMP is only weakly bonded to TS, the addition of LV increases the level of reduced folate (CH2THF) which enables binding and the formation of a stable tertiary complex(*4*). FOLFIRI relies on its ability to induce p53-dependent cell growth arrest and apoptosis (Figure 2B) however, about half of all colorectal cancer patients have p53 mutations and the underlying mechanism is poorly understood (*5*). Additionally, FOLFIRI treatment results in toxic side effects such as loss of appetite, difficulty swallowing, severe diarrhea, nausea and vomiting. Besides causing morbidity and occasional mortality, the side effects of systemic therapy have a major impact on the quality of life of the patients. The administration of fluoropyrimidines and irinotecan is frequently accompanied by gastrointestinal toxicity (*2*). Adjunct antibody treatments can potentially be more

effective due to their reduced side effects when compared to higher doses of chemotherapeutic drugs alone.

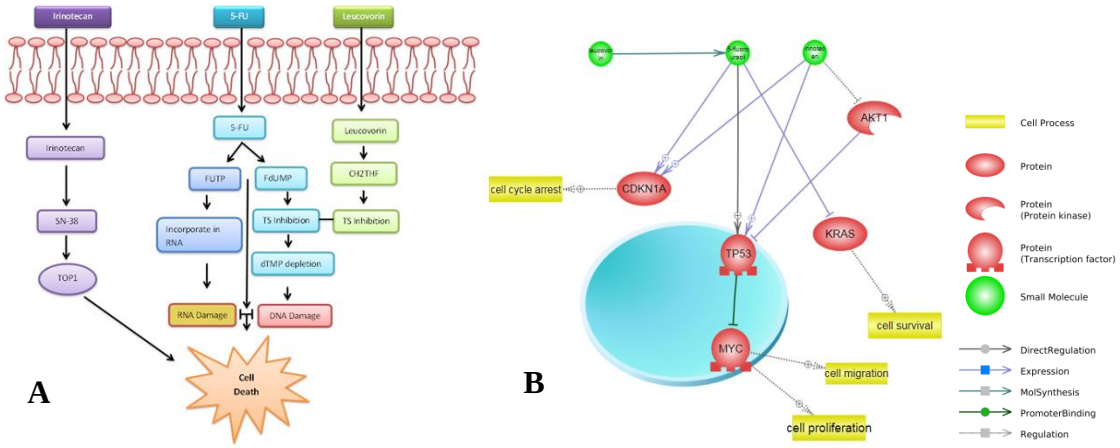


Figure 2: FOLFIRI. 2A: Mechanism of Action. 2B: FOLFIRI Pathway.

Despite advances in surgical and medical therapies, cure rates and long-term survival in colorectal cancer patients have changed little in the past several decades (*1*). In recent years, inhibitors of immune checkpoints such as antibodies against CTLA4, PD1, and PD-L1 demonstrated great clinical successes in a number of tumor types. However, the effectiveness of those immunotherapies has been limited in colorectal and pancreatic cancer. In colorectal cancer, immunotherapy is only effective in patients with microsatellite instability (MSI) which accounts for 15% of the patients. In addition to CTLA4/B7 and PD1/PD-L1, there are several novel checkpoint pathways that have been identified, including VISTA, LAG-3, TIGIT, TIM-3, and CEACAM6 (CD66c). These newly discovered checkpoint proteins are emerging as the next generation immunotherapy targets that hold a huge promise in producing more potent and broadly

applicable therapeutics. Several inhibitors of these new checkpoint proteins are currently being tested in the clinic. Stromatis Pharma has developed a humanized monoclonal antibody, CT109, against CEACAM6 (new checkpoint inhibitor) and has demonstrated its promising antitumor activity against pancreatic and colorectal cancer in preclinical models. In a previous *in-vivo* study it was shown that CT109, at 2.5mg/kg given twice per week in a LS-174T xenograft model, attenuated tumor growth (Figure 3).

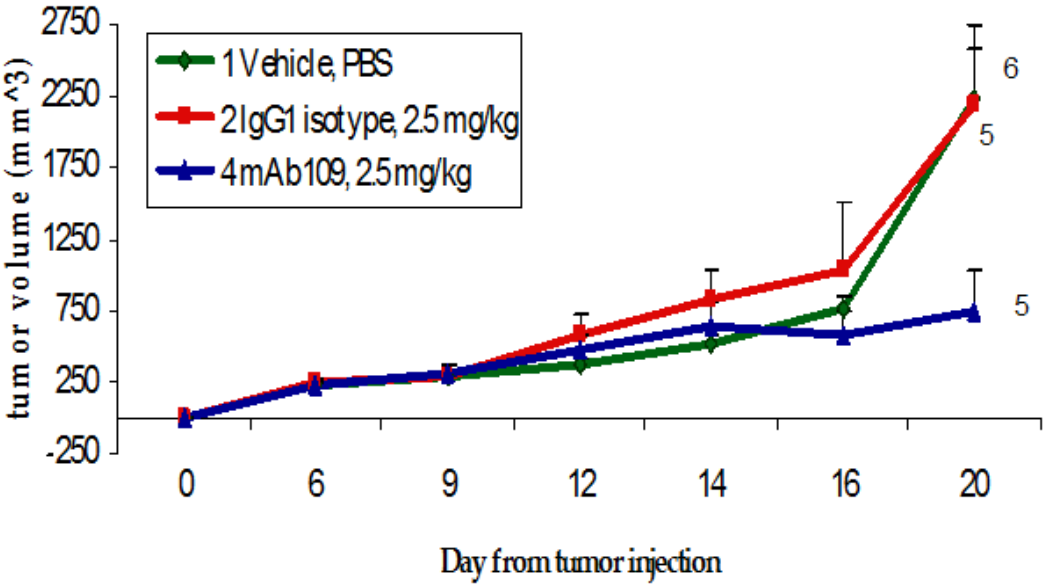


Figure 3: LS-174T Xenograft. Vehicle (PBS), Isotype control at 2.5mg/kg and CT109 at 2.5mg/kg were all given via IP (intraperitoneal) injections twice a week.

Monoclonal antibodies (moAbs), such as CT109, can be used in combination with drugs to treat cancer as adjunct therapy. MoAbs minimize treatment toxicity and are more effective at shrinking tumors, inhibiting tumor growth, prolonging life, and/or improving quality of life. CT109 attaches to the CEACAM6 antigen on cancer cells and by doing so

disrupting signal transduction (Figure 4). Increasing evidence indicates that CEACAM6 plays an important role in gastrointestinal cancer progression, and CEACAM6 is more widely distributed than CEA (CEACAM5) in normal tissues, with significant expression in many epithelia (*6*). CEACAM6 is significantly overexpressed in colorectal cancer tissues and is closely associated with poor prognosis, suggesting CEACAM6 may be used as a potential therapeutic target to control malignancy and metastasis of colorectal cancer (*6*). CEACAM6 induces tumor cell migration, invasion and adhesion, and formation of distant metastases (*7*) (Figure 4A). CT109 inhibits CEACAM6 regulation of oncogenic signaling pathway within the cancer cell to promote tumor progression (Figure 4B). Additionally, CEACAM6 is a novel immune checkpoint protein. During tumorigenesis, tumor cells develop mechanisms to evade the host immunity. CEACAM6 on a cancer cell binds to CEACAM1 on a T cell to block the function of the T cell (Figure 4C). Checkpoint proteins are emerging as the next generation immunotherapy targets that hold great promise in producing more potent and broadly applicable therapeutics. CT109 has a dual effect on the tumor itself and the immune system by enhancing its response. Some inhibitors of those checkpoint proteins are currently being tested in the clinic. CT109 has the potential to be used as a novel immunotherapeutic agent for the treatment of colorectal cancer.

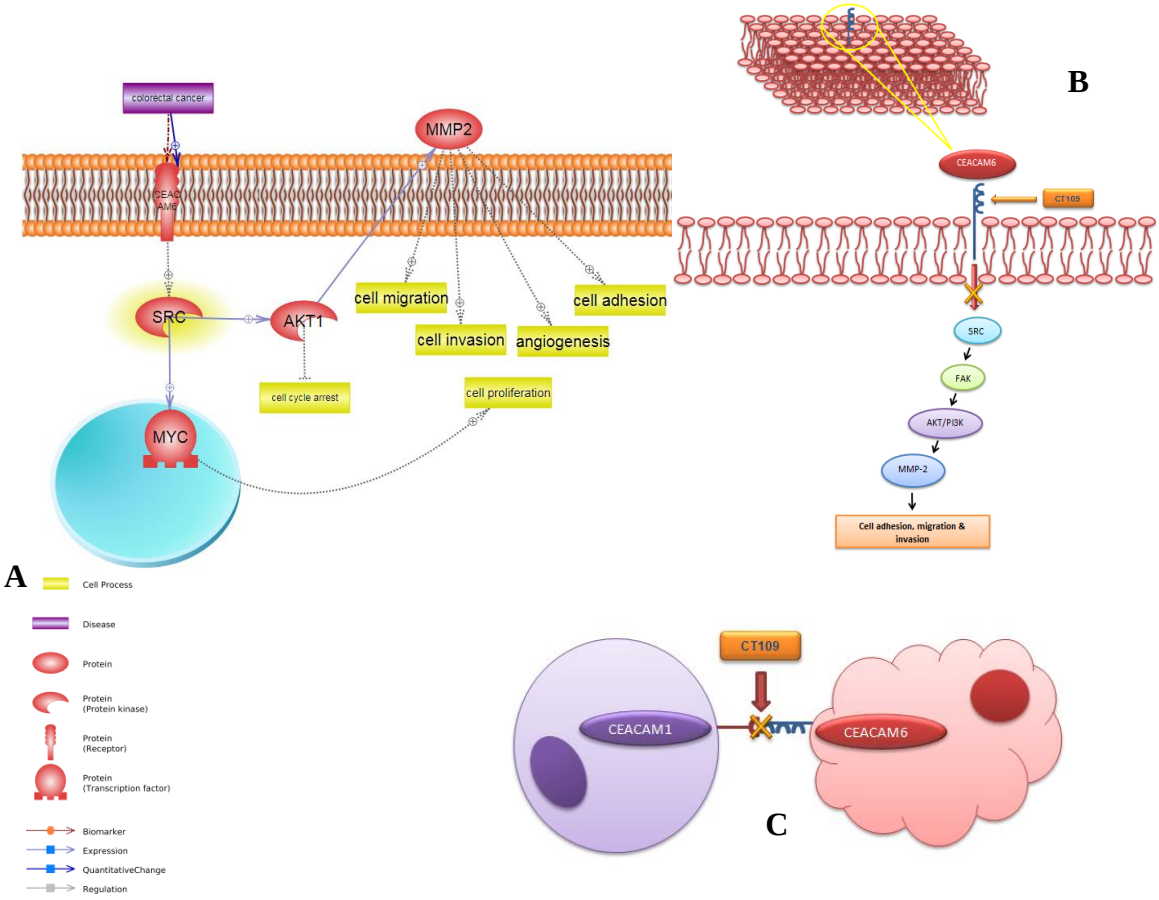


Figure 4: CT109 TARGET. A: CEACAM6 Pathway. B: CT109 disrupts tumor cell autonomous regulation. C. CT109 inhibiting binding of CEACAM6 (cancer cell) to CEACAM1 (T-cell).

By using Stromatis' proprietary subtractive immunization technologies, CT109 is directed against malignant but not healthy epitopes. CT109 was shown to specifically target a unique cancer-specific epitope on CEACAM5/6. It also has a high binding affinity to CEACAM6. For this project, CT109 is being studied as a novel immunotherapeutic agent in combination with FOLFIRI and Bortezomib for the treatment of colorectal cancer. Bortezomib (Velcade/PS-341) is a first-in-class, reversible, proteasome inhibitor that has proven to be highly effective in some hematological malignancies. BTZ overcomes conventional chemoresistance, directly

induces cell cycle arrest and apoptosis, and also targets the tumor microenvironment (*8*). Bortezomib inhibits 26S proteasome that binds to and inhibits the 20S catalytic core. After stimulation of cells by cytokines and/or growth factors, IκB is phosphorylated by the IKK complex, leading to degradation of IκB by the 26S proteasome and this allows translocation of the NF-κB complex to the nucleus where it can activate transcription of a number of genes that can promote cancer progression (*9*). NF-κB is overexpressed in several tumors and regulates the expression of genes involved in apoptosis (including Bcl-2 and Bcl-xL), cell cycle progression (p21), inflammation, and angiogenesis (VEGF); through inhibition of NF-κB Bortezomib not only promotes apoptosis of cancer cells but also sensitizes these cells to chemotherapy, radiation, or most importantly immunotherapy (*10*). Several clinical trials targeting colorectal cancer using BTZ have been performed or are ongoing.

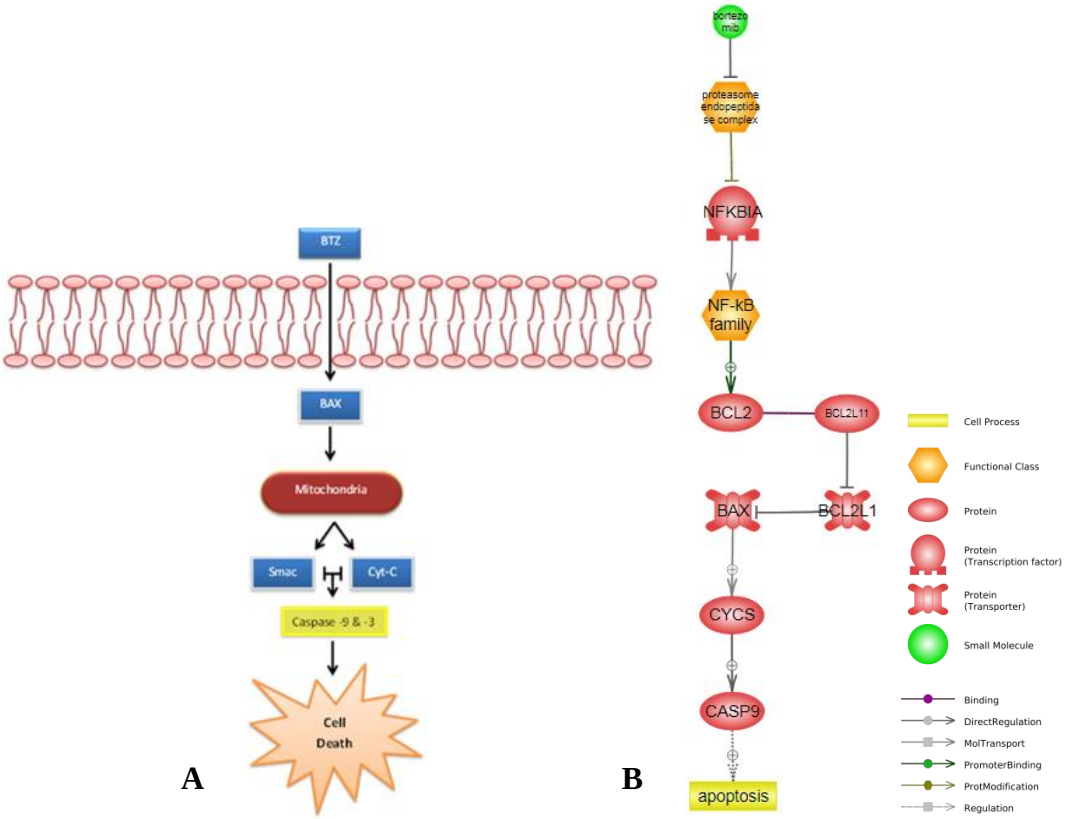


Figure 5: Bortezomib. 5A: Mechanism of Action. 5B. Pathway of Interest.

Targeting CEACAM6 is a viable approach for the treatment of patients with colorectal cancer. Pancreatic cell lines are included in this study for their CEACAM6 level of expression. Bx-PC3, a high CEACAM6 expressing pancreatic cancer cell line, will be used as a positive control and PANC-1, a non-expressing CEACAM6 pancreatic cancer cell line, will be used as the negative control. Targeted molecular therapy with CT109, a monoclonal antibody against CEACAM6, offers new treatment potentials for patients with colorectal cancer. Disruption of CEACAM6 oncogenic signaling pathway by CT109 in combination with chemotherapeutic agents will lead to a greater attenuation of cancer progression than chemotherapy alone.

Aim 1

Aim 1 part A is to demonstrate the antitumor activity of CT109 in ADCC assays with CEACAM6 expressing pancreatic and colorectal cancer cell lines (Bx-PC3, HT29 and LS-174T). Aim 1 part B will be testing the apoptotic effects of CT109, Bortezomib, and FOLFIRI in HT29 cells through Western Blotting and Cell Viability Assays.

Aim 2

Aim 2 part A is to validate the efficacy of CT109 seen in previous preclinical studies displaying potent activity in a colorectal cancer cell line. Aim 2 part B is to study the antitumor efficacy of CT109 in combination with the current standard-of-care (FOLFIRI) chemotherapy as well as Bortezomib in a nude mouse xenograft model for colorectal cancer. By combining CT109 and chemotherapy, antitumor activity will be increased in the xenograft model given that CT109 has already displayed potent antitumor activity in LS-174T cells by itself.

CHAPTER TWO

Methodological Approach- Specific Aim 1

Cell Culture

Four cancer cell lines, two pancreatic and two colorectal, were maintained in order to test the direct killing efficacy of CT109 alone, chemotherapy alone and combination therapy. All cell lines were obtained from American Type Culture Collection (ATCC). Cell lines were maintained in their respective ATCC recommended complete growth media.

Western Blot

Western blots were performed to compare the CEACAM6 expression level of cancer cell lines as well as the direct cell killing effects of BTZ, FOLFIRI and CT109. All Western blots performed for this project used iBlot™ Transfer Stack (nitrocellulose) regular size by Invitrogen™ accompanied with MOPS buffer. The membranes were blocked, and antibodies diluted in 5% nonfat dry milk in PBS (Phosphate-buffered saline) with 0.05% Tween-20. The optimal colorectal cell line for in-vivo studies will be determined by its level of CEACAM6 expression. All cell lines (Bx-PC3, PANC-1, LS-174T, and HT-29) were incubated in 6-well plates at concentration of 4x10^5 cells per well. Cells were plated on day 1 and then rested overnight for lysing and supernatant collection on day 2. RIPA buffer (Thermo Fisher Scientific) in combination with a

Protease Inhibitor Cocktail (Thermo Fisher Scientific) was used to lyse the cells. Commercially available CEACAM6 recombinant (Thermo Fisher Scientific) was used as a positive control. CT109 (1:1000) was used as the primary antibody and rabbit anti-human IgG HRP-linked (1:3000) was used as the secondary antibody. The Apoptosis Antibody Sampler Kit by Cell Signaling Technology was used for measuring apoptotic markers. The primary antibodies used were Cleaved caspase 3, Cleaved caspase 9, and Cleaved PARP (all rabbit) at a 1:1000 dilution; the secondary antibody was anti-rabbit IgG, HRP-linked at a 1:3000 dilution. The drug treatments concentrations were: 1.25ug/mL 5-FU, 80 ug/mL Irinotecan, 50uM Leucovorin, 5nM Bortezomib and CT109 at 2ug/mL and 10ug/mL. Lastly, the negative control Human IgG was at a concentration of 10ug/mL. GAPDH was used as the loading control.

Cell Viability Assay

To determine the direct cell killing effect of the chemotherapy drugs as well as CT109, a cell viability assay was performed. The commercially available Cell-Titer Glo kit, by Promega, was used to compare the cell death percentage of each treatment either alone or in combination with CT109. Tests were performed in triplicates. DTX 880 Multimode Detector was used for the luminescence detection.

ADCC Reporter Bioassay

To demonstrate the antitumor activity of CT109 in cancer cell lines an ADCC (Antibody Dependent Cell-mediated Cytotoxicity) assay was performed. The ADCC Reporter Bioassay Core Kit Protocol from Promega was used. The bioluminescent reporter assay was used to quantify the biological activity on pathway activation by the

humanized monoclonal antibody CT109 in an ADCC mechanism of action assay. The readout was measured in luminescence signal from NFAT (nuclear factor of activated T-cells) response element driving expression of firefly luciferase. The target cells expressing CEACAM6 antigen bind to CT109 which then binds to the FcγRIIIa receptor on the Jurkat effector cells. Readout occurs during activation of gene transcription through the NFAT pathway in the effector cells. Target cells were plated and incubated overnight during day 1. On day 2 the antibody serial dilutions and effector cells (1:6 target to effector cells) were added to the plate and incubated for overnight. Lastly, on day 3 the Bio-Glo Luciferase Assay Reagent was added and after 5-minute incubation, at room temperature, the luminescence was measured. Tests were performed in triplicates.

Methodological Approach- Specific Aim 2

Nude mouse xenograft model

To test CT109's ADCC activity and ability to block CEACAM6 signaling on tumor cells that may lead to the augmented antitumor activity of two chemotherapeutic treatments (FOLFIRI and Bortezomib) in colorectal cancer. The animal study was conducted using a cell line based nude mouse xenograft model. The HT-29 cell line was selected based on the ADCC assay results and CEACAM6 expression when compared to LS-174T. To generate xenograft tumors, using subcutaneous implantation, athymic nude mice were used. The 6-week-old, female mice (Foxn1nu/Foxn1+) were purchased from Envigo. Athymic nude mice lack T cells but still have plenty of B and NK cells that can mediate the ADCC activity and are therefore suitable for use in testing antibodies. Thirty-one female nude mice were injected with 5x10^6 cells on the right flank and tumor

volume was measured twice a week. Tumors were measured using a digital caliper for length (L), width (W) and height (H) 2-3 times a week until mean average of all mice reached about 80mm^3. Tumor volume (mm^3) was calculated using the following formula:

Equation 1.Tumor Volume

$$V = \left(\frac{\pi}{6}\right) * L * W * H$$

Once tumor volume reached an average of 80mm^3, for all the mice, they were divided into 6 groups. The groups are as follows: G1 Human IgG isotype control, G2 H109, G3 FOLFIRI, G4 FOLFIRI and H109, G5 BTZ and G6 BTZ and H109. Day 0 of the study was defined as the day the mice reached the desired tumor volume and the first day of chemotherapy and immunotherapy. The injection schedule can be found on Table 1. The concentration regimen is as follows: HIgG isotype control and H109 at 2.5mg/kg, Irinotecan 40mg/kg, 5-FU 30mg/kg, LV 90mg/kg and BTZ at 0.5mg/kg. Tumors were measured twice a week starting on Day 0 and tumor growth rate plotted (Figure 9). The mice were checked for tumor ulceration, weight loss and tumor size (≥1000mm^3) as end points. The animal welfare guidelines were strictly adhered to. Some tumors reached 1,000 mm^3 on day 21 from the start of chemotherapy treatment. On day 22 mice were euthanized then analyzed by gross necropsy to evaluate tumor volume, presence of metastases, and any treatment toxicity-associated effects on host organs. Since all mice started treatments at the same time with different tumor volumes, the percent change from baseline per mouse was used to determine the antitumor efficacy. GRAPHPAD Prism 6 was used for data entry, graphing and analysis. Unpaired t-tests were used to determine the significance of the tumor growth rates and One-way ANOVA with

multiple comparisons was used to determine the significance for tumor volume increase percentage. $P \leq 0.05$ was considered statistically significant.

CHAPTER THREE

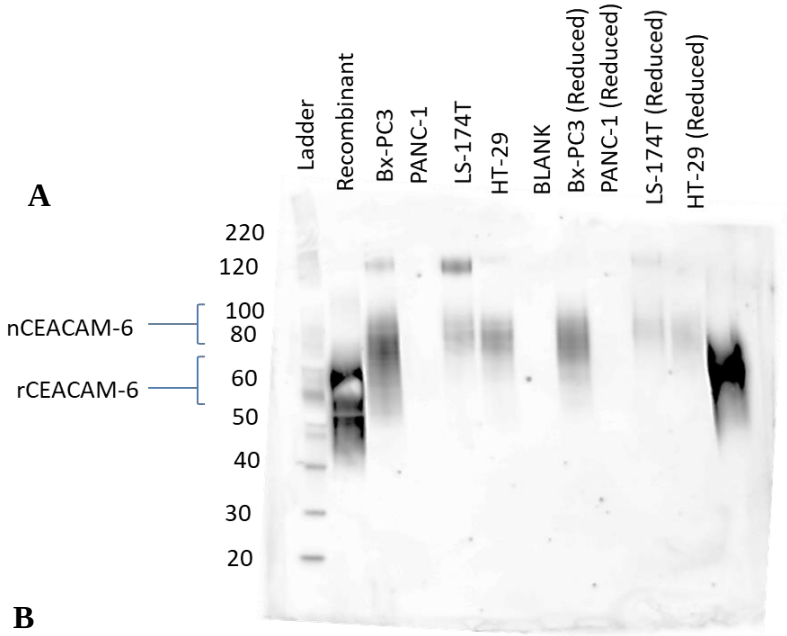
Discussion- Aim 1

Characterization of Colorectal Cancer Cell Line

Bx-PC3, the positive CEACAM6 expressing cell line, expressed the highest amount of CEACAM 6. PANC-1, the negative CEACAM6 expressing cell line, did not express any CEACAM6. A CEACAM6 recombinant was used at a lower concentration as a control to test the efficacy of CT109 as the primary antibody. When comparing the colon cancer cell lines, HT-29 expressed higher levels of CEACAM6 than LS-174T (Figure 6A) making HT-29 the optimal cancer cell line to research for future in-vitro and in-vivo studies.

The ADCC assay showed the most killing by CT109 in Bx-PC3, the positive control, and the least in PANC-1, the negative control (Figure 6B). When comparing the colon cancer cell lines CT109 can induce the highest amount of killing through ADCC in the HT-29 cells than LS-174T (Figure 6C). The data from the ADCC assay is consistent with the CEACAM6 expression levels measured in the Western blot. The higher the CEACAM6 expression level the greater ADCC killing effect CT109 has on the cancer cell line. HT-29 cells express higher levels of CEACAM6 when compared to LS-174T and will be further studied.

A


Ladder
Recombinant
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PANC-1
LS-174T
HT-29
BLANK
Bx-PC3 (Reduced)
PANC-1 (Reduced)
LS-174T (Reduced)
HT-29 (Reduced)
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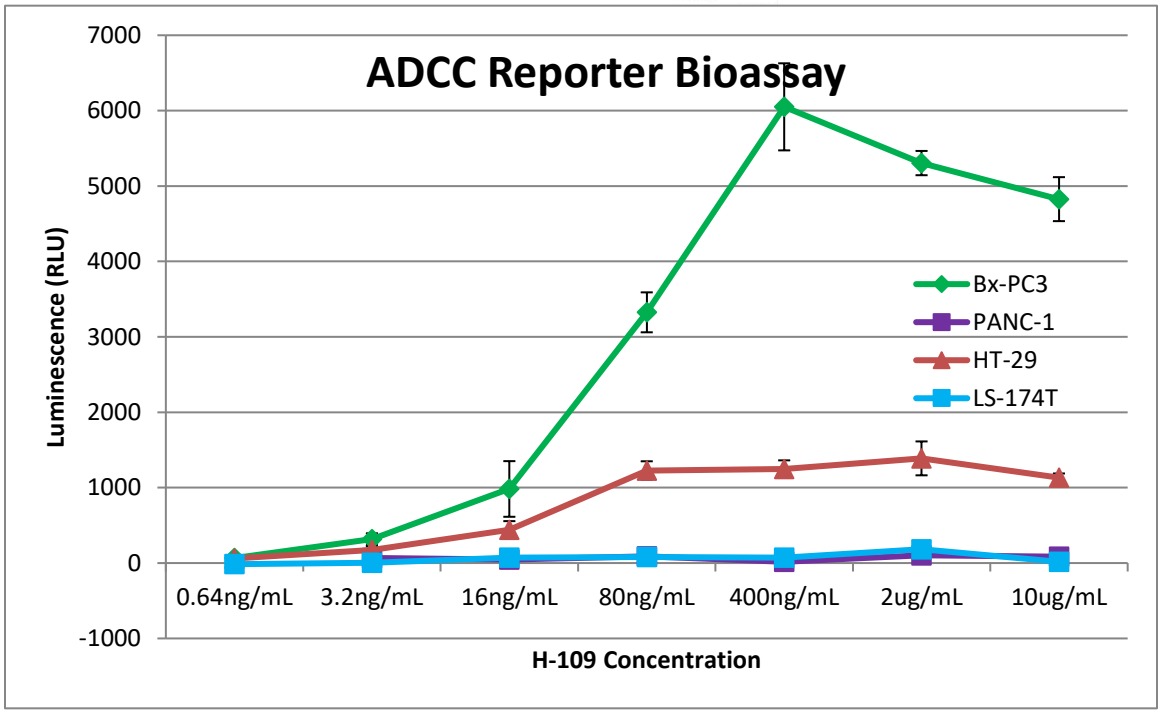
nCEACAM-6
rCEACAM-6

B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5000
4000
3000
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3.2ng/mL
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2ug/mL
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H-109 Concentration
Bx-PC3
PANC-1
HT-29
LS-174T

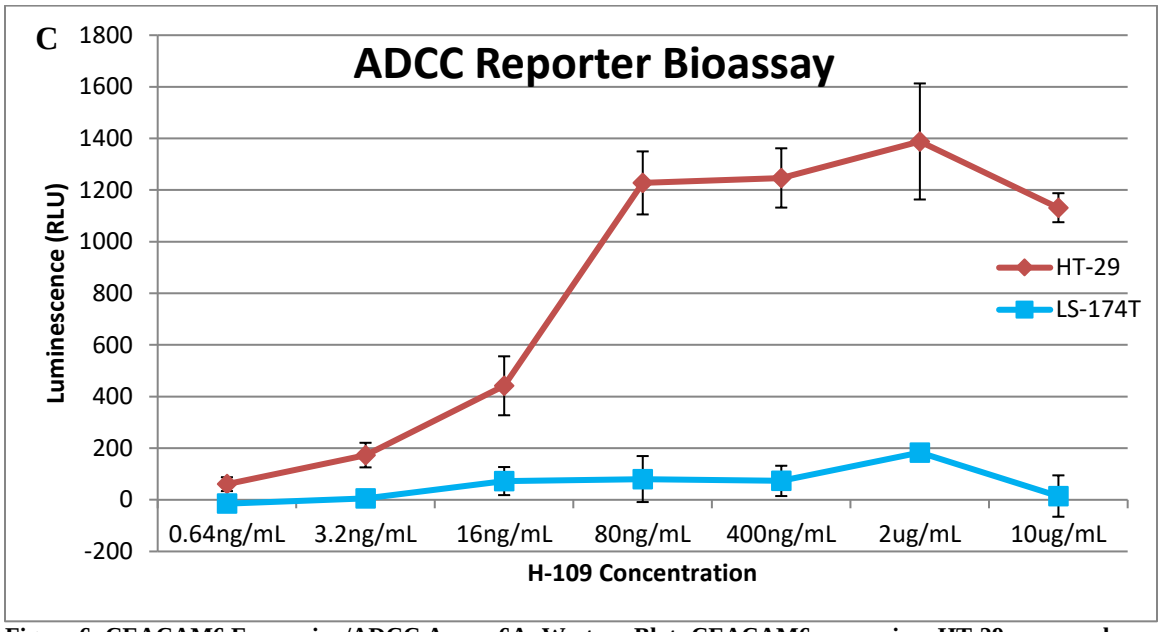


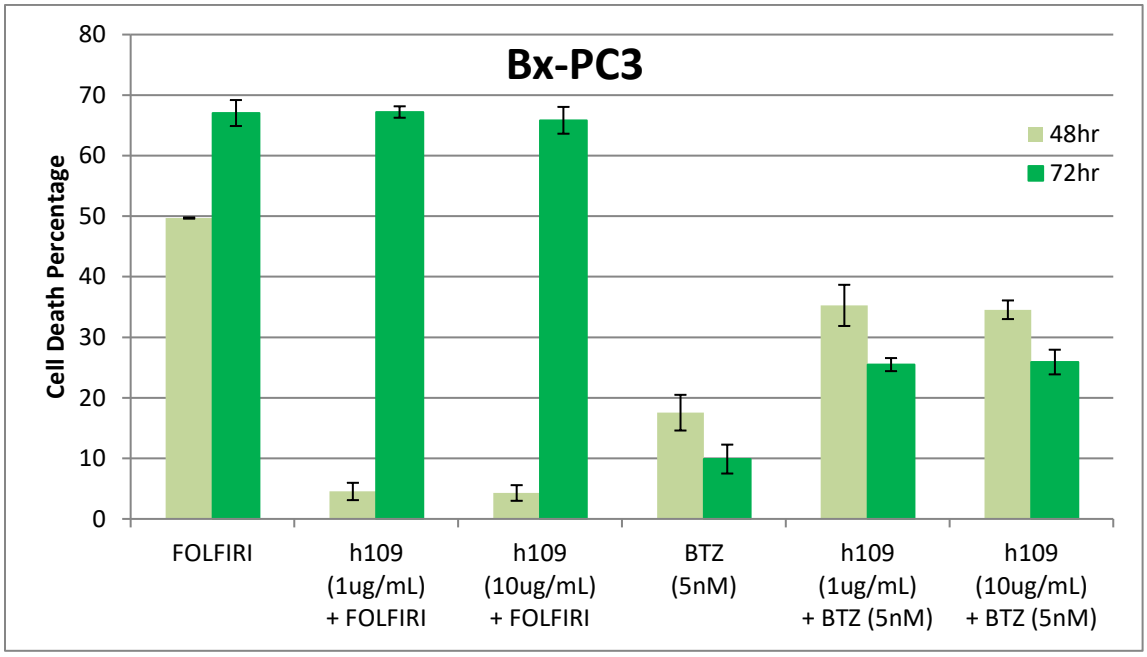
Figure 6: CEACAM6 Expression/ADCC Assay. 6A: Western Blot: CEACAM6 expression. HT-29 expressed higher levels of CEACAM6. 6B-C ADCC Reporter Bioassay (CT109). CT109 induced a greater ADCC in HT29 cells than LS-174T.

Bortezomib and CT09 synergistically inhibited the viability of CRC cells

A Cell Titer-Glo assay was performed for time-course and dose-response analysis of BTZ and FOLFIRI either alone or in combination with CT109. Cell viability assay was performed in Bx-PC3 (Figure 7A) and HT-29 (Figure 7B) cells; cell death percentage was plotted. Mean untreated number of cells was subtracted from mean treated number of cells and that number was divided by mean untreated number of cells to equal the cell death percentage. On Bx-PC3 cells FOLFIRI had a greater effect at increasing cell death percentage than BTZ. However, CT09 had a greater impact at synergistically increasing cell death percentage in combination with BTZ, in Bx-PC3 cells, than FOLFIRI in which CT109 had no impact. Furthermore, CT109 drastically

lessened FOLFIRI's direct killing effect at the 48hr time point. For the HT-29 cells the combination of BTZ with a higher dose of CT109 was the most effective; FOLFIRI only had slight effect with combination having no effect. CT109 has a greater impact at direct cell killing when combined with BTZ.

A



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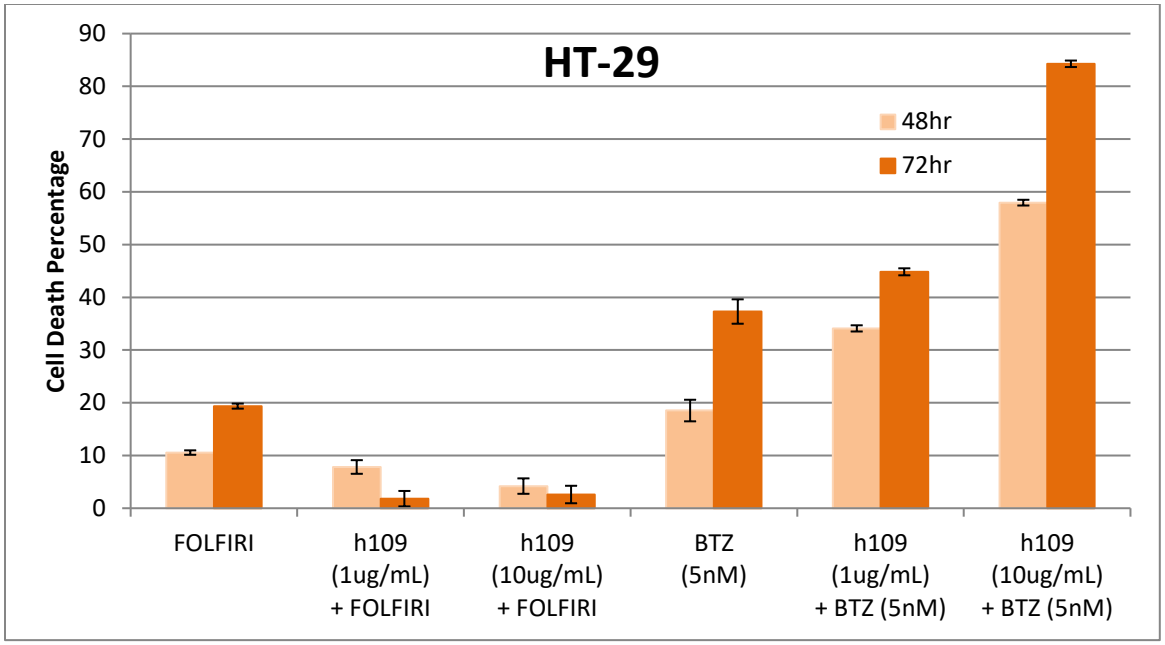


Figure 7: Cell Viability Assay. 48hr & 72hr Incubation. Figure 7A: FOLFIRI has a greater direct cell killing effect on Bx-PC3 than BTZ. CT109 increases BTZ effect only. Figure 7B: BTZ in combination with CT109 showed the highest cell death percentage in HT-29 cells. CT109 lowered the effect of FOLFIRI.

Synergistic induction of apoptosis by BTZ and CT109 is mediated through an increase in caspase activation

The effects of BTZ, FOLFIRI and CT109 on the expression of caspases and PARP proteins (Figure 8) were studied. Once activated (cleaved), caspase 9 cleaves and activates downstream effector caspases (including 3, 6 and 7), which in turn cleave cytoskeletal and nuclear proteins such as PARP and induce apoptosis. For this assay the expression of cleaved Caspase 9 (37kDa) and cleaved PARP (89Kda) were measured in HT-29 cells. At 24-hour incubation (Figure 8A) it can be seen that the treated cells have increased cleaved Caspase-9 and cleaved PARP expression levels however, those same proteins were not significantly changed among the different kinds of treatments. About half of the treated cells are going through early apoptosis (cleaved Caspase-9 expression)

and the other are going through late apoptosis (cleaved PARP expression). At 48hr most of the cells in the treated groups are going through late apoptosis. At this time point it can be seen that the combination of a lower dose of CT109 with BTZ (5nM) in HT-29 cells was more effective at direct cell killing than either agent alone. CT109 seemed to lower the apoptotic effect of FOLFIRI. From these results BTZ should be considered as a promising candidate to be used in *in-vivo* studies as a chemotherapeutic treatment in combination of CT109. FOLFIRI, despite having a greater apoptotic effect on HT-29 cells alone it will also move on to be tested in combination with CT109 in *in-vivo* studies due its high efficacy as current standard care of treatment in colorectal cancer patients.

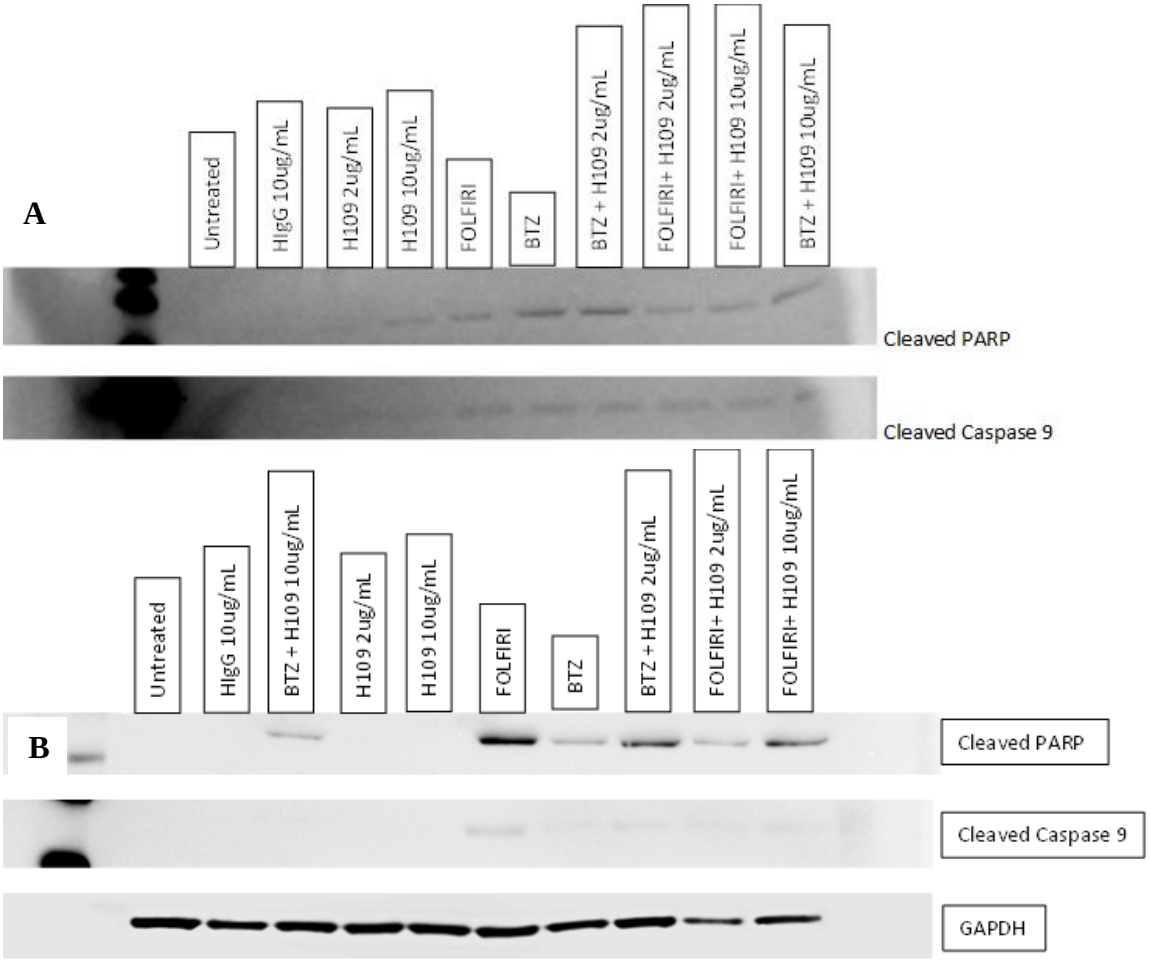


Figure 8: Apoptotic Markers Western Blot. Figure 8A: 24 hour incubation. Cells are equally distributed a early apoptosis and late apoptosis among the treated groups. Figure 8B: 48hr incubation. Cleaved PARP and cleaved Caspase 9 levels of expression were higher in HT-29 cells treated with BTZ combination with CT109 for 48 hours at a concentration of 2ug/mL than Bortezomib (BTZ) alone. Bortezomib in combination with CT109 at 2ug/mL induces a higher apoptotic death of HT29 cells via mitochondria-mediated apoptotic pathway when compared to Bortezomib alone.

Discussion- Aim 2

CT109 and FOLFIRI attenuate HT-29 xenograft tumorigenesis

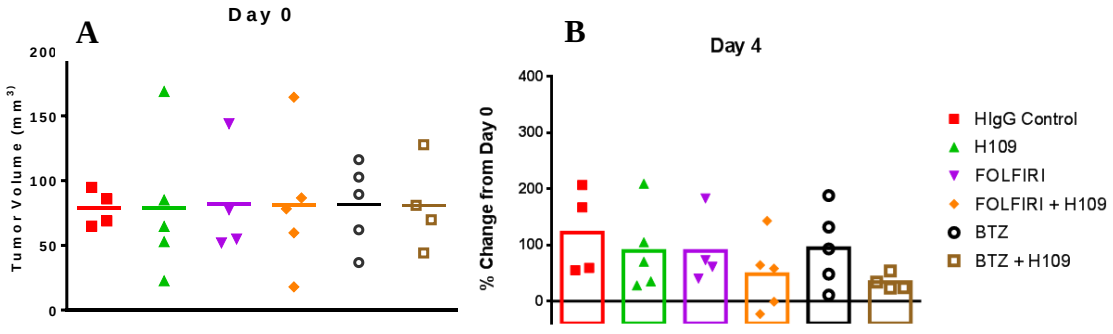
A nude mouse xenograft model was used to validate the efficacy of CT109 seen in previous preclinical studies displaying potent activity in a colorectal cancer cell line. Additionally, the antitumor efficacy of CT109 in combination with the current standard-of-care (FOLFIRI) chemotherapy as well as Bortezomib was studied. Day 0 of the study was defined as the day the mice reached the desired tumor volume and the first day of chemotherapy and immunotherapy. The injection schedule can be found on Table 1.

Table 1 Injection schedule of monoclonal antibody CT109 in combination of FOLFIRI and Bortezomib.

Day	HIgG	H109	5-FU + LV	IRI	H109 (FOLFIRI)	BTZ	BTZ + H109
0	✓	✓	✓		✓	✓	✓
1				✓			
2							
3							
4	✓	✓			✓	✓	✓
5							
6			✓				
7	✓	✓		✓	✓	✓	✓
8							
9							
10	✓	✓	✓		✓	✓	✓
11							
12				✓			
13							
14	✓	✓	✓		✓	✓	✓
15				✓			
16							
17							
18	✓	✓			✓	✓	✓
19							
20			✓				
21	✓	✓		✓	✓	✓	✓

22	END OF STUDY

On day 0, the mice were measured, and groups divided accordingly to obtain a relative equal mean volume across all groups (Figure 9A). Since all mice started treatments at the same time with different tumor volumes, the percent change from baseline per mouse was used to determine the antitumor efficacy and the mean of that percentage was plotted (Figure 9B-G). FOLFIRI in combination with CT109 showed the least tumor volume increase over time and it even shows that there is tumor volume regression for some of the mice in that group from Days 0-11 (Figure 9B-D). Tumor volumes were recorded on a growth rate curve (Figure 9H-I). FOLFIRI in combination with CT109 consistently showed the lowest tumor growth rate within a 21 day period. When running an unpaired t-test comparing the HIgG isotype control group with the combination treatment of FOLFIRI with CT109 with a p-value ≤ 0.05 the results are significant and are shown on the graph as an * (Figure 9I). CT109 in combination with FOLFIRI was the most effective at attenuating tumor growth *in-vivo*.



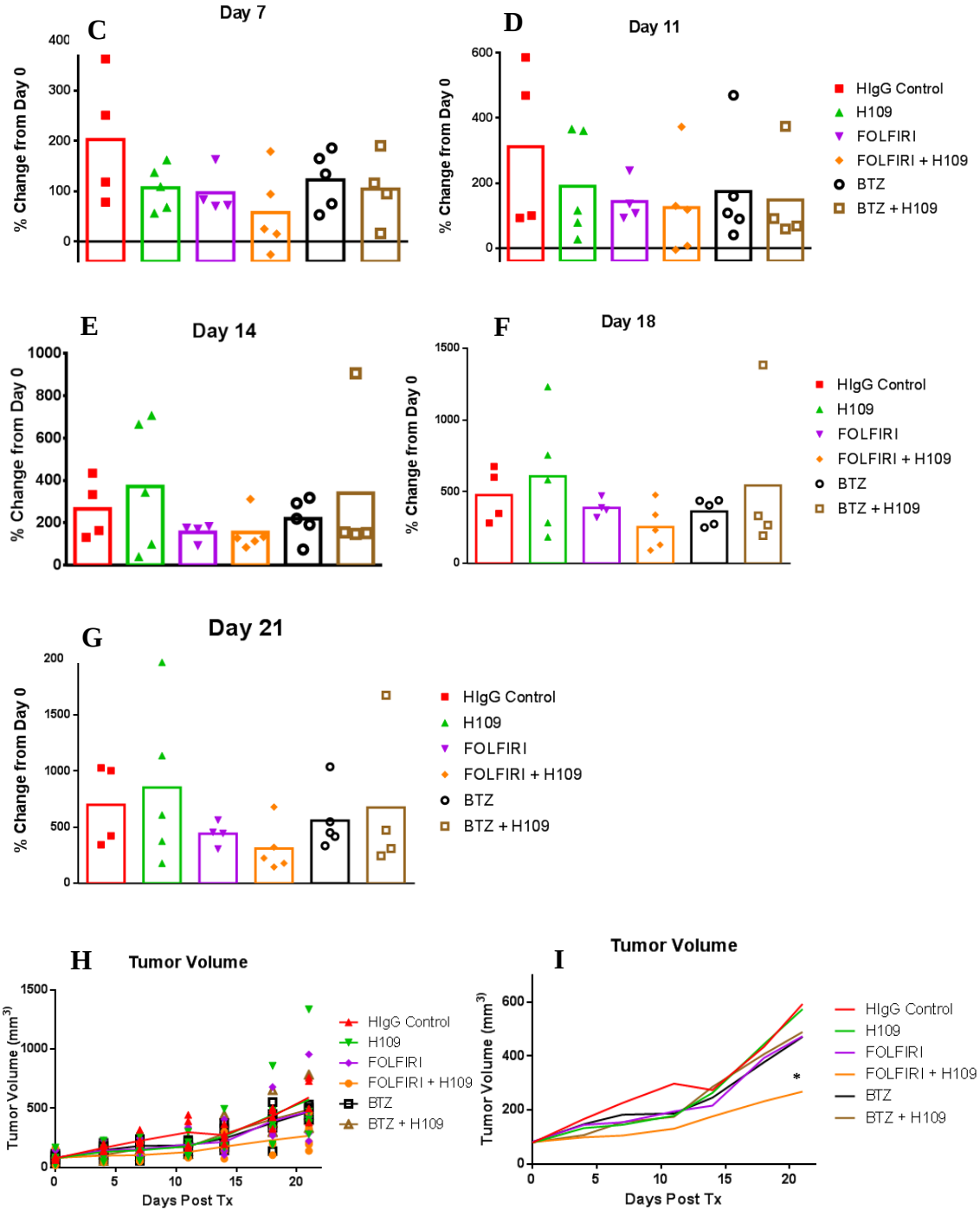


Figure 9. Tumor Volume Increase Percentage. Change in Tumor Volume from Baseline Measurement. The combination of FOLFIRI and CT109 showed the lowest percent tumor volume growth and it even shows that there was regression for some mice during Day 4-11.

CHAPTER FOUR

Conclusion

CT109, an anti-CEACAM6 humanized Moab, is able to create a dual tumor effect on the cancer cell itself and the immune system by inhibiting the binding of CEACAM1 antigen receptor in immune cells to the CEACAM6 antigen receptor in colorectal cancer cells. CT109 is able to inhibit the cancer's defense against the immune system and by doing so increasing its response. Additionally, CT109 is able to disrupt the tumor cell autonomous regulation by binding to the CEACAM6 receptor which results in inhibition of survival and invasion. CT109 inhibits the cancer cell's ability to migrate, invade, adhere, proliferate, induce cell cycle arrest and start angiogenesis. FOLFIRI the current standard care of treatment for colorectal cancer patients relies on its ability to induce p53-dependent cell growth arrest and apoptosis. Immunotherapy in combination with chemotherapy offers complimentary and additive effects of multiple agents with different mechanisms of action to better target the key pathways to inhibit cancer cell proliferation and induce apoptosis. Ongoing experimental studies and clinical trials have revealed that, as a single agent or in combination with other conventional anti-cancer drugs, BTZ has anti-tumor effects on colorectal cancer tumors. BTZ is approved by the FDA for the treatment of patients with multiple myeloma or patients with mantle cell lymphoma. For

this study BTZ was able to inhibit growth in HT-29 and Bx-PC3 cancer cell lines in *in-vitro* studies. Furthermore, the combinatorial treatment of CT109 with BTZ had a synergistically effect at direct cell killing which surpassed BTZ alone. In the *in-vivo* xenograft model the combination of FOLFIRI and CT109 consistently showed the lowest percent tumor volume growth from the first dose until the end of the study and it even showed that there was tumor volume regression for some of the mice during the first 11 days of treatment. These data have demonstrated that CEACAM6 is a novel and viable therapeutic target for colorectal cancer. CT109 is highly selective to its target with exceedingly high affinity, and highly specific to cancer cells. With its demonstrated *in-vitro* and *in-vivo* activity CT109 presents an exceptionally promising drug candidate to be given in combination to patients for whom FOLFIRI alone has failed.

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BIOGRAPHY

Kelly Arias Cardenas graduated from Gar-Field Senior High School, Woodbridge, Virginia, in 2011. She received her Bachelor of Science from George Mason University in 2015. She interned at Serpin Pharma located at the Prince William County Science Accelerator from 2018-2019.