Cetuximab DM1 Antibody-Drug Conjugate Cytotoxicity with Lung Cancer Cell Lines

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Thesis submitted to the Department of Chemistry and Biochemistry and Honors College in Partial Fulfillment of the Bachelor of Science degree in Molecular Cellular Biology and with Honors in Biochemistry

Department of Chemistry & Biochemistry
University of Arizona, Tucson
Spring 2020

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Apr 9, 2020
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Apr 13, 2020
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ABSTRACT

The goal of the study was to create an antibody-drug conjugate which would be potentially more effective than conventional chemotherapeutic treatments. Through DNA transduction a plasmid was introduced to *Escherichia coli* (*E. coli*). The plasmid containing *E. coli* was selected with hygromycin. The plasmid was then extracted and purified from *E. coli* then grown in TB broth. Expi293™ cells were then transfected with the purified DNA to create the Cetuximab antibody. Then through a fluorescein label, the binding affinity of Cetuximab to various cancer cell lines were determined through flow cytometry. The two highest bindings were A549 and HCC827 and the lowest binding was H2170. The three cell lines were selected for further tests. Through the conjugation of Cetuximab with SMCC-DM1 the antibody drug conjugate of Cetuximab-DM1 (Cet-DM1) was created at the Drug-to-Antibody ratio of 7.5:1, 15:1, and 30:1. From cytotoxicity assays it was determined the Cet-DM1 Drug-to-Antibody ratio of 15:1 was the best to continue experimentation. The IC50 of the Cet-DM1 15:1 ADC with A549 cells was 4.113e-2 mg/mL and with H2170 cell was 8.284e-3 mg/mL. This indicated that the Cet-DM1 15:1 ADC was more cytotoxic than naked Cetuximab and more selective than just DM1 for A549 and H2170 cell lines.

INTRODUCTION

The discovery of chemotherapeutic drugs has led to clinical success in treatments for cancer. Different drugs have been discovered to combat the various hallmarks of cancer: angiogenesis, survival, metastasis, immortality, loss of anti-growth signals, and excessive proliferation. The drug of interest for the study was mertansine (DM1) which targets microtubule assembly and prevents cell proliferation[1]. This drug has shown to be a promising chemotherapeutic

treatment in previous studies[2]. However, systemic chemotherapy could potentially cause side effects in a patient, which limits its ability to be a therapy on its own.

Antibodies are used to provide specificity for targeting antigens and are often seen in the immune response[3]. Due to the specificity by which antibodies can bind to their antigen, monoclonal antibodies (mAb) have been used to target proteins overexpressed on the extracellular matrix of cancer cells. One such overexpressed protein is vascular endothelial growth factor (VEGF) which when constitutively active promotes cell growth. Through the use of a mAb the activity of VEGF has been inhibited thus causing induction of apoptosis[4]. Endothelial growth factor receptors (EGFR) can also be overexpressed on the surface of some cancer cells. Therefore research has been done to find targeting molecules for this receptor in the hopes of finding more treatment options. One such targeting molecule is the mAb Cetuximab (Erbitux®) which both blocks ligand binding and dimerization of EGFR through binding to domain 3 of the receptor[5]. With the inhibition of EGFR there can be some cytotoxicity induced to the cancer cells. However, Cetuximab alone is not as effective as chemoradiotherapy and must be paired with radiotherapy to achieve a similar effect[6].

The antibody-drug conjugate (ADC) takes the highly selective antibody and combines it with a cytotoxic drug through the use of a chemical linker. The ideal antibody would be one whose target is a protein which is expressed highly on individual cancer cells but has little to no expression on normally regulated cells. The ideal cytotoxic drug is one which is small enough to be linked to the antibody but cytotoxic enough to induce cell death upon absorption into tumors. The linker must also be stable enough to not prematurely disassemble the ADC before reaching its target but still able to release once absorbed into the targeted tumor. The success of antibody drug conjugates can be seen by the $HER2^+$ targeting T-DM1, marketed as Kadcyla[7]. Additional ADCs

for CD19, CD22 and others are still in ongoing clinical trials[8]. For this study, an ADC of both Cetuximab and DM1 was promising since it would prevent the systemic off target effects of mertansine while also allowing for a more cytotoxic antibody. The method by which to produce this drug was to generate Cetuximab and chemically link the DM1 through SMCC-DM-1.

The tested cell lines were HCC827, H1975, H1299, A549, MDA-MB-231, and H2170 which are available from ATCC. HCC827 and H1975 were selected since they were lung epithelial cells with kinase domain mutations to EGFR[9]. A549, H1299, and H2170 were selected since they were lung epithelial cells with wild-type EGFR[9,10,11]. MDA-MB-231 was selected since it was a mammary gland/breast; derived from metastatic site cell with epidermal growth factor receptor expression. The cells were selected since Cetuximab was a EGFR binding mAb.

The purpose of the experiment was to first generate an antibody from its corresponding plasmid. Then from the antibody, be able to characterize its binding to various breast and lung cancer cell lines. A final test was to determine the appropriate dosage of the ADC through cytotoxicity assays. These tests would allow for the determination of the dosage and profile of the potential target for Cet-DM1.

METHODS

Plasmid Preparation. Research done on the light and heavy chain of the antibody of interest. A plasmid was then ordered from GenScript™ which was optimized for humans and with a resistance to Hygromycin B Gold.

Bacterial Transformation. 4 µg of plasmid was initially in a plastic vial. The vial was then centrifuged at 200 G for 30 seconds. To the vial was added 40 µL of TE buffer, which was shaken inside of the vial. The vial was then left to sit for 30 minutes. During the 30 minutes TOP10 *E.*

coli was taken out of a -80ºC freezer and put on ice. When the 30 minutes had passed 25 µL of TOP10 *E. coli* was put into two 1.5 mL tubes on ice. 4 µL of TE buffer and plasmid mixture was added to each of the tubes and then gently agitated. The tubes were then put onto ice for 30 minutes. After the 30 minutes both tubes were put into a tube vial warmer at 42ºC for 30 seconds and then put immediately back on ice. To each of the tubes was added 150 µL of S. O. C. Medium. The tubes were then incubated in a 37ºC incubator at 120 rpm for 1 hour. 20 µL of the respective vials were added to agar plates with 100 µg/mL of Hygromycin B Gold. The plates were then incubated overnight at 37ºC and 120 rpm.

Bacterial Selection and Growth. 2 mL of S. O. C. Medium was added to a round-bottom 5 mL tube. The agar plates were removed from the incubator. With the end of a 10 µL pipette tip, an isolated colony was scrapped off a plate. The pipette tip was then ejected into the tube containing S. O. C. Medium. The tube was then incubated in a 37ºC incubator at 120 rpm for 2 hours. During the 2 hours incubation 1.2 L of TB broth was prepared in 2 L capped flasks. To the TB broth was added 1.2 mL of 100 mg/mL Hygromycin B Gold with constant swirling during addition. After the 2-hour incubation at 37ºC and 120 rpm all the contents of the tube was added to the TB broth and then incubated overnight at the same temperature and speed.

E. Coli DNA Extraction and Purification. Procedures for extraction and materials were in accordance with the NucleoBond® Endotoxin-free plasmid DNA purification User manual. Following from section 8 for Maxi preps, the flask was centrifuged and the supernatant removed. The pellet was then resuspended and then lysed. Lysate was neutralized and then pelleted at 4696 G for 12 minutes. Supernatant was run through an equilibrated NucleoBond® Xtra Column with an inserted filter and emptied by gravity filtration. The filter was washed with a buffer. Then the filter was then removed, and the column was washed with an endotoxin free buffer and a wash

buffer. Elution buffer was added to the column to remove the DNA and collected in a 50 mL centrifuge tube. Following from section 8.3 for Maxi- NucleoBond® Finalizer Large, the DNA was then precipitated out of the elution with 100% isopropanol. The mixture was vortexed and let rest at room temperature for 2 minutes. The mixture was then run through a NucleoBond® Finalizer slowly. The finalizer is washed with 70% ethanol. The finalizer is then dried until there is no more ethanol present. DNA on the Finalizer is eluted out with 1 mL of TE buffer and labeled.

DNA Quantification. BioTek Take3 Micro-Volume Plate was opened and cleaned with 70% ethanol and dried. 4 µL of H_2O is added to a well. The Take3 was gently closed and placed on a Multiwell microplate reader. A blank was run and the Take3 cleaned. 4 µL of purified DNA was added to the same well. Absorbance at 260 nm was taken and the concentration of DNA determined.

Transfection. Procedures for transfection and materials were in accordance with the Gibco™ Expi293™ Expression System User Guide. 2 L flasks were filled with 400 mL of Expi293™ Expression Medium at 37ºC with 600 x 10^6 cells and swirled. 400 mg of purified DNA was added and mixed with 24 mL of Opti-MEM™ I Reduced Serum Medium. The mixture was added and swirled with dilute ExpiFectamine™ 293 and then incubated after 20 minutes. After 20 minutes the transfection complex was formed. The complex was then slowly added to a 2 L flask containing the Expi293™ cells. The flasks were then incubated at 37ºC at 120 rpm. 18 hours after incubation began 2.4 mL of Transfection Enhancer 1 and 24 mL of Transfection Enhancer 2. The flasks were incubated at 37ºC with 8% CO_2 at 120 rpm for 4 days. After 4 days of incubation the cells were then centrifuged at 4696 g for 30 minutes. A filter .22 µm filter was then used on the supernatant and collected.

FPLC purification. Proteins purified using affinity chromatography using Protein A resin. The purified antibody was then put into a hydrated 20,000 MWCO Dialysis Cassette in DPBS, no calcium, no magnesium. The cassette was gently spun overnight covered from light.

Cetuximab Quantification. Procedures for protein quantification and materials were in accordance with the Pierce™ BCA Protein Assay Kit. Protein standards were created of Bovine Albumin Standard with (µg/mL): 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0. 10 µL of each standard was added to respective wells of a 96 well plate. Dilution factors of 2, 4, and 8 were created for the purified Cetuximab and 10 µL of each dilution was added to respective wells of a 96 well plate. 200 µL/well of BCA Reagent A was added to 4 µL/well of BCA Reagent B. After mixing the two reagents 200 µL was added to each well containing standard and samples. The plate was incubated at 37ºC for 30 minutes. Absorbance at 560 nm recorded and sample concentration calculated from the standard curve. Data found in Table 1.

Cetuximab NHS-Fluorescein Labeling. Procedures for Fluorescein labeling were in accordance with ThermoScientific™ User Guide: NHS-Fluorescin. A mini stir bar was added to 3 mL capped glass vial. The mini stir bar was washed in 100 µL of PBS, pH 7.2, then the PBS was removed. 1 mg of Cetuximab was added to the glass vial. Glass vial was put on a stir plate and a gentle stir started. To the vial was slowly added 300 µL of PBS, pH 7.2. To the vial was slowly added .04685 mg of NHS-Fluorescein. The vial was capped and covered in tin foil and left for 1 hour with gentle stirring. Then the contents of the vial were put into a hydrated 20,000 MWCO Dialysis Cassette. The cassette was put into a beaker with 800 mL of DPBS, no calcium, no magnesium. A stir bar was added to the beaker. The beaker was put on a stir plate, covered in tin foil, and a gentle stir started. 3 hours later the DPBS was removed, replaced with more DPBS, and left overnight with gentle stir.

Cetuximab-Fluorescein Quantification. Procedures for protein quantification and materials were in accordance with the Pierce™ BCA Protein Assay Kit. Protein standards were created of Bovine Albumin Standard with (µg/mL): 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0. 10 µL of each standard was added to respective wells of a 96 well plate. Cetuximab-Fluorescein was removed from dialysis and ran through a .22 µm filter. Dilution factors of 2, 4, 8, and 16 were created for the purified Cetuximab-Fluorescein and 10 µL of each dilution was added to respective wells of a 96 well plate. 200 µL/well of BCA Reagent A was added to 4 µL/well of BCA Reagent B. After mixing the two reagents 200 µL was added to each well containing standard and samples. The plate was incubated at 37°C for 30 minutes. Absorbance at 560 nm recorded and sample concentration calculated from the standard curve. Data found in Table 2.

Cetuximab-Fluorescein FACS Analysis. The day before testing, cell lines were plated. The cell lines used for labeling were HCC827, H1975, H1299, A549, MDA-MB-231, and H2170. A 6 well plate for HCC827 was plated at $5x10^6$ cells/well in 2 mL/well of RPMI 1640 Medium with added fetal bovine serum (FBS) to a final concentration of 10% and added 10,000 U/mL Penicillin-Streptomycin (P/S) to a final concentration of 1%. A 6 well plate for H1299 was plated at $5x10^6$ cells/well in 2 mL/well of RPMI 1640 Medium-10% FBS & 1% P/S. A 6 well plate for H1299 was plated at $5x10^6$ cells/well in 2 mL/well of RPMI 1640 Medium-10% FBS & 1% P/S. A 6 well plate for A549 was plated at $5x10^6$ cells/well in 2 mL/well of DMEM-10% FBS & 1% P/S. A 6 well plate for MDA-MB-231 was plated at $5x10^6$ cells/well in 2 mL/well of RPMI 1640 Medium-10% FBS & 1% P/S. A 6 well plate for H2170 was plated at $5x10^6$ cells/well in 2 mL/well of RPMI 1640 Medium-10% FBS & 1% P/S. All 6 well plates were placed in a 37°C and 5% CO_2 incubator overnight. The next day, 3 testing conditions were determined: IgG antibody, Cet-Fluorescein, and media only. From each of the 6 well plates 3 wells had media removed. 1 mL/well

DPBS was used to wash each of the respective wells and then removed. 500 µL/well of TrypLE™ was added to each of the respective wells and incubated at 37ºC and 5% CO_2 for 5 minutes. The plates were gently tapped to remove the cells from the bottom of the plates. 500 µL/well of DPBS was added to each of the respective wells and then all contents of wells were collected into individual 1.5 mL tubes. 500 µL/well of DPBS was added to each of the respective wells and then collected into their respective 1.5 mL tubes. All tubes were centrifuged at 200 G for 5 minutes. Then all the supernatants were removed to about 100 µL left in the tubes. 100 µL/tube of DPBS was added to each of the respective tubes and the pellets were broken. To 1 tube per cell line was added 10 µg of IgG antibody and incubated at room temperature for 30 minutes in the dark. To 1 tube per cell line was added 10 µg of Cet-Fluorescein and incubated at room temperature for 30 minutes in the dark. After incubation, 300 µL/tube of DPBS was added to each of the tubes. All tubes were centrifuged at 200 G for 5 minutes. Then all the supernatants were removed to about 100 µL left in the tubes. 300 µL/tube of DPBS was added to each of the respective tubes and the pellets were broken. All tubes were centrifuged at 200 G for 5 minutes. Then all the supernatants were removed from the tubes. 300 µL/tube of DPBS was added to each of the respective tubes and the pellets were broken. Contents of 1.5 mL tubes were transferred to their respective round-bottom 5 mL tube with cap. All tubes were run through a FACS machine and analyzed to determine binding.

Creation of Cet-DM1 Reaction Buffer. The reaction buffer was 50 mM KH_2PO_4, 50 mM NaCl, and 2 mM EDTA. 200 mL of reaction buffer was created. .8 mL of .5 M EDTA was added to 199.2 mL Deionized H_2O. To the mixture was added 1.3609 g KH_2PO_4 and .5844 g NaCl.

Cetuximab-DM1 Synthesis. A mini stir bar was added to three 3 mL capped glass vials labeled 7.5:1, 15:1, and 30:1. Each mini stir bar was washed in 100 µL of PBS, pH 7.2, then the

PBS was removed. 10 mg of Cetuximab was added to each glass vial vial. The glass vials were put on their respective stir plate and a gentle stir started. To each vial was slowly added 1.14 mL of 50 mM KH_2PO_4, 50 mM NaCl, and 2 mM EDTA reaction buffer. To the vial was slowly added .06 mL of DMSO. Then slowly added was .5518, 1.104, and 2.207 mg of 20 mg/mL SMCC-DM1 to their respective vials. The vials were capped and covered in tin foil and left for 19 hours with gentle stirring. Then the contents of the vials were put into a hydrated 20,000 MWCO Dialysis Cassette. The cassette was put into a beaker with 800 mL of DPBS, no calcium, no magnesium. A stir bar was added to each beaker. The beaker was put on a stir plate, covered in tin foil, and a gentle stir started. 3 hours later the DPBS was removed, replaced with more DPBS, and left overnight with gentle stir.

Cetuximab-DM1 Quantification. Procedures for protein quantification and materials were in accordance with the Pierce™ BCA Protein Assay Kit. Protein standards were created of Bovine Albumin Standard with (µg/mL): 2000, 1500, 1000, 750, 500, 250, 125, 25, and 0. 10 µL of each standard was added to respective wells of a 96 well plate. Cetuximab-DM1 samples were removed from dialysis and ran through a .22 µm filter. Dilution factors of 2, 4, and 8 were created for the purified Cet-DM1 and 10 µL of each dilution was added to respective wells of a 96 well plate. 200 µL/well of BCA Reagent A was added to 4 µL/well of BCA Reagent B. After mixing the two reagents 200 µL was added to each well containing standard and samples. The plate was incubated at 37°C for 30 minutes. Absorbance at 560 nm recorded and sample concentration calculated from the standard curve. Data found in Table 3.

Cytotoxicity Assay with Cetuximab-DM1 without Removing Media. The day before testing, cell lines were plated. The cell lines used for testing were HCC827, H2170, and A549. Five 96 well plates for HCC827 were plated at $4x10^3$ cells/well in 100 µL/well of RPMI 1640 Medium

with added fetal bovine serum (FBS) to a final concentration of 10% and added 10,000 U/mL Penicillin-Streptomycin (P/S) to a final concentration of 1%. Five 96 well plates for H2170 were plated at $4x10^3$ cells/well in 100 µL/well of RPMI 1640 Medium-10% FBS & 1% P/S. Five 96 well plates for A549 were plated at $4x10^3$ cells/well in 100 µL/well of DMEM Medium-10% FBS & 1% P/S. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator overnight. The next day five testing conditions were determined: Cetuximab-DM1 7.5:1, Cetuximab-DM1 15:1, Cetuximab-DM1 30:1, Cetuximab, and DM1. The concentrations of the treatments for Cetuximab-DM1 7.5:1, Cetuximab-DM1 15:1, Cetuximab-DM1 30:1, Cetuximab, and DM1 were (mg/mL): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 0. Dilution was done to achieve the dilutions required for the treatments with RPMI 1640-10% FBS & 1% P/S. 50 µL of the respective dilution was added in accordance with Fig. 7. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator for 72 hours. After incubation the amount of media inside of the wells was determined. To each of the wells containing samples, 10x PrestoBlue™ Cell Viability Reagent was added in low light so the final mixture in each well was 90% media and 10% PrestoBlue™ Cell Viability Reagent. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator for 2 hours. After incubation the fluorescence excitation was measured at 560 nm and the emission was measured at 590 nm. The cell viability was determined from the data.

Cytotoxicity Assay with Cetuximab-DM1 with Removing Media. The day before testing, cell lines were plated. The cell lines used for testing were HCC827, H2170, and A549. Three 96 well plates for HCC827 were plated at $4x10^3$ cells/well in 100 µL/well of RPMI 1640 Medium with added fetal bovine serum (FBS) to a final concentration of 10% and added 10,000 U/mL Penicillin-Streptomycin (P/S) to a final concentration of 1%. Three 96 well plates for H2170 were plated at $4x10^3$ cells/well in 100 µL/well of RPMI 1640 Medium-10% FBS & 1% P/S. Three 96

well plates for A549 were plated at $4x10^3$ cells/well in 100 µL/well of DMEM Medium-10% FBS & 1% P/S. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator overnight. The next day three testing conditions were determined: Cetuximab-DM1 15:1, Cetuximab, and DM1. The concentrations of the treatments for Cetuximab-DM1 15:1, Cetuximab, and DM1 were (mg/mL): 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 0. Dilution was done to achieve the dilutions required for the treatments with RPMI 1640-10% FBS & 1% P/S. 50 µL of the respective treatments were added to the plates without gentle mixing. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator for 4 hours. After incubation, all media was removed. For the plates containing HCC827, 100 µL/well of RPMI 1640-10% FBS & 1% P/S was added to each well containing sample. For the plates containing H2170, 100 µL/well of RPMI 1640-10% FBS & 1% P/S was added to each well containing sample. For the plates containing A549, 100 µL/well of DMEM-10% FBS & 1% P/S was added to each well containing sample. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator for 72 hours. After incubation the amount of media inside of the wells was determined. To each of the wells containing sample 10x PrestoBlue™ Cell Viability Reagent was added in low light so the final mixture in each well was 90% media and 10% PrestoBlue™ Cell Viability Reagent. All 96 well plates were placed in a 37ºC and 5% CO_2 incubator for 2 hours. After incubation the fluorescence excitation was measured at 560 nm and the emission was measured at 590 nm. The cell viability was determined from the data.

RESULTS

Determining Concentration of Cetuximab

Table 1 shows the data from the BCA Assay of Cetuximab. The concentration of Cetuximab was calculated to be 8.7976 mg/mL with the use of equation (1). Where A was the

absorbance of the sample, Y was the y-intercept of the standard, and S was the slope of the standard. Since the Cetuximab was diluted to determine its concentration, therefore the concentration of undiluted Cetuximab had to be calculated using equation (2). Where D was the diluted sample concentration, DF was the dilution factor of the sample which was in the range of the BSA standard, and UD was the undiluted sample concentration.

1) $(A - Y)/(S) = [D]$

2) $[D](DF) = [UD]$

Table 1: Concentration of Cetuximab Taken from BCA Assay Standard with a Slope of .0005, y-intercept of .0853, and R^2 of .9992. If the Absorbance was Out of Range of the Standard, Then the Concentration was Determined by Doubling the Concentration of the Next Higher Dilution Factor Until the Absorbance was in the Range. The Concentration of Cetuximab was Determined to be 8.7976 mg/mL.

	Dilution Factor			
Samples	1	2	4	8
Cetuximab (µg/mL)	8797.6	4398.8	2199.4	1269.4

Fluorescein Labeling to Select Cell Lines of Interest

Table 2 shows the data from the BCA Assay of Cetuximab-Fluorescein. The concentration of Cetuximab-Fluorescein was calculated to be 2.0472 mg/mL with the use of equation (1). Since the Cetuximab was diluted to determine its concentration, therefore the concentration of undiluted Cetuximab had to be calculated using equation (2).

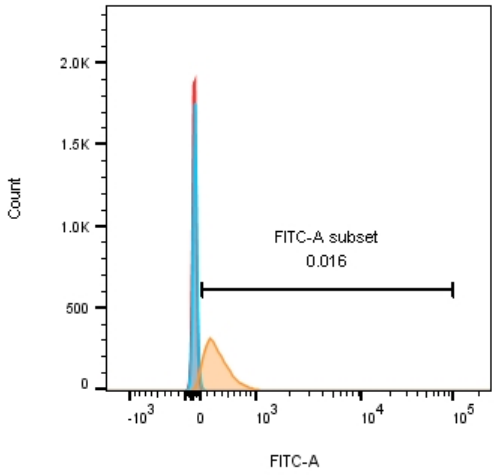
Table 2: Concentration of Cetuximab-Fluorescein Taken From BCA Assay Standard with a Slope of .0005, y-intercept of .0982, and R^2 of .9974. If the Absorbance was Out of Range of the Standard Then the Concentration was Determined by Doubling the Concentration of the Next Higher Dilution Factor Until the Absorbance was in the Range. The Concentration of Cetuximab was Determined to be 2.0472 mg/mL.

	Dilution Factor				
Samples	1	2	4	8	16
Cetuximab-Fluorescein (µg/mL)	2047.2	1023.6	539.6	255.6	111.6

Table 3 shows the FACS analysis which was performed on the cells of interest with Cetuximab-Fluorescein. The analysis was performed on the data in Fig 1-6. The percent binding of Cetuximab to A549, HCC827, H1299, H1975, H2170, and MDA-MB-231 was 92.4, 83.4, 22.6, 64.4, 4.99, and 79.5 respectively.

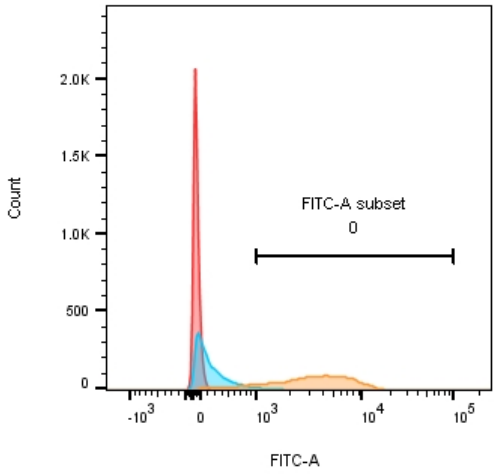
Table 3: FACS Analysis Performed on the FACS Data of Cetuximab-Fluorescein.

Cell Line	Control	Positive control	Cetuximab (EGFR)
A549	0.016	0.48	92.4
HCC827	0	2.57	83.4
H1299	0	0.30	22.6
H1975	0.14	0.45	64.4
H2170	0.075	0.48	4.99
MDA-MB-231	0	0.15	79.5



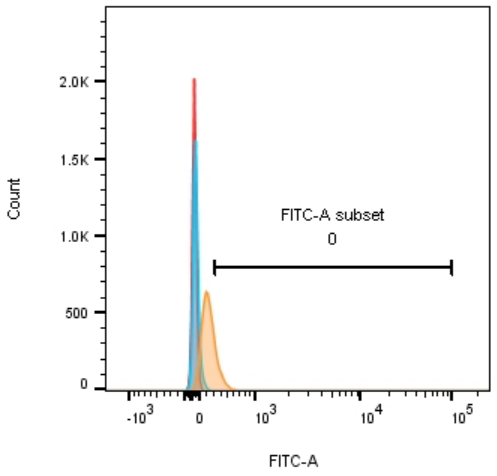
	Sample Name	Subset Name	Count
	Target cells_A549_CEX_009.fcs	Lymphocytes	6143
	Target cells_A549_POS_008.fcs	Lymphocytes	5670
	Target cells_A549_Control_007.fcs	Lymphocytes	6253

Figure 1: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to A549.



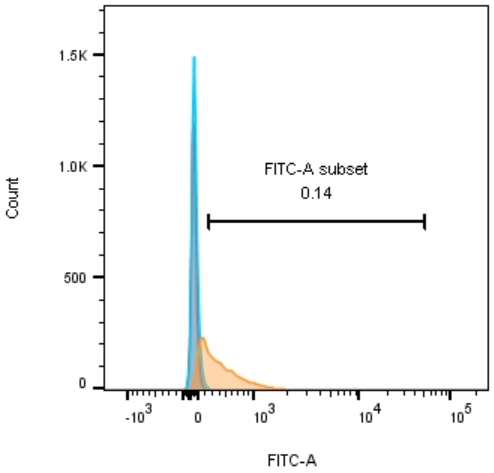
	Sample Name	Subset Name	Count
	Target cells_HCC827_CEX_012.fcs	Lymphocytes	7088
	Target cells_HCC827_POS_011.fcs	Lymphocytes	6033
	Target cells_HCC827_Control_010.fcs	Lymphocytes	8605

Figure 2: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to HCC827.



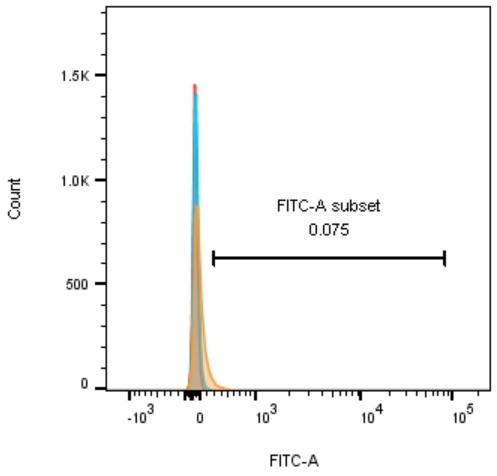
	Sample Name	Subset Name	Count
	Target cells_H1299_CEX_006.fcs	Lymphocytes	7235
	Target cells_H1299_POS_005.fcs	Lymphocytes	6776
	Target cells_H1299_Control_004.fcs	Lymphocytes	6702

Figure 3: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to H1299.



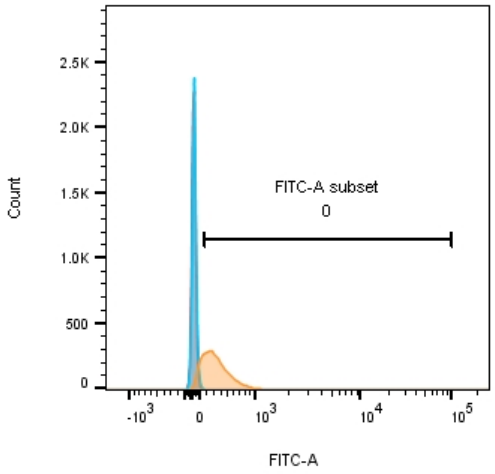
	Sample Name	Subset Name	Count
	Target cells_H1975_CEX_015.fcs	Lymphocytes	5206
	Target cells_H1975_POS_014.fcs	Lymphocytes	6460
	Target cells_H1975_Control_013.fcs	Lymphocytes	4888

Figure 4: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to H1975.



	Sample Name	Subset Name	Count
	Target cells_H2170_CEX_003.fcs	Lymphocytes	5967
	Target cells_H2170_POS_002.fcs	Lymphocytes	5810
	Target cells_H2170_Control_001.fcs	Lymphocytes	5351

Figure 5: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to H2170.



	Sample Name	Subset Name	Count
	Target cells_MDAMB231_CEX_001_019.fcs	Lymphocytes	6846
	Target cells_MDAMB231_POS_017.fcs	Lymphocytes	7508
	Target cells_MDAMB231_Control_016.fcs	Lymphocytes	7371

Figure 6: FACS data comparing FITC-A with the cell count of Cetuximab-Fluorescein when added to MDA-MB-231.

Conjugation of DM1 to Cetuximab with Different Drug-to-Antibody Ratios and Testing

Table 4 shows the data from the BCA Assay of the various Cet-DM1 conjugates. The Cet-DM1 ADC was created with 3 different drug-to-antibody ratios (DAR) of 7.5:1, 15:1, and 30:1. The concentration of Cet-DM1 7.5:1 was calculated to be 1.718 mg/mL with the use of equation (1). The concentration of Cet-DM1 15:1 was calculated to be 3.018 mg/mL with the use of equation (1). The concentration of Cet-DM1 30:1 was calculated to be 2.634 mg/mL with the use of equation (1). Since the Cet-DM1 was diluted to determine its concentration, therefore the concentration of undiluted Cetuximab had to be calculated using equation (2).

Table 4: Concentration of Cetuximab-DM1 with a Drug-to-Antibody Ratio of 7.5:1, 15:1, and 30:1 Taken from BCA Assay Standard with a Slope of .0005, y-intercept of .0955, and R^2 of .9987. If the Absorbance was Out of Range of the Standard, Then the Concentration was Determined by Doubling the Concentration of the Next Higher Dilution Factor Until the Absorbance was in the Range. The Concentration of Cetuximab-DM1 7.5:1 was Determined to be 1.718 mg/mL, 15:1 was Determined to be 3.018 mg/mL, and 30:1 was Determined to be 2.634 mg/mL.

	Dilution Factor			
Samples	1	2	4	8
Cetuximab-DM1 7.5:1 (µg/mL)	1718	859	505	263
Cetuximab-DM1 15:1 (µg/mL)	3018	1509	861	485
Cetuximab-DM1 30:1 (µg/mL)	2634	1317	763	419

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0	

Figure 7: Concentrations used for plating of Cet-DM1 7.5:1, Cet-DM1 15:1, and Cet-DM1 30:1, Cetuximab, and DM1 during cytotoxicity assays. Concentrations in mg/mL.

The cytotoxicity of the DAR of 7.5:1, 15:1, and 30:1 was compared to the cytotoxicity of naked Cetuximab antibody and DM1. Fig. 8-10 shows the graphs of the data for HCC827, A549, and H2170 cancer cell lines. Table 5 shows the IC50 values determined by GraphPad Prism 8 for the treatments. Without the removal of treatment over the 72-hour incubation period the IC50 of the naked Cetuximab was the least cytotoxic treatment whereas the DM1 was the most cytotoxic. For HCC827 the IC50 of the Cet-DM1 DAR 7.5:1, 15:1, and 30:1 was 1.657e-4, 1.031e-5, and 6.645e-5 mg/mL respectively. For A549 the IC50 of the Cet-DM1 DAR 7.5:1, 15:1, and 30:1 was 2.061, 9.376e-3, and 3.104e-4 mg/mL respectively. For H2170 the IC50 of the Cet-DM1 DAR 7.5:1, 15:1, and 30:1 was 3.872e-2, 1260e-3, and 3.979e-5 mg/mL respectively.

Table 5: IC50 for Cet-DM1 7.5:1, Cet-DM1 15:1, Cet-DM1 30:1, Cetuximab, and DM1 with 72 Hour Incubation without Removal of Treatment.

Cell Line	Cet-DM1 7.5:1	Cet-DM1 15:1	Cet-DM1 30:1	Cetuximab	DM1
HCC827	0.0001657	1.031e-005	6.461e-005	9.988e-005	Ambiguous
A549	~ 2.061	0.009376	0.0003104	Ambiguous	Ambiguous
H2170	0.03872	0.001260	3.979e-005	Interrupted	Ambiguous

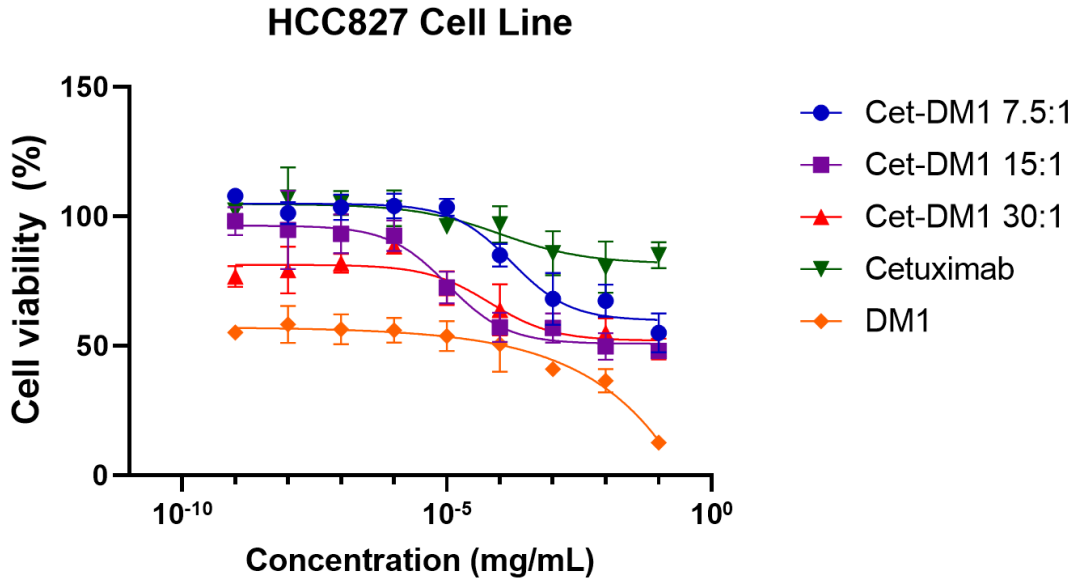


Figure 8: Graph of the cytotoxicity activity against the HCC827 cancer cell line of Cet-DM1 7.5:1, Cet-DM1 15:1, Cet-DM1 30:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

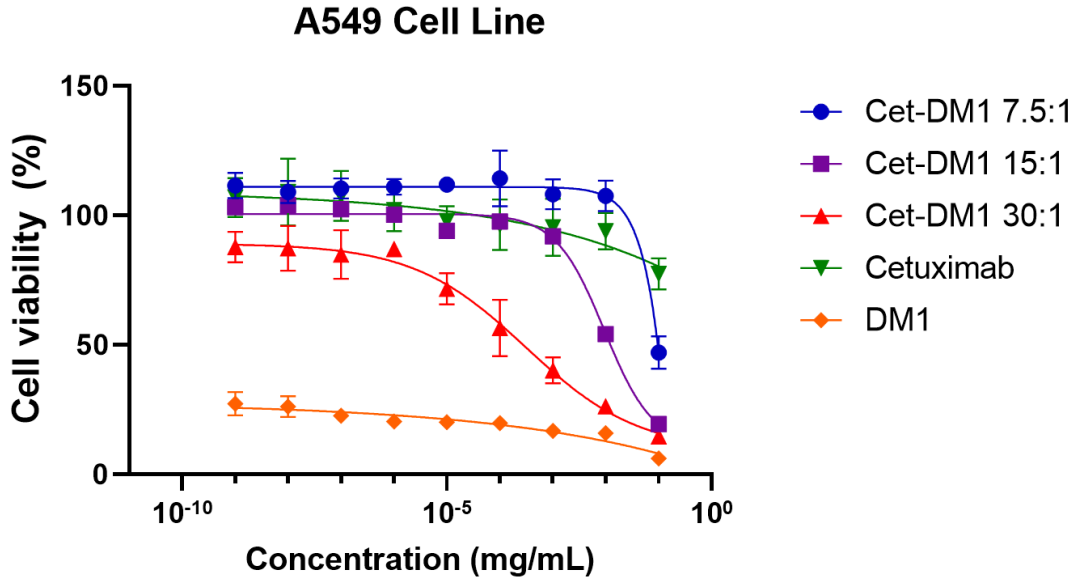


Figure 9: Graph of the cytotoxicity activity against the A549 cancer cell line of Cet-DM1 7.5:1, Cet-DM1 15:1, Cet-DM1 30:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

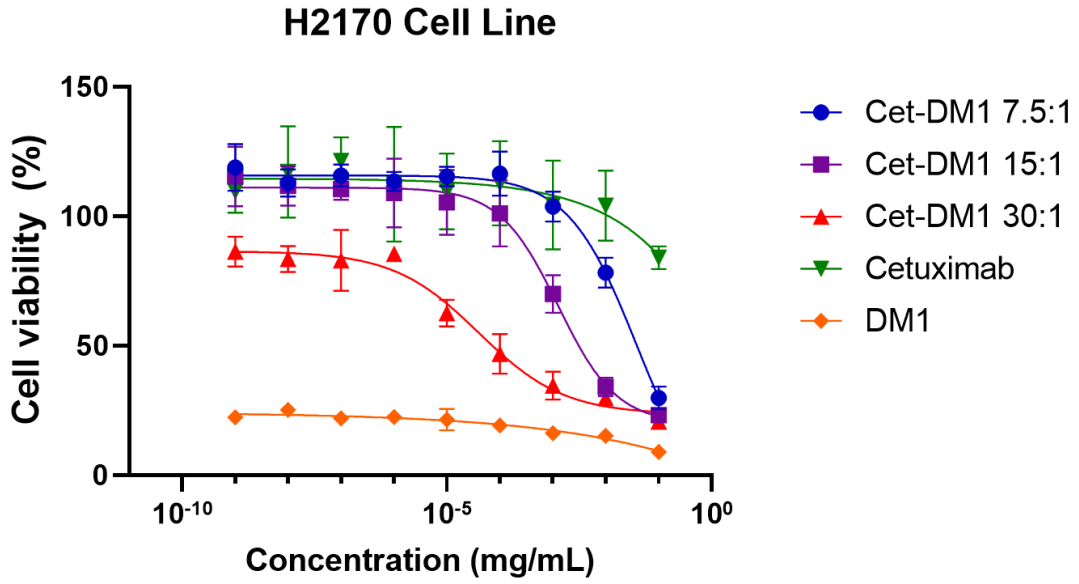


Figure 10: Graph of the cytotoxicity activity against the H2170 cancer cell line of Cet-DM1 7.5:1, Cet-DM1 15:1, Cet-DM1 30:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

The cytotoxicity of the DAR of 15:1 was compared to the cytotoxicity of naked Cetuximab antibody and DM1. Fig. 11-13 shows the graphs of the data for HCC827, A549, and H2170 cancer cell lines. Table 6 shows the IC50 values determined by GraphPad Prism 8 for the treatments. With the removal of treatment after 4 hours, followed by a 72-hour incubation period the IC50 of the naked Cetuximab was the least cytotoxic treatment whereas the DM1 was the most cytotoxic. For HCC827 the IC50 of the Cet-DM1 DAR 15:1 could not be determined. For A549 the IC50 of the Cet-DM1 DAR 15:1 was 4.113e-2 mg/mL. For H2170 the IC50 of the Cet-DM1 15:1was 8.284e-3 mg/mL.

Table 6: IC50 for Cet-DM1 15:1, Cetuximab, and DM1 with 72 Hour Incubation with Removal of Treatment.

Cell Line	Cet-DM1 15:1	Cetuximab	DM1
HCC827	Ambiguous	Ambiguous	0.001073
A549	0.04113	Ambiguous	2.920e-005
H2170	0.008284	0.03548	0.0006994

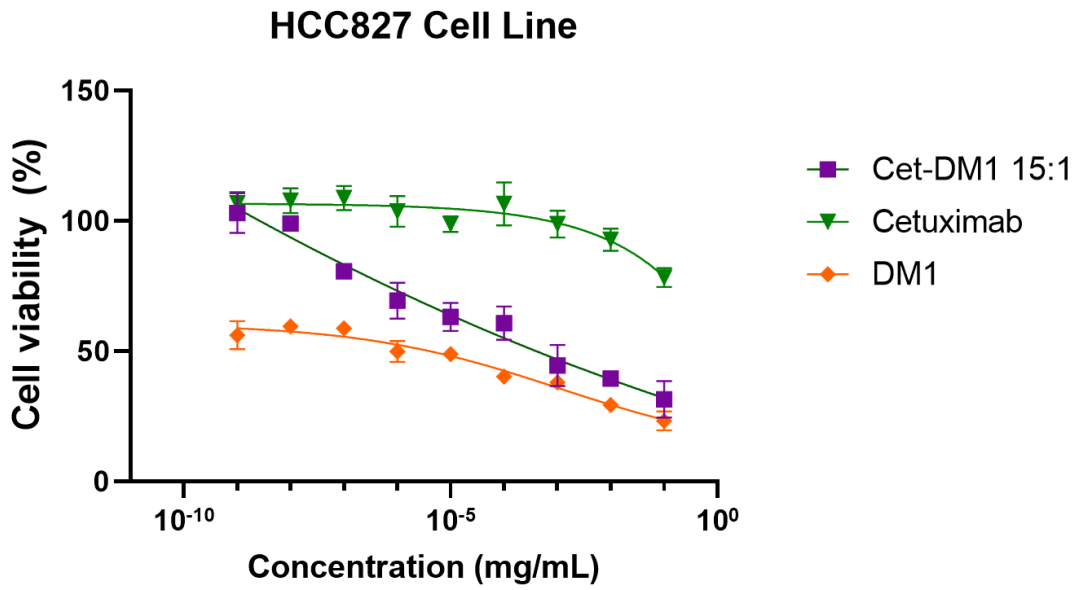


Figure 11: Graph of the cytotoxicity activity against the HCC827 cancer cell line of Cet-DM1 15:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cells exposed to treatment for 4 hours. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

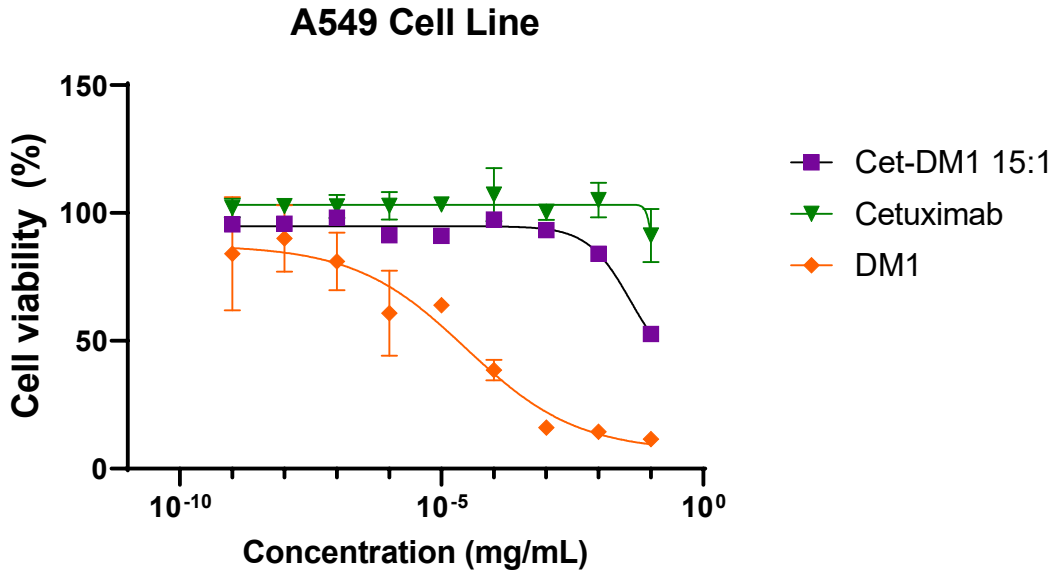


Figure 12: Graph of the cytotoxicity activity against the A549 cancer cell line Cet-DM1 15:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cells exposed to treatment for 4 hours. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

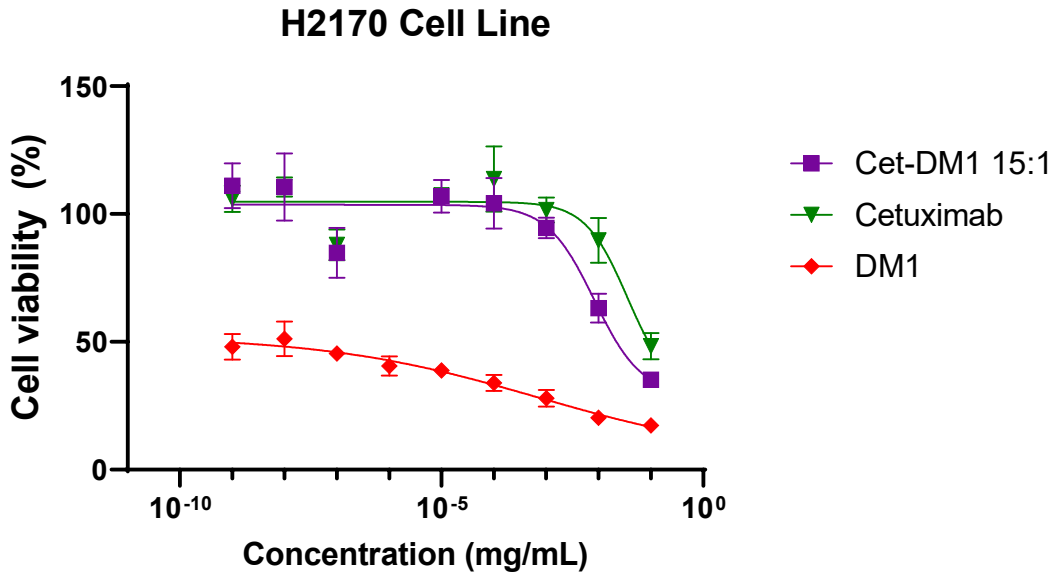


Figure 13: Graph of the cytotoxicity activity against the H2170 cancer cell line of Cet-DM1 15:1, Cetuximab, and DM1. Cell plated with $4x10^3$ cells/well at the bottom of flat-bottom 96 well plate. Cells exposed to treatment for 4 hours. Cell viability was determined after 72 hours of incubation, with PrestoBlue™ Cell Viability Reagent. Made using GraphPad Prism 8.

DISCUSSION

Fluorescein Labeling to Select Cell Lines of Interest

The cancer cells which were selected for the initial test were cell lines which were available in the laboratory storage and had expression of EGFR. Through that criteria the cell lines A549, HCC827, H1299, H1975, H2170, and MDA-MB-231 were used for flow cytometric analysis. The volume of 300 μL was chosen to give more rapid reads during the FACS run. From the flow cytometry analysis data found on Table 3 it was seen that H2170 had the lowest percentage of cells bound with the antibody with 4.99%. Whereas A549 had the highest percentage of cells bound with 92.4%. HCC827 was the second highest with 83.4%. And the rest of the cell lines were MDA-MD-231, H1975, and H1299 with 79.5%, 64.4%, and 22.6% cells bound to Cetuximab respectively. From these results H2170 was selected since it had the lowest binding to Cetuximab, so it would provide a comparison to a cell line which had weak affinity to the naked antibody. Both A549 and HCC827 were selected since they had the highest affinity, but also due to their binding patterns. Fig. 1 indicated A549 had the most cells with Cetuximab binding to EGFR so it would be an ideal target for the Cet-DM1 treatment. Fig. 2 indicated that even though HCC827 did not have as many cells with Cetuximab binding to EGFR it did indicate there was multiple Cetuximab binding to the cells. For this reason, both HCC827 and A549 were selected to see what the cytotoxic difference would be between these two cell lines. The other cell lines tested did not stand out as significant but in the future testing cytotoxicity could potentially be tested on them.

Conjugation of DM1 to Cetuximab with Different Drug-to-Antibody Ratios and Testing

An important aspect to the ADC is the amount of cytotoxic drug, which is conjugated to the targeted antibody, known as the drug-to-antibody ratio (DAR). Based on previous studies the

DAR of 7.5:1 was used to test cytotoxicity[12]. However, the DAR of 15:1 and 30:1 was added to compare with how the effectiveness of the ADC changed as more DM1 was added during Cet-DM1 synthesis. What was expected as the DARs increased the IC50 value would decrease to indicate for a smaller concentration the drug would have a similar cytotoxicity.

To perform the antibody drug conjugation a reaction plan had to be created. The reaction buffer made for the synthesis of the Cet-DM1 was modified from the reaction buffer used in the literature[12]. The reaction buffer would hopefully allow for the DM1 to properly mix with the Cetuximab and prevent the DM1 from reacting on only a few antibodies. The reaction buffer was added after the antibody addition and before the SMCC-DM1 to create a more controlled reaction. SMCC-DM1 was highly reactive, if it was added directly onto the Cetuximab it had the potential to only react with the antibodies closest to the site of addition, which would create a mixture with naked Cetuximab. By diluting the Cetuximab with the reaction buffer the SMCC-DM1 would not be able to react so easily with the antibodies closest to the site of addition and would mix more with all the antibodies in solution and provide a more uniform DAR between individual antibodies.

The cytotoxicity studies were the primary result to determine the effectiveness of the created ADCs. The design of the plate layout was to minimize the variation within a duplicate but also across the entire treatment. To achieve this goal the outermost edge of the plate was not used and only the middle four rows were utilized to minimize the different rates of evaporation. The range of the concentrations were determined from previous attempts (data not included) where it was found the IC50 was between 10^{-4} and 10^{-1} mg/mL. Anything higher was not viable due to the low starting concentration and low volume of the Cet-DM1 collected from synthesis. The choice to first start without removing the media after treatment was to determine whether the ADC was cytotoxic to the cancer cells. Then to confirm whether the ADCs were targeted to the cancer cells

the treatment was removed 4 hours after initial exposure. These 4 hours were to ensure the antibodies had time to bind to the cancer cells.

The first cytotoxicity study was with the ADC treatment exposed to the cancer cells for all 72 hours of incubation. Table 5 shows the results of this study, however naked Cetuximab and DM1 are more accurately shown through Figure 8-10. The cause of the Ambiguous and Interrupted results was due to naked Cetuximab and DM1 having a more linear shape than sigmoidal, so the program was unable to determine an accurate IC50. With the combination of Table 5 and the corresponding figures it was determined that the DAR of 15:1 was the most optimal ADC to continue the study. The cell lines HCC827, A549, and H2170 when the selected ADC was added had an IC50 of 1.031e-5, 9.376e-3, and 1.260e-3 mg/mL respectively. The DAR 15:1 was selected over 7.5:1 since 15:1 was more cytotoxic than 7.5:1. The more cytotoxic ADC would be preferable since it would require a lower concentration of the ADC to achieve a similar effect. The DAR 15:1 was selected over 30:1 since 15:1 at the lowest concentration had near complete cell viability, but 30:1 had some cell death even at the lowest concentration. This could indicate that either the addition of DM1 to antibody at that scale was not stable or caused off target effects, which was not ideal. With the Cet-DM1 15:1 ADC selected; the study could continue.

The second cytotoxicity study was like the first however with the additional step of replacing the media containing the treatment after 4 hours. In the first study the major factor tested was the cytotoxicity of the ADCs, however in the second study the main focus was on the binding and specificity of the selected ADC with the same cell lines. The treatment was added to the media to prevent cell death from the process of removing and adding new media. The 4-hour incubation was selected since it allowed for enough time for the treatment to diffuse into the old media and have enough time to bind to the cells. After the 4 hours the media was gently removed, and new

media was added to wash any ADC which had not bound in the initial incubation. Table 6 shows the results of this study. The cause for the ambiguous results for naked Cetuximab was due to it not being cytotoxic enough, as seen in Figure 11-12 so it produced a more horizontal line which the program was unable to determine an accurate IC50 for HCC827 and A549. HCC827 had an ambiguous IC50 for Cet-DM1 15:1. When comparing Figure 11 to Figure 8 it can be seen that in the first cytotoxicity assay the ADC caused a more sigmoidal shape, whereas that shape was lost in the second assay. This indicates the experiment must be redone since there was more cell death than expected which could be due to unnecessary cell death during the removal of the treatment. Cet-DM1 15:1 when treated on A549 cells had an IC50 of .04113 mg/mL. This value was higher than DM1 with an IC50 of 2.92e-5 mg/mL, which indicated the ADC was binding with specificity, which was ideal for an ADC. Cet-DM1 15:1 when treated on H2170 cells had an IC50 of 8.284e-3 mg/mL. This value was higher than DM1 with an IC50 of 6.994e-4 mg/mL. However, the reason this IC50 was so high could be since H2170 was very sensitive to DM1, seen in Figure 13. The Cet-DM1 15:1 IC50 in A549 was lower than naked Cetuximab with an IC50 of 3.549e-2 mg/mL. Since the Cet-DM1 15:1 had a lower IC50 value than the Cetuximab, it showed that it was more cytotoxic to the cancer cells than just the antibody, and since it had a higher IC50 value than the DM1 it showed there was more specificity of the ADC to its targets.

CONCLUSION

Throughout the experiment an antibody-drug conjugate of Cetuximab and DM1 was created for targeting of the Epithelial Growth Factor Receptor which can be overexpressed in cancers such as breast and lung cancer. Then the ADC was optimized with the drug-to-antibody ratio of 15:1 through investigation of the cytotoxic effects *in vitro* when compared to the naked

antibody and the cytotoxic drug. The ADC showed it was more cytotoxic than the Cetuximab antibody and more selective than DM1. However, the study was not conclusive as to if the ADC cytotoxicity was directly correlated to the expression of the EGFR on the cell's surface. Without the complete data from the HCC827 cell line it is uncertain whether the amount of EGFR on the cell's surface is a factor in the ADC's effectiveness on cancer cells. From the available data, the original hypothesis of a conjugate between an antibody and a cytotoxic molecule would have both the benefit of selectivity from the antibody and reduced cytotoxic effect which would be used to target cancers overexpressing EGFR. For future testing, the results found could be further validated through additional series of the procedures described, or potentially to be tested in a model organism to further validate the selectivity of the ADC.

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