

ANALYSIS OF THE EFFICACY AND CYTOTOXICITY OF A
SYNTHESIZED ANTI-HER2 ANTIBODY-DRUG CONJUGATE

by

Brody Blackburn

Copyright © Brody Blackburn 2020

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfilment of the Requirements

For the Degree of

MASTER OF SCIENCE

MEDICAL PHARMACOLOGY

PERFUSION SCIENCES

In the Graduate College

THE UNIVERSITY OF ARIZONA

2020

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Master's Committee, we certify that we have read the thesis prepared by Brody Blackburn, titled *Analysis of the Efficacy and Cytotoxicity of a Synthesized Anti-HER2 Antibody-Drug Conjugate* and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.


Date: April 21, 2020
Dr. Young-Wook Won, PhD


Date: April 24, 2020
Dr. David Bull, MD


Date: April 21, 2020
Dr. Raymond Wong, PhD, CCP

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

We hereby certify that we have read this thesis prepared under our direction and recommend that it be accepted as fulfilling the Master's requirement.


Date: April 21, 2020
Dr. Young-Wook Won, PhD
Master's Thesis Committee Co-Chair
Department of Surgery


Date: April 24, 2020
Dr. David Bull, MD
Master's Thesis Committee Co-Chair
Division of Cardiothoracic Surgery

Table of Contents

• List of Figures and Tables	4
• Abstract	5
• Chapter 1: Introduction	6
Prevalence and Relevance of Non-Small Cell Lung Cancer.....	6
Basis of an Antibody-Drug Conjugate.....	7
Selecting a Molecular Target in HER2.....	9
Trastuzumab as the ADC Selective Element.....	16
Existing Trastuzumab Antibody Drug Conjugates; T-DM1.....	21
Potential of Exatecan-Based Antibody Drug Conjugates.....	27
Proposal of HER2 Selective ADC Conjugated to Exatecan.....	31
• Chapter 2: Objectives, Hypothesis, and Aims	33
Project Aims.....	34
• Chapter 3: Materials and Methods	35
Trastuzumab Reduction.....	35
Exatecan-SMCC Complex Formation.....	38
Reduced Trastuzumab-SMCC-Exatecan (T-Exa) ADC Reaction.....	39
Mass Spectrometry Analysis.....	39
Mammalian Cell Culturing.....	40
MTT Assay for Cytotoxicity Verification.....	42
• Chapter 4: Results	45
Trastuzumab Reduction Verification.....	45
T-Exa Synthesis Verification.....	47
T-Exa Cytotoxicity Analysis.....	49
Comparing Cytotoxicities of DM1 and Exatecan.....	50
Comparing Cytotoxicities of T-DM1 and T-Exa.....	52
• Chapter 5: Discussion	54
• Chapter 6: Future Directions	56
• Chapter 7: Conclusion	58
• References	60

List of Figures

1	Antibody Structure.....	7
2	Epidermal Growth Factor Receptor (EGFR) Family.....	9
3	HER2 Extracellular Domain (ECD).....	11
4	HER-Mediated Signal Transduction.....	12
5	PI3K Activated Akt Pathway.....	14
6	Trastuzumab and Pertuzumab Binding Locations on HER2 Receptor.....	17
7	ADC Mechanism of Action.....	20
8	Cellular Replication Visuals.....	21
9	T-DM1 Constituents.....	22
10	T-DM1 Mechanisms of Action via HER2 Receptor Binding.....	23
11	Topoisomerase I Function.....	28
12	Proposed Constituents of a Synthesized Trastuzumab-MCC-Exatecan ADC.....	32
13	DTT Disulfide Reduction Chemical Reaction.....	35
14	MTT Conversion to Formazan.....	43
15	Ellman's Reaction Results.....	45
16	BCA Assay Results.....	46
17	Mass Spectrometry Results.....	48
18	MTT Assay Results for Trastuzumab, Reduced Trastuzumab, and T-Exa.....	49
19	MTT Assay Results for DM1 and Exatecan.....	51
20	MTT Assay Results for T-DM1 and T-Exa.....	52

List of Tables

1	Day 3 MTT Assay Results Summary.....	53
---	--------------------------------------	----

ABSTRACT

With biological immunotherapy becoming an increasingly prominent means of treating multiple forms of carcinoma, such immunotherapies are now the focal point of ongoing research. Traditional chemotherapeutic agents are considered cytotoxic in that they interfere with mitosis with the objective of inducing apoptosis in rapidly dividing cells, ideally cancerous cells. Despite the curative intent, there is a concerning lack of specificity of these agents. Healthy cells in the body that normally display increased rates of division are likely to suffer damage consequently. Treatments designed to treat malignant disease target DNA synthesis or replication, mitotic spindles, inhibit protein synthesis or induce cell differentiation.¹ The cytotoxic actions of traditional chemotherapies currently used may prove to be effective in some cancer cases, however, such chemotherapeutics are notorious for causing deleterious side effects. Myelosuppression, or reduced production of cells originating from the bone marrow such as erythrocytes, leukocytes, and thrombocytes can manifest as a result. Anemia, immunosuppression, and thrombocytopenia-derived coagulopathy can then respectively ensue.² It is further documented that known chemotherapeutic agents such as mercaptopurine and azathioprine are associated with myelosuppression, though genetic variation dictates severity.^{3,4} Developing is an ever-expanding trend to deviate from traditional chemotherapies and pursue more target-based therapies tailored to one's pathology of disease. The implementation of monoclonal antibodies in the production of antibody-drug conjugates (ADC) is an ideal means of targeting various forms of carcinoma, with the over-arching goal to reduce systemic cell susceptibility to cytotoxic agents. The objective of this work is to produce an ADC to target non-small cell lung carcinoma (NSCLC), that is known to over express the surface antigen human epidermal growth factor receptor-2 (HER2). A monoclonal antibody specific for HER2, trastuzumab, is to be conjugated to a DNA topoisomerase I inhibitor, exatecan. Such an ADC

will be tested in vitro against HER2 positive and negative mammalian cells in order to see if this additional element of specificity is effective in inducing cell death selectively.

CHAPTER 1: INTRODUCTION

Prevalence and Relevance of Non-Small Cell Lung Cancer

Biological therapy is an ever-expanding topic of research for various forms of disease with growing prevalence for clinical implementation. Immunotherapy can be considered a relatively newer approach of treating multiple forms of carcinoma with promising potential in the clinical setting. According to the American Society of Clinical Oncology (ASCO), lung cancer is the second most common cancer and the leading cause of cancer death for both men and women. It is estimated that over 140,000 deaths in 2019 were associated with this disease. ASCO further goes on to suggest that the 5-year survival rate for non-small cell lung cancer (NSCLC) is 23%, compared to 6% for small cell lung cancer.⁵ Efforts are being made to develop more efficacious therapies specifically for NSCLC, which according to the American Cancer Society, accounts for nearly 80-85% of all lung carcinoma.⁶ Though there are several types, NSCLCs can be generalized as any type of epithelial lung cancer apart from small cell lung cancer (SCLC).⁷ The US National Library of Medicine describes three particular subtypes that are most prevalent; adenocarcinoma predominates in mucus-secreting cells, squamous cell carcinoma effects flat squamous cells that line the inside surface barrier of the airway, while large cell carcinoma can appear in any part of the lung.⁸ Despite being derived from different types of lung cells, they are associated together due to similarities in prognosis and treatment options.

Neoadjuvant or adjuvant chemotherapy are commonly implemented before or after surgical resection respectively, however, it is noted that NSCLCs are largely insensitive to conventional

chemotherapy and radiation therapy in comparison to small cell carcinoma.^{7,9} This conclusion heightens the prospective potential of immunotherapeutics and selective biological agents as they are becoming more relevant in terms of cancer treatment. Despite there being many variations, our research pertains to the utilization of monoclonal antibodies as a biologically selective means of targeting various carcinoma depending on the expression of certain molecular markers that are commonly over expressed in cancerous cells.

Basis of an Antibody-Drug Conjugate

The premise of an antibody drug conjugate (ADC) as a biopharmaceutical drug, is to be utilized under the designation of targeted therapy. An ADC can be divided into 3 main constituents; the monoclonal antibody, the cytotoxic drug, and the cross-linker molecule conjugating the two.

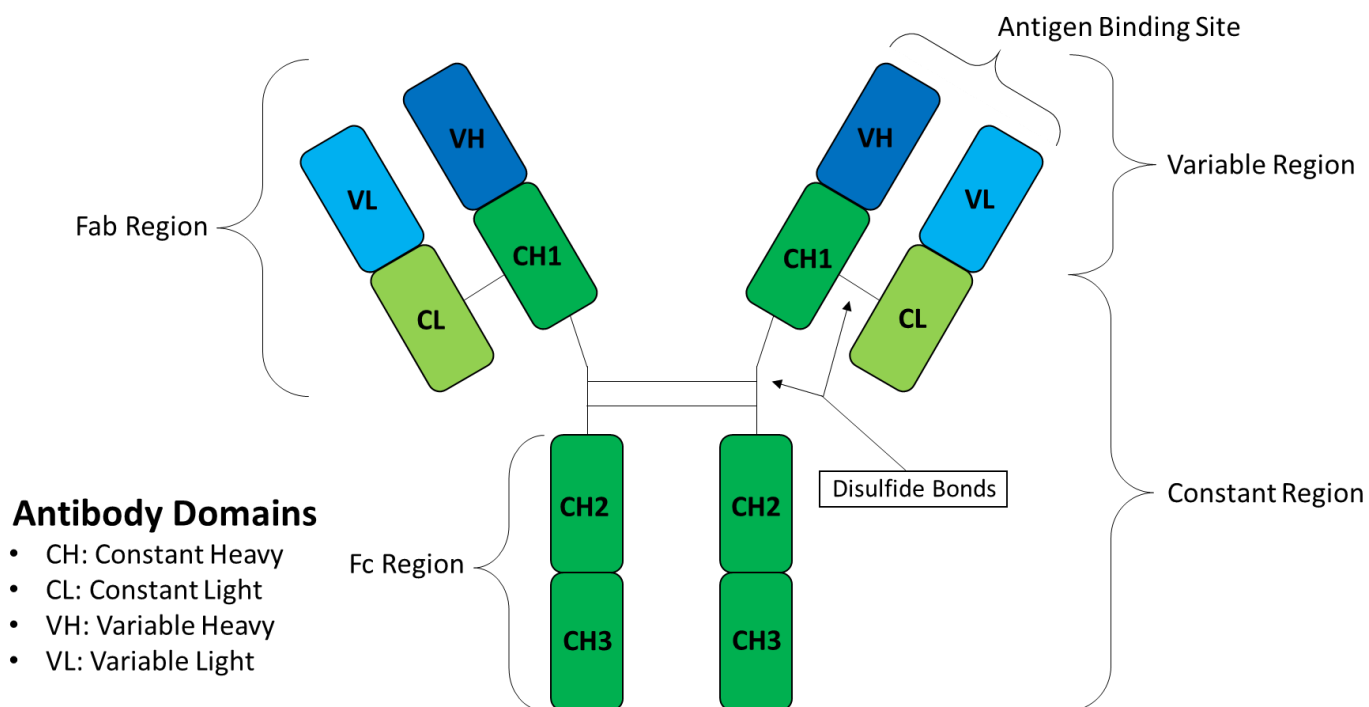


Figure 1. Antibody Structure: Depiction of an antibody, as well as the orientation of functional and structural regions of a typical antibody. Also included are the locations of the fragment crystallizable (Fc) region, fragment antigen-binding (Fab) region, and disulfide bonds.

The antibody is made up of two main fragments. The first being the fragment crystallizable region, or Fc region, which is a portion of the antibody tertiary structure that is constant between antibody classes, but variant enough to differentiate isotypes.¹⁰ This region found in the heavy chains, normally binds to Fc receptors (FcR) on cell surfaces to elicit signal transduction or interacts with components of the classical complement pathway to induce inflammation and activate an immune response. The Fc-FcR interactions link the humoral and cellular immunity.¹¹ The other region of relevance is the fragment antigen-binding (Fab) region, which contains both light chains and terminal portions of the heavy chains. The terminal portion of the Fab fragment is designated as the variable region, in which the variable heavy and variable light domains (V_H and V_L , respectively) pair to form the antigen-binding site of the antibody.¹² Please refer to Figure 1 for a visual representation. The paratope, or antigen-binding site, is classified as the variable region, and it is within the variable region of the Fab fragments where the specificity remains in terms of antigen binding.¹³ The specificity offered within the paratope makes an antibody the ideal biological element to guide a drug virtually exclusively to carcinoma that can be differentiated by molecular targets. Variations in carcinoma are commonly characterized by the up regulation of specific molecular markers. Such molecular markers are overexpressed as a consequence of genomic mutations that render otherwise normal healthy cells into tumor cells. The target-based treatment of an ADC can exploit the up regulation of molecular targets to effectively deliver cytotoxic drugs to carcinoma. This is a significant deviation from general chemotherapeutic agents that lack specificity, prompting systemic cell death due to their non-selective nature.

Selecting a Molecular Target in HER2

In terms of NSCLC, it is known that HER2, or human epidermal growth factor receptor 2, mutations and over expression are commonly associated with adenocarcinoma, as well as breast and gastroesophageal cancers.¹⁴ The HER2 receptor is coded for within the *HER2* or *ERBB2* (erythroblastic (erb)-b2 receptor tyrosine kinase) gene and is within the epidermal growth factor receptor (EGFR) family, including HER1 (ERBB1), HER3, and HER4.¹⁵ The *ERBB2* gene is located on the long arm of chromosome 17 (17q21).¹⁴ HER2 resides on the cell surface to act as a receptor tyrosine kinase. Please refer to Figure 2 for a visual representation of the EGFR family, activating ligands, and subsequent pathways that then ensue.

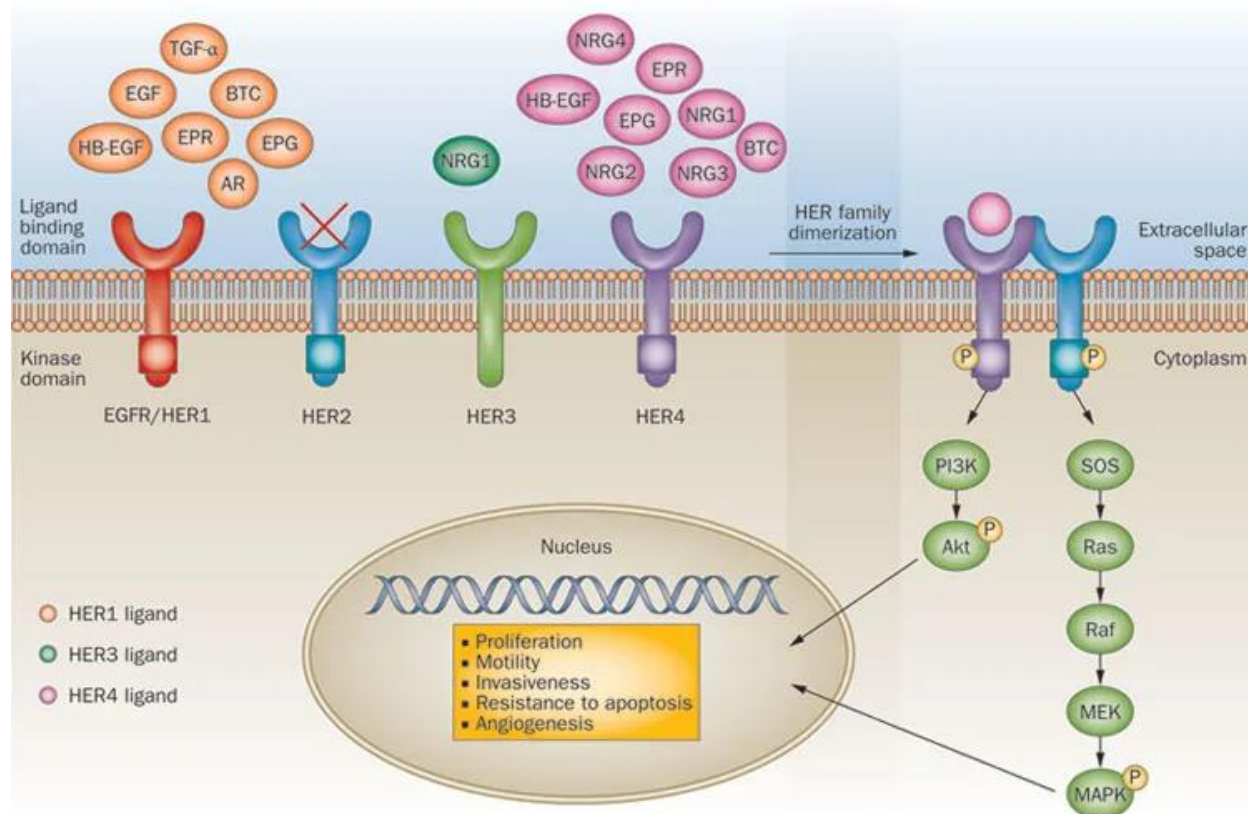


Figure 2. Epidermal Growth Factor Receptor (EGFR) Family: Demonstration of heterodimerization to initiate signal transduction. Abbreviations: AR, amphiregulin; BTC, betacellulin; EPG, epigen; EPR, epiregulin; HB-EFG, heparin-binding EGF-like ligand; NRG, neuregulin.¹⁶ Reproduced with permission.

HER2 primarily exists as a monomer, but in the presence of ligands, it is known to preferentially dimerize with other related tyrosine kinases such as HER1, HER3, and HER4.¹⁷ Interestingly, HER2 does not have an identified ligand, but rather stays in the open configuration to accommodate dimerization. Ligand exposure to the extracellular ligand-binding domains of either HER1, HER3, or HER4 results in the formation of a heterodimer with HER2 in which the intracellular HER kinase domains become phosphorylated, with the exception of HER3 that does not contain an intracellular kinase domain.¹⁶ Common ligands include epidermal growth factor (EGF) and transforming growth factor α (TGF α). These heterodimers initiate signal transmission responsible for controlling and regulating normal cell growth and proliferation under normal conditions. It is also thought that heterodimers containing HER2 exhibit a higher transduction potency when compared to other HER heterodimers or homodimers without HER2.¹⁸ The transmembrane glycoprotein HER2 is divided within three distinct domains: an N-terminal extracellular domain, a single α -helix transmembrane domain, and the previously discussed intracellular tyrosine kinase domain.¹⁹ The most prominent region of HER2, the N-terminal extracellular domain, contains roughly 600 residues and contributes 90-110 Kda to the total molecular weight and can be further divided into four subdomains (I-IV).¹⁹ Subdomains I and III interact to form a binding site for potential ligands,²⁰ though there are currently no known endogenous ligands exclusively for the HER2 receptor. Subdomains II and IV are thought to have a heavy cysteine residue contribution and are integrally involved in homodimerization and heterodimerization. Subdomain II is thought to be the main contributor to dimerization, as it contains what is termed to be a 'dimerization arm'.²¹ This dimerization arm is classified as a hairpin loop that protrudes outward to interact with the dimerization arm of a partnering HER receptor to complete dimerization.²² Please refer to Figure 3.

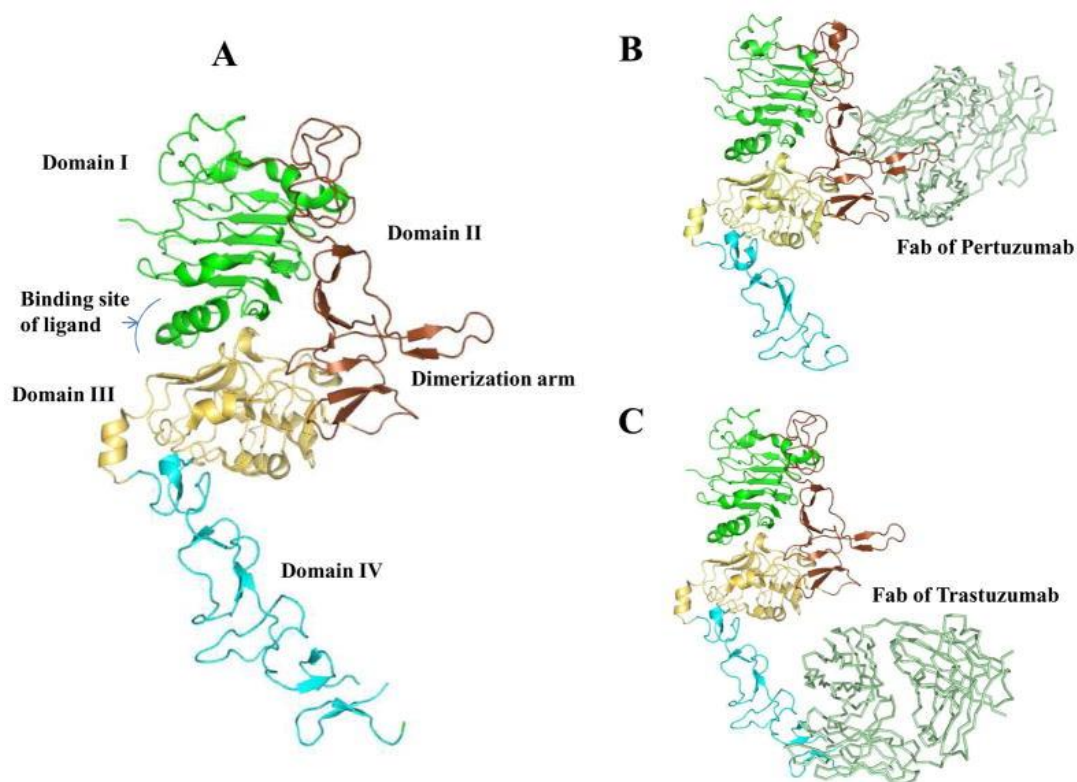


Figure 3. HER2 Extracellular Domain (ECD): (A) Locations of the four ECD subdomains. (B) Pertuzumab binding to the dimerization arm of domain II. (C) Trastuzumab binding to domain IV of the ECD of HER2.¹⁹ Reproduced with permission.

The carboxyl-terminal tail resides in the intracellular region and contains the enzymatic active site for the tyrosine kinase (TyK) domain.²³ A total of six tyrosine residues are present in the TyK domain and participate in transphosphorylation to then provide a docking site for signaling molecules that have phosphotyrosine binding domain.²⁴ After dimerization and transphosphorylation have occurred on HER receptors, a signaling cascade can then undergo to initiate activation of the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide-3-kinase (PI3K) activated protein kinase B (Akt) pathway.¹⁵ The MAPK pathway, Akt pathway, stress-activated protein kinase cascade, and protein kinase C (PKC) pathways ultimately translate their effect with the recruitment of nuclear transcriptional factors that can then initiate the transcription of oncogenes, or genes that can transform a conventional

healthy cell into a tumor cell. Oncogenes include *fos*, *jun*, and *myc*.²⁵ The overall effect of signaling pathway activation is integrally related to the cells involved, the extracellular ligands present, the constituents that make up the heterodimer, and the transduction pathways being activated. Please refer to Figure 4 for a visual representation of HER-induced signal transduction.

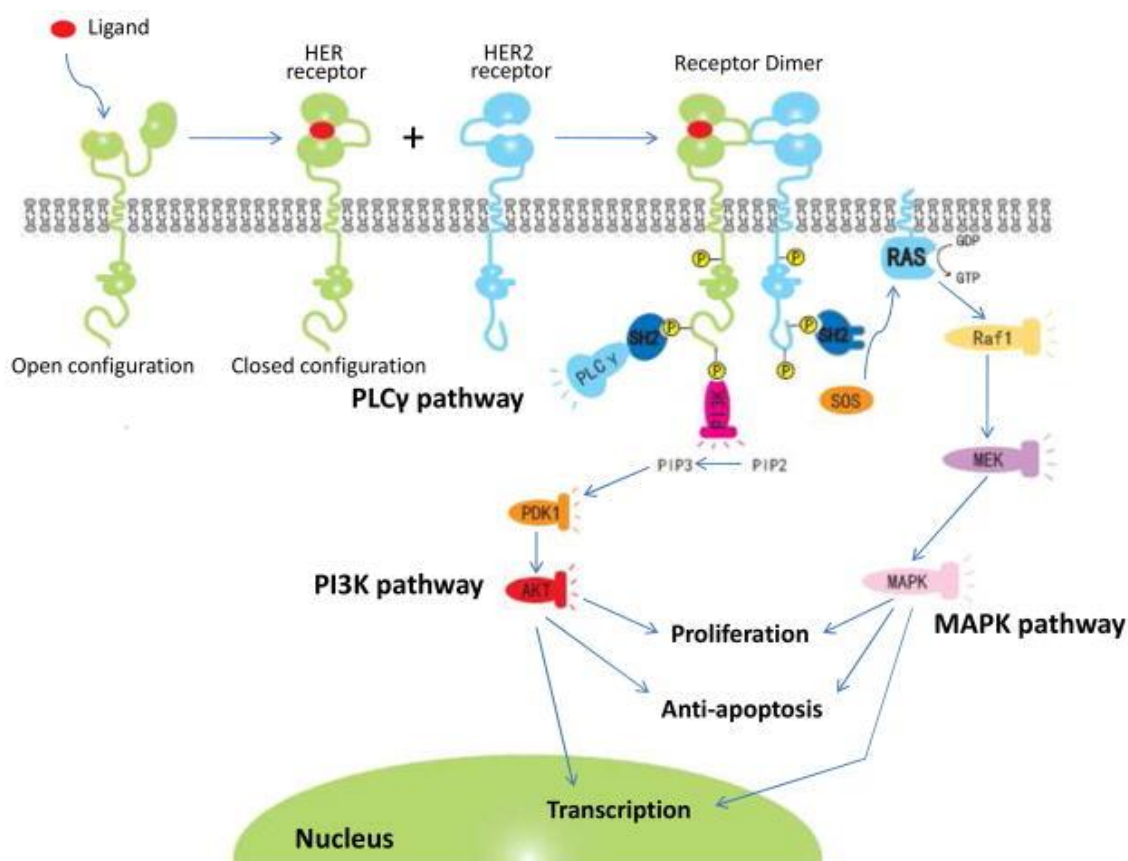


Figure 4. HER-Mediated Signal Transduction: The HER heterodimerization is conducive to cellular survival through multiple pathways including MAPK, PLC, and PI3k.¹⁹ Reproduced with permission.

To date, there are at least three known members of the MAPK family: the extracellular signal-regulated kinase (ERK), Jun kinase (JNK), and the p38 kinase. A variety of extracellular activators can transduce a signal via an elaborate network of many mediators to stimulate cell proliferation, differentiation, apoptosis, or aid in an inflammatory response.²⁶ The Akt pathway is reliant on the activation of PI3K via insulin receptor substrate (IRS), an adapter molecule. IRS

has an N-terminal phospho-tyrosine binding (PTB) domain,²⁷ allowing it to bind to the intracellular phosphorylated tyrosine residues on the HER heterodimers. The carboxy terminus of IRS contains serine and tyrosine phosphorylation sites that can be bound by the PTB domains of src-homology 2 (SH2) proteins, such as p85, where it regulates the effects of PI3K.²⁸ PI3K is a heterodimer composed of a regulator p85 subunit and catalytic p110 subunit. The p85-p110 complex is predominately inactive in its native state. However, HER dimerization and phosphorylation recruits PI3K, where the SH2 domain on the p85 subunit interacted with the phosphorylated tyrosine residues on the receptor tyrosine kinase.²⁹ This interaction removes the regulation by the p85 subunit, granting the p110 subunit to acquire catalytic activity. Activated PI3K then acts on lipid substrates due to now being within proximity to the plasma membrane. More specifically, PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3).^{30,31} PIP3 has both 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt binding domains. Considering PIP3 is a docking phospholipid as it is adherent to the cell membrane, the binding domains of PIP3 not only recruit PDK1 and Akt to the plasma membrane, but also bring them within close proximity for PDK1 to carry out its kinase function and phosphorylate Akt for activation. The phosphorylated Akt then relies on a multitude of downstream signaling pathways, mediators, and transcription factors to upregulate select gene transcription and potentiate tumorigenesis via enhanced cell survival, cell proliferation, and cell growth.³² Please refer to Figure 5 for a visual representation of the preceding description.

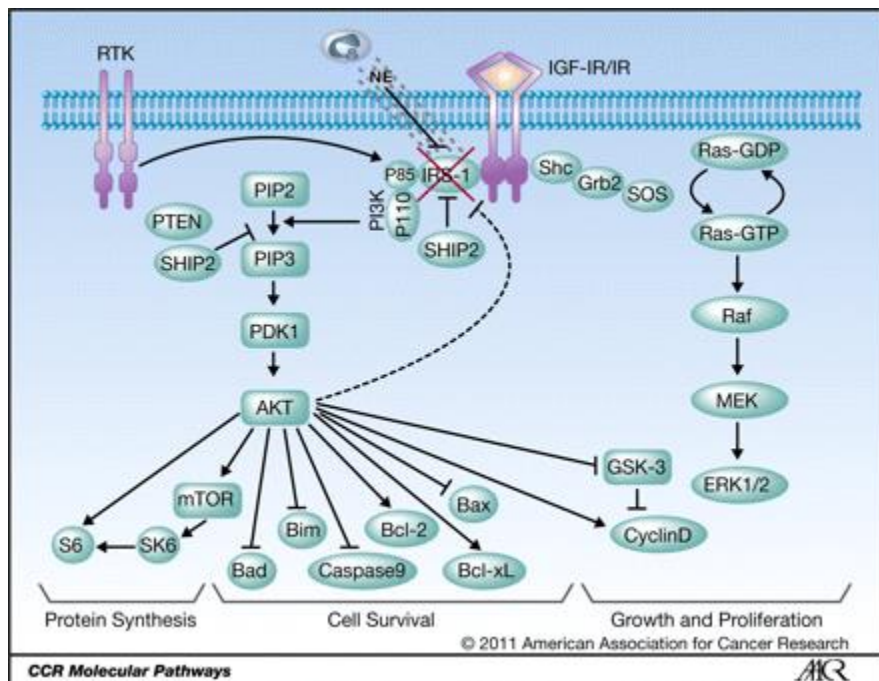


Figure 5. PI3K Activated Akt Pathway: Representation of RTK stimulation of PI3K (p85-P110) complex, leading to the conversion of PIP2 to PIP3 to activate PDK1, which phosphorylates Akt yielding cellular responses.²⁸ Reproduced with permission.

Having provided the background of the potential physiological cellular responses to HER receptor dimerization, one can gain the intuitive inclination that excessive stimulation through ligand-mediated dimerization of members within the epidermal growth factor receptor (EGFR) family, including HER1 (ERBB1), HER2, HER3, and HER4, can lead to tumorigenesis and mitogenesis. Publications argue that the most transforming combination of HER dimers is the formation of the HER2-HER3 heterodimer. Considering HER-2 has no currently identified extracellular ligand for activation, and HER3 is absent of an intracellular kinase domain, homodimers of HER2 or HER3 alone lack the ability to elicit signal transduction.³³ Yet, the HER2-HER3 heterodimer is claimed to be the most mitogenic receptor complex.³⁴ It has also been suggested that HER1-HER2 dimerization complexes greatly potentiates mitogenesis in the presence of EGF ligand.³⁵ Previous publications analyzing the transformation capacity of HER

proteins conclude that the co-existence of HER1 and HER2 proteins undergo more effective transformation with either HER1 alone, or HER1 and HER3 co-existence.³⁶ The HER receptors are normally internalized at varying rates in which they are degraded and recycled within intracellular endosomes and lysosomes. This internalization can be triggered by ligand-binding, dimerization, signal termination, or normal recycling at a basal rate. Furthermore, HER1 is known to have a considerably higher rate of internalization and downregulation compared to other EGFR family members, while HER3 is constitutively recycled.³⁷ It is worthy of acknowledging that in comparison, HER2 is known to be less expeditiously internalized due to a reduced rate of endocytosis.³⁸ One theory correlating HER2 overexpression with carcinogenesis is that heterodimer formations involving HER2 with other HER proteins reduces the frequency in which the other receptors are endocytosed and recycled assuming ligand binding and dimerization occurs. This could prolong the duration of HER1 and HER3 being on the cell surface and extend the time in which signal transduction is occurring as instances of internalization and deactivation maybe less frequent when HER2 participates in the dimerization. Not only does HER2 exhibit a slower rate of internalization, it is also said to contribute to cellular proliferation in an oncogenic capacity due to a presumptive increase in basal tyrosine kinase activity compared to other HER proteins, perhaps contributing to a more intense signaling response.³⁹ Existing publications also support the claim that HER2 coupling to the MAPK pathway is extremely efficient, further prompting mitogenesis through signal transduction.⁴⁰ As previously alluded to earlier, HER2 is a preferred partner within other EGFR members. This implies that HER2 functions as an amplifier of signaling initiated by EGF-like ligands.⁴¹ With all considered, one can surmise that HER2 over expression via mutation or overactivation can manifest in overwhelming signaling for cellular proliferation and growth, ultimately associated

with carcinoma. Publications within Clinical Cancer Research suggests that HER2 protein is overexpressed in approximately 10-15% of NSCLC, with reported prevalence as high as 30% in adenocarcinoma, another form of NSCLC.^{42,43} As previously mentioned, NSCLC makes up approximately 80% of all lung carcinoma. Therefore, HER2 overexpression affects a substantial portion of those affected with lung cancers. A study published within the British Journal of Cancer investigated 238 non-small cell lung cancer cases for the prevalence of HER2 overexpression by the utilization of immunohistochemistry. Of those included within the study, 35% of those with adenocarcinomas, 20% of those with large cell carcinoma, and only 1% of those with squamous cell carcinoma were considered to have substantial overexpression of HER2.⁴⁴ Though not all cases of lung carcinoma are HER2 positive, this tyrosine kinase receptor still has prevalence in the disease and can be considered a distinguishing molecular target for a target-based therapy. In addition, HER2 positive carcinoma is associated with poorer prognosis and linked to shortened survival.⁴⁵ This emphasizes the significance of a targeted therapy specific for HER2 overexpression.

Trastuzumab as the ADC Selective Element

With an ideal molecular target of interest, the basis is provided for the development of an antibody-drug conjugate (ADC) specific for HER2 proteins. This would involve using a monoclonal antibody, trastuzumab, specific for HER2 receptors. Trastuzumab, or also referred to by the brand name Herceptin, is an already existing therapy used for HER2 positive breast and gastroesophageal cancers. As previously alluded to earlier, HER2 positive tumors are associated with a significant increase in growth rate as well as cellular proliferation.⁴⁶ Trastuzumab can be used for either adjuvant or neoadjuvant therapy towards these tumors. Adjuvant therapy can be considered treatments provided in conjunction with the primary therapy, either surgical

interventions or initial chemotherapeutics, in the efforts to maximize the disease-free duration. For instance, an example of a primary chemotherapy treatment regimen for various forms of cancers involves chemotherapeutic agents Adriamycin and Cytosin as a combination, then administration of Taxol and Herceptin also in combination (AC-TH).⁴⁷ Neoadjuvant therapy is classified as treatments prior to primary therapy with the intent of enhancing the effectiveness of the primary intervention or aiding in the chances of disease-free survival. In this capacity, Herceptin or pertuzumab monoclonal antibodies can be administered in conjunction with chemotherapeutic agent Taxotere (docetaxel) pre-operatively.⁴⁸

As previously emphasized the HER2 receptor is composed of extracellular, transmembrane, and intracellular kinase regions. The extracellular region contains an L-domain, which includes domains I and III (~190 amino acids each), and a cysteine-rich domain, which includes domains II and IV (~120 amino acids each). Please refer to Figure 6.

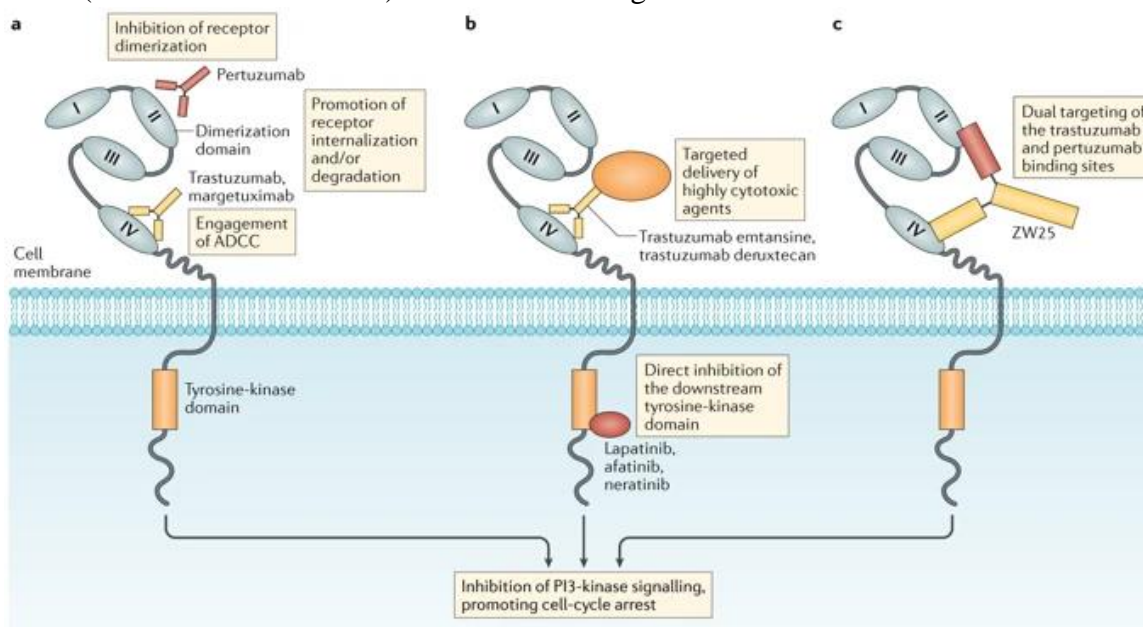


Figure 6. Trastuzumab and Pertuzumab Binding Locations on HER2 Receptor: A) Trastuzumab binds to domain IV while Pertuzumab binds to domain II, both preventing dimerization and signal transduction. B) Trastuzumab ADC with a cytotoxic payload. C) ZW25 produced antibody display dual-paratopes for recognizing two different epitopes of the HER2 receptor.⁴⁹ Reproduced with permission.

The variable regions of Trastuzumab (VL and VH) bind to domain IV of HER2, or also known as the juxtamembrane trastuzumab-binding domain, which is normally occupied by the extending fingers of domain II of adjacent HER proteins upon dimerization.^{50,51} Three distinct locations within the c-terminal end of domain IV of HER2 are recognized by the antigen-binding site of trastuzumab via electrostatic and hydrophobic interactions: HER2 residues 557-561, 570-573, and 593-603.⁵¹

The stand-alone trastuzumab monoclonal antibody displays anti-tumor effects by several proposed mechanisms, such as antibody-dependent cellular cytotoxicity.⁵² Other phenotypic changes that are observed upon trastuzumab binding to HER2 include downmodulation of HER2 receptors, inhibition of tumor cell growth, reversed cytokine resistance, restored E-cadherins expression levels correlative to tumor suppression, and reduced production of vascular endothelial growth factor.⁵³ Other hypothesized anti-tumorigenesis mechanisms of interest include prevention of HER2-containing heterodimer formation, initiation of cell cycle G1 stage arrest, inhibition of angiogenesis, and indication of immune mechanisms with the potential of targeting tumor cells.⁵⁴ The prevention of HER2-HER3 dimerization is also said to be key to significantly decrease downstream signaling.⁵⁵ With respect to immune-based tumor suppression, trastuzumab binds to hyper-proliferating cells via the overexpressed HER2 receptor. The Fc region of the antibody is recognized by immune cells, typically natural killer cells, to induce lysis of the cancer.⁵²

Despite trastuzumab being FDA-approved for chemotherapy, it is emphasized that before the initiation of trastuzumab for treatment, HER2 protein overexpression or gene amplification must be confirmed, ideally by further FDA-approved diagnostic assays specific for tumor type identification.⁴⁶ The rationale for this confirmation is correlated to the claim that trastuzumab is

only effective in tumors that are deemed HER2 positive. However, deleterious side effects of trastuzumab are possible during the utilization of any form of therapy and are worthy of consideration. Infusion reactions, pulmonary toxicity, cardiomyopathy, and even embryo-fetal toxicity can manifest as unintended effects during therapy.⁵⁶

The premise of an ADC is to integrate an antibody that selectively binds a molecular target that is ideally upregulated, overexpressed, or exclusive to a particular carcinoma of interest. The monoclonal antibody is to be covalently bound to a cytotoxic agent via a cleavable or non-cleavable crosslinker molecule. The selectivity of the antibody and cytotoxicity of the drug underscores the significance of an ADC used as therapy to achieve disease free survival.⁵⁷

Considering the specificity of trastuzumab for HER2 receptors, an ADC containing trastuzumab would be ideal for HER2 positive carcinoma, more specifically for HER2 positive NSCLC. The crosslinker of choice to conjugate the antibody to the drug plays an integral role in terms of the functionality of the ADC. Non-cleavable crosslinkers are incorporated in conjugation with the intent of the ADC binding to the cell of interest via binding of cell-specific surface receptors, becoming internalized into an endosome by binding-mediated endocytosis, and subsequent fusion of the endosomal vesicle with a lysosome. Lysosomal degradation metabolites include intact drug compounds and linkers attached to select amino acid functional groups such as amines or thiols. Much of the immunoglobulin is degraded due lysosomal proteolytic activity, yielding cytotoxic metabolites to carry out their intended function.⁵⁸ The chemotherapeutic agent primarily acts on the cell for which they were internalized and have low probability of affecting neighboring cells. Because many drugs are charged species, they have a low probability of crossing the cellular membranes of other cells with a lack of fascination. Furthermore, ADC with non-cleavable crosslinkers and charged drug species can be said to exhibit more selective cell

targeting and would be ideal for carcinoma that express a more homogenous population of target molecules.⁵⁹ The alternative method of ADC production involves the utilization of a cleavable crosslinker that can undergo autocleavage at a certain pH or becomes cleaved by a specific protease that detaches the drug from the monoclonal antibody. Although most cleaving of the ADC linker has been shown to occur intracellularly, the liberated drug compounds can leave the cytoplasm of one cell and cross the membrane of a neighboring cells assuming the compound is relatively nonpolar and has increased membrane permeability. Other studies indicate that extracellular tumor microenvironments can provide a reducing environment for cleavable disulfide linkers, or increased acidity for pH-dependent cleavable linkers, suggesting that ADC containing cleavable crosslinker are less stable and more susceptible to extracellular dissociation, further enhancing the ‘bystander effect’ where neighboring cells are vulnerable.^{60,61} The significance of this named ‘bystander effect’ is that antibody-facilitated internalization is not required for drug entrance assuming the ADC payload has increased membrane permeability.⁶² Please refer to Figure 7 for a depiction of ADC mechanisms of action.

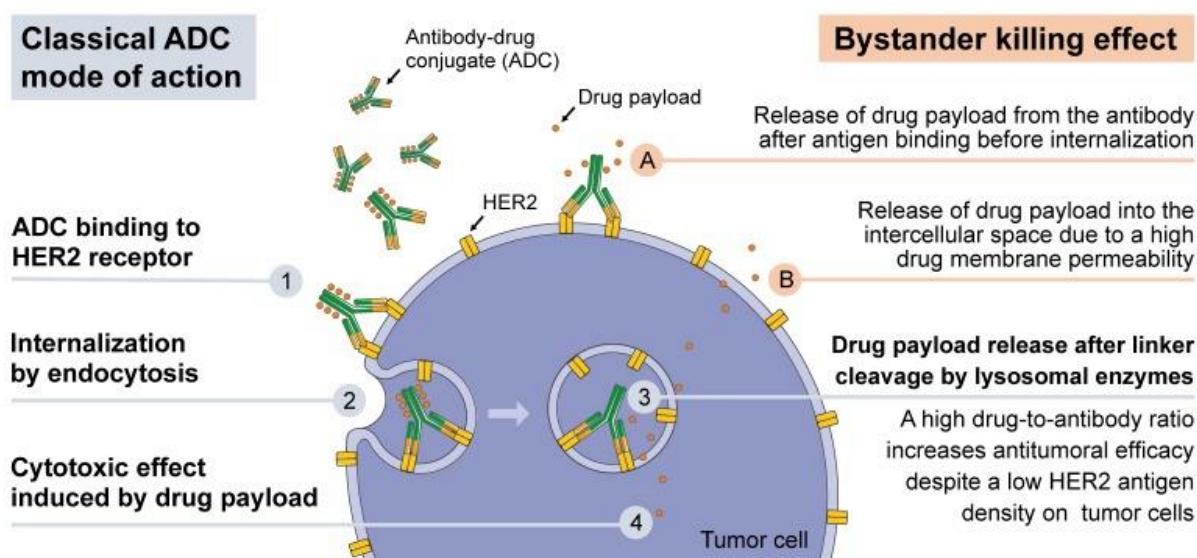


Figure 7. ADC Mechanism of Action: Classical ADC mode of action requiring internalization, as well as the bystander killing mechanism that potentiate the extracellular liberation of the chemotherapeutic agent.⁶³ Creative Commons Attribution 4.0 International License.

Existing Trastuzumab Antibody Drug Conjugates; T-DM1

To date, T-DM1, or Kadcyla is the only approved ADC for the treatment of advanced HER2 positive breast cancer.⁶³ T-DM1 is a second-generation ADC containing an IgG1 monoclonal antibody specific for HER2 proteins, trastuzumab. The drug DM1, or mertansine, is derived from its parent compound maytansine, and is differentiated by the addition of a thiol group to a terminal amide functional group. DM1 and maytansinoid compounds displays high affinities to soluble tubulin and have the ability to inhibit microtubular polymerization, leading to mitotic arrest.⁶⁴ This potent inhibition of microtubule assembly not only revealed mitotic catastrophe by the discovery of aberrant mitotic states, but also is associated with cellular apoptosis.⁶⁵ The prevention of microtubule elongation hinders the formation of functional mitotic spindles, unattachment to kinetochores on sister chromatids at the centromeric region, and ultimately limits the ability to transition from metaphase to anaphase of the mitotic cycle resulting in multinucleated cell.⁶⁶ Please refer to Figure 8.

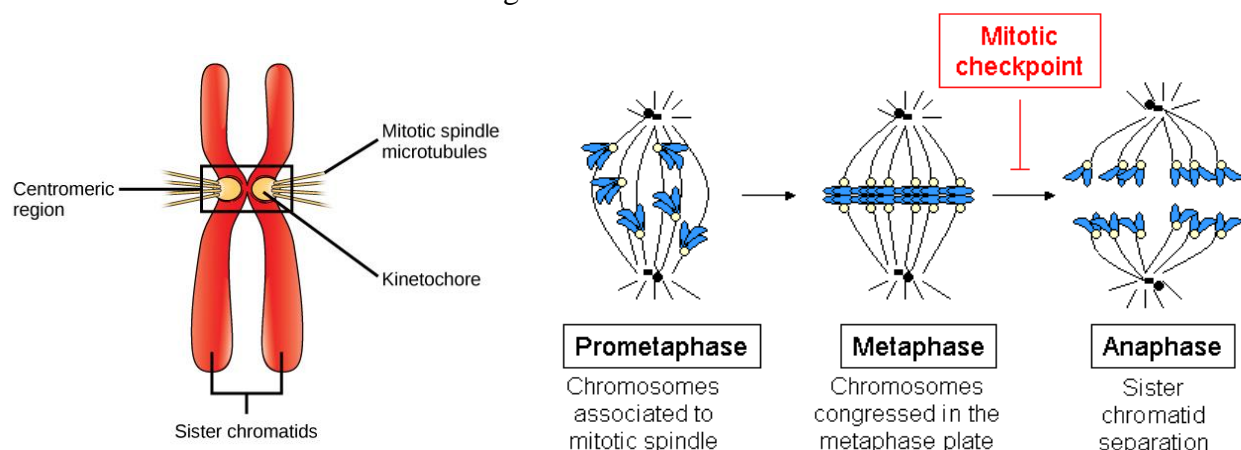


Figure 8. Cellular Replication Visuals: Centromeric region of replicated sister chromatids contain the kinetochore in which non-elongating microtubules cannot attached due to the inhibitory binding of DM1. Metaphase arrest manifests as a result.^{67,68} Creative Commons Attribution 4.0 International License.

Trastuzumab and DM1 is conjugated together via the non-cleavable crosslinker SMCC, or succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate. A free thiol or sulfhydryl

group from DM1 acts on the maleimide group on SMCC to form a stable thioether bond. This DM1-SMCC conjugated compound is termed emtansine. A primary amine functional group from lysine residues within the immunoglobulin structure can act as a nucleophile by attacking the electrophilic carbon atom of the ester group of emtansine SMCC to transform the ester into an amide. This results in the dissociation of the succinimide group of SMCC, rendering the maleimidomethyl cyclohexane-1-carboxylate (MCC) thioether linker conjugating trastuzumab with DM1.⁶⁶ Please refer to Figure 9 for a visual representation of the described conjugation.

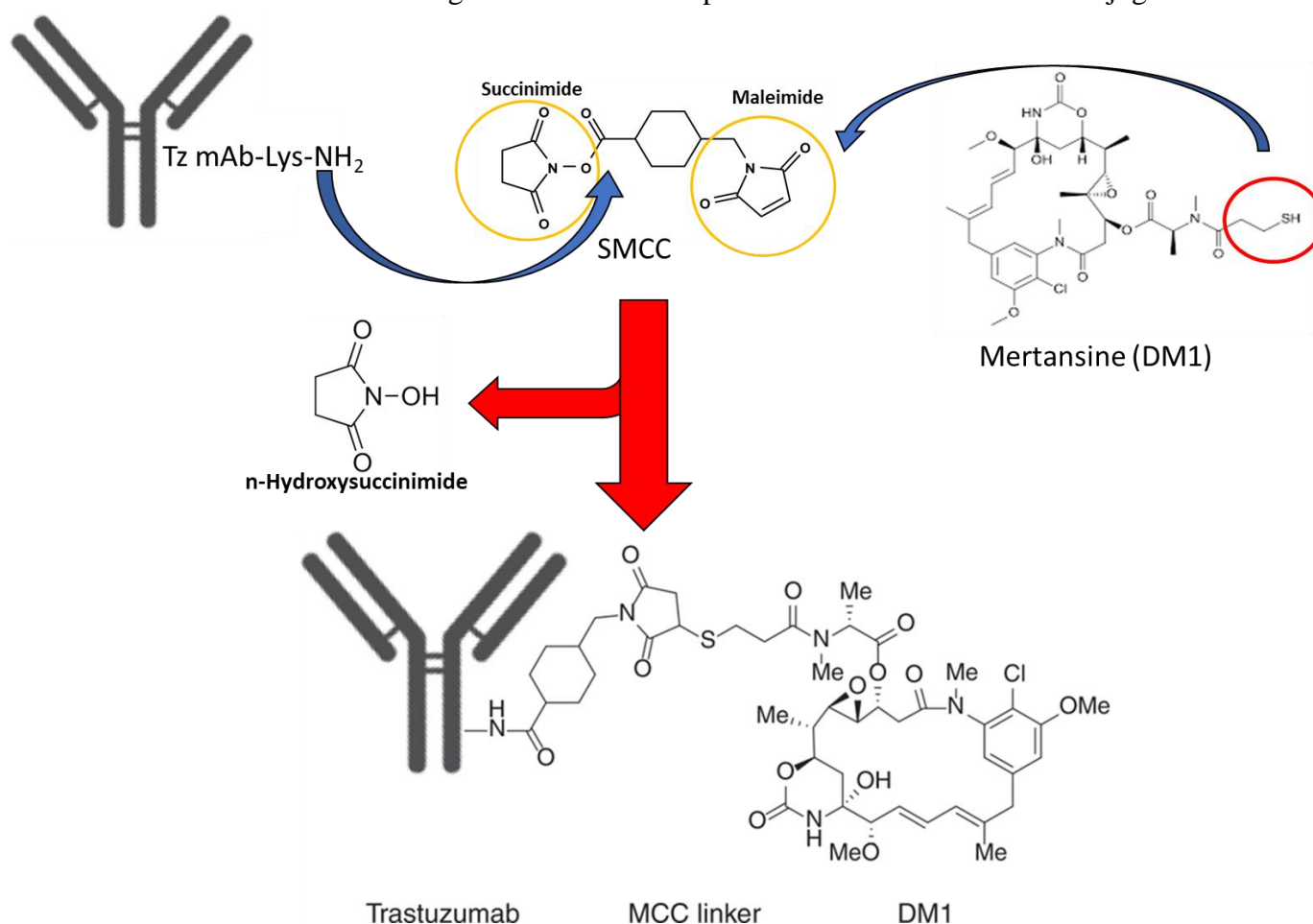


Figure 9. T-DM1 Constituents: T-DM1 is composed of the trastuzumab monoclonal antibody, non-cleavable SMCC linker, and DM1 maytansinoid. The final MCC linker of the ADC, after the loss of n-hydroxysuccinimide, is covalently bound to DM1 via a thioether bond, and covalently bound to trastuzumab via an amide bond with the amine provided by immunoglobulin lysine residues.^{66,69,70} Note: This figure is not intended to portray a chemical reaction, but merely a depiction of the T-DM1 constituents and to which molecular structures they bind. Components reproduced with permission.

T-DM1 is considered an effective antitumor agent by its involvement in at least three mechanisms. The first mechanism involves the simple inhibition of HER receptor dimerization with the binding of trastuzumab to the domain IV of the HER2 receptor, a favored dimer partner proven to elicit greater downstream signaling. The second mechanism involves the prevention of the extracellular HER2 domain shedding upon antibody binding. This prolongs the duration for which the molecular target for T-DM1 is presented by the tumor cells and increases the ability of innate immune effector cells to recognize the Fc region of trastuzumab. In this aspect, the patient's own immune system can aid in the efforts to reduce the tumor viability.⁷¹ The last mechanism discussed pertains to the conventional functions of an ADC with a non-cleavable crosslinker. This involves the internalization of T-DM1 via receptor-mediated endocytosis.⁶⁶

Please refer to Figure 10 for a visual representation of the three discussed mechanisms.

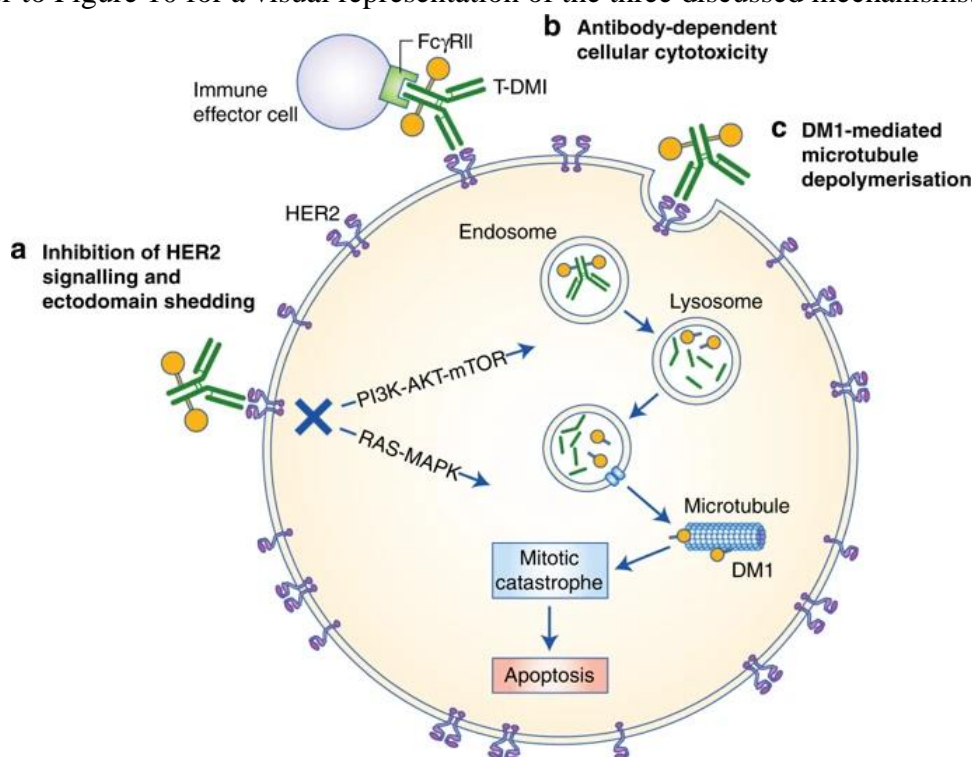


Figure 10. T-DM1 Mechanisms of Action via HER2 Receptor Binding: A) Trastuzumab of T-DM1 binding HER2, preventing extracellular shedding of the receptor and preventing dimerization. B) The Fc region provided by T-DM1 can elicit the activation of immune effector cells. C) T-DM1 receptor-mediated internalization.⁶⁶ Reproduced with permission.

After receptor-mediated endocytosis, the endocytic vesicles fuse with lysosomes to permit the lysosomal degradation of antibody component of the ADC. The liberated metabolites contain lysine-*N*^E-MCC-DM1 products. These compounds are considered charged cytotoxic metabolites at physiological pH ranges due to the presence of charged amino and carboxyl groups of the lysine residues, making them largely impermeable to plasma membranes.⁷² It is also suggested that this charged lysine and crosslinker-yielding metabolite is considerably less cytotoxic than the DM1 (mertansine) drug alone, potentially contributing to a beneficial effect in terms of tolerable doses.⁷³ Interestingly, the charged nature of these metabolites are said to necessitate active transport in order to exit the lysosomal membrane, which strengthens the claim that these charged metabolites have lower probability of freely crossing the cellular plasma membrane. This lessens the risk of the ‘bystander effect’ in which neighboring HER2 negative cell could be subjected to cytotoxic T-DM1 metabolites.⁷⁴ Once in the cytoplasm, the maytansinoid-containing metabolite can then execute its intended function of microtubule polymerization inhibition, ultimately leading to decreased intracellular trafficking required for normal cell functions and metaphase arrest of dividing cells causing death of ideally cancerous cells.⁷⁵

Despite HER2-targeted therapies being standard in breast and gastric cancer, with T-DM1 being the only approved ADC for the treatment of HER2 positive breast cancer, there currently are no standard therapies specific for the targeting of HER2 proteins in NSCLC. However, there are growing ambitions to test the efficacy in HER2 positive NSCLC patients by the initiation of undergoing clinical trials. A publication within *Clinical Cancer Research* medical journal from January 2019, described a phase II study analyzing the effect of HER2-targeted ADC trastuzumab emtansine (T-DM1) on HER2 positive NSCLC. Subjects within the study were divided by HER2 positive status, either immunohistochemistry (IHC) 2+ or 3+. A total of 49

patients (29 IHC 2+, 20 IHC 3+) were eligible to participate in the study, in which all patients received T-DM1 (3.6 mg/kg intravenously every 3 weeks) until investigator-assessed disease progression, detection of unmanageable toxicity, or study termination. Survival status was assessed every 3 months, while radiographic evaluations of tumor assessment were conducted every 6 weeks. Of those involved in the study, no treatment responses were observed in the IHC 2+ group, however, four of the 20 IHC 3+ patients were said to reveal responses to T-DM1 treatment, resulting in a 20% overall response rate (ORR).⁷⁶ T-DM1 is also said to display potent inhibition of *in vitro* growth of HER2 positive IHC 3+ Calu3 and H2170 NSCLC cells and IHC 2+ H1781 NSCLC cells.⁷⁷ Mouse xenograft tumor models of Calu3 and H1781 cells also revealed robust antitumor activity when treated with T-DM1.⁷⁶ These results suggest that the potentiation for a HER2-targeted therapy, such as an ADC, is very relevant for the treatment of NSCLC and should be pursued further.

Despite the known beneficial therapeutic effects of T-DM1 in the treatment of advanced HER2 positive breast cancers, it is reported that patients can eventually develop T-DM1 resistance where the anti-tumorigenesis effect becomes lost.⁷⁸ A study published within the International Journal of Cancer analyzed the effects of T-DM1 against T-DM1-resistant gastric cancer cell line N87-TDMR. It was noted that N87-TDMR cells acquired upregulated ATP-binding cassette (ABC) transporters ABCC2 and ABCG2. However, the addition of MK-571, a leukotriene D4 antagonist and is also said to be a multidrug resistance protein 1 (MDR1) inhibitor, caused N87-TDMR to regain T-DM1 sensitivity. This observation suggests that ABC transporter expression has a direct correlation with T-DM1 resistance. This assumption correlates with other publications stating that maytansinoids, such as DM1, among other cytotoxic compounds are substrates for MDR1 or ABC transporters, thus may exhibit reduced efficacy due to cellular

efflux.⁷⁹ It was noted that T-DM1 mediated cytotoxicity can be considered a threshold event. In other words, a given intracellular concentration threshold of Lys-MCC-DM1 must be achieved to elicit cell death.⁸⁰ The upregulation of efflux transporters such as MDR1 can prevent normal ADC therapeutic doses from achieving the intracellular threshold. Furthermore, an exatecan-derived topoisomerase inhibiting compound, deruxtecan (Dxd), was able to inhibit the growth of T-DM1 resistant cell line N87-TDMR.⁷⁸ The suggestion was made that the Dxd compound may be a less effective substrate for ABC transporters and other efflux proteins. T-DM1 resistance can also arise from impaired lysosomal release mechanisms. The lysosomal degradation product of T-DM1 after internalization is Lys-MCC-DM1, which is charged and largely membrane impermeable. A functional genomic screen of transporter genes identified *SLC46A3* which encodes for a lysosomal membrane protein capable of transporting maytansine-derived drug linker metabolites from the lysosome to the cytoplasm in order to avert the inability to passively diffuse across the lysosomal membrane.⁸¹ The decreased expression of *SLC46A3* reduces the potency of T-DM1, as the drug linker complex is not readily released from the lysosome. Interestingly, it was noted that ADCs with alternative payloads were not affected by the expression levels of *SLC46A3*, suggesting DM1 is the primary substrate for this lysosomal membrane transport protein.⁶⁶ *SLC46A3* expression levels have been proposed as a selection biomarker for those who should receive T-DM1 as a cancer therapy, or those who should not due to low expression causing reduced lysosomal efflux and subpar therapeutic effects.⁸²

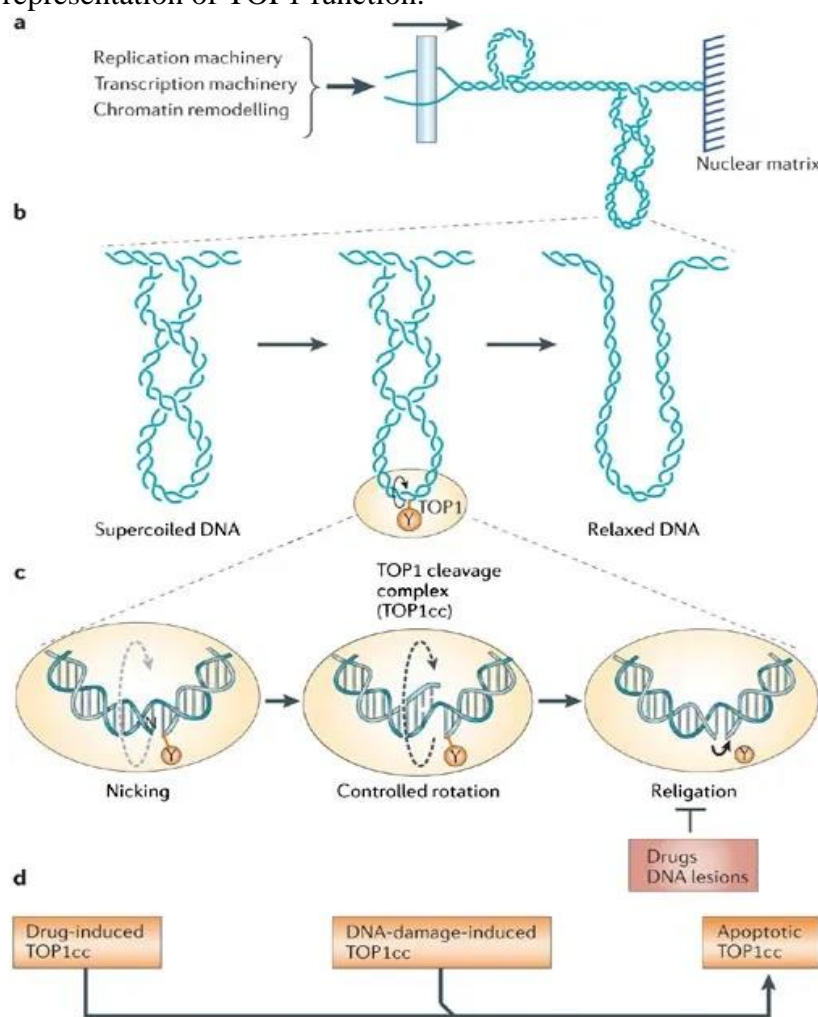
Another mechanism in which cancer cells can become resistant to DM1 and other maytansinoid derivatives is through the reduced expression of cellular cyclin B1 which is required for the progression into the M-phase, or mitosis, of the cell cycle via the activation of cyclin-dependent kinase 1 (CDK1).⁸³ Normally, cyclin B1/CDK1 becomes degraded, promoting the exit of mitosis

to enter the next phase of the cell cycle, G1 phase. Cellular checkpoints during mitosis requires all kinetochores on each sister chromatid be attached to microtubule. The cyclin B1- cyclin-dependent kinase 1 (CDK1) acts as the checkpoint regulator, and only upon its degradation can progression into anaphase and thus mitosis exit occur.⁸⁴ Reduced induction of cyclin B1/CDK1 not only results in the malfunction of mitotic spindles, but lower levels also simulate the physiological stimulus to exit mitosis, hindering the ability to halt cell cycle progression. Mitotic exit and cytokinesis, or division of cellular cytoplasm occurs, resulting in the avoidance of mitotic catastrophe-mediated apoptosis. The products resulting from the atypical mitotic event manifest in altered daughter cell karyotype inheritance due to improper chromosome separation, leading to genetically instable progeny.^{66, 85} Though some cells may ultimately be inviable, others may contribute to a greater magnitude of malignancy all the while avoiding the cytotoxic effect of DM1.

Potential of Exatecan-Based Antibody Drug Conjugates

Even though approved ADCs containing tubulin polymerization inhibiting-based payloads are considered effective in terms of reducing cancer cell proliferation, there are other preclinical antitumor drug candidates currently under development that show extraordinary promise. One of the novel drug candidates under current research are exatecan-derived agents. In particular, DS-8201a is a promising HER2 selective ADC that contains a human monoclonal IgG1 produced with reference to the same amino acid sequence as trastuzumab.⁸⁶ The payload is a potent topoisomerase I inhibitor, deruxtecan (Dxd), which is conjugated to the anti-HER2 antibody via a cleavable peptide linker; maleimidocaproyl-glycine-glycine-phenylalanine-glycine-aminomethylene (mc-GGFG-am).⁸⁷ This cleavable linker was designed in efforts to be cleaved by lysosomal enzymes cathepsin B and L, where T-DM1 has a non-cleavable linker.⁸⁸

Topoisomerase I (TOP1) is a conserved enzyme essential for relaxing supercoiled DNA to alleviate helical constraints. Supercoiling can manifest as a consequence of DNA or RNA polymerases unwinding double stranded DNA (dsDNA) for DNA replication or transcription.⁸⁹ TOP1 binds to supercoiled double stranded DNA and cleaves one strand to permit the relaxation of the coil, and aids in the reannealing process. If TOP1 is not functioning, supercoils can halt replication forks, inhibiting further transcription, or cause dsDNA breaks.⁹⁰ Please refer to Figure 11 for a representation of TOP1 function.



Copyright © 2006 Nature Publishing Group
Nature Reviews | Cancer

Figure 11. Topoisomerase I Function: A) Generation of dsDNA supercoils. B) TOP1 cleavage complex (TOP1cc) formation. C) DNA uncoiling mechanism via nicking, unravelling and religation. D) TOP1cc stabilizing mechanisms leading to inhibition.⁹¹ Reproduced with permission.

Camptothecins are naturally occurring TOP1 inhibitors, in which TOP1 is the only known target. Camptothecin derivatives topotecan and irinotecan are TOP1 inhibitors that have recently been approved by the FDA for the treatment of ovarian and colorectal cancers respectively. Irinotecan (CPT-11) must be hydrolyzed by carboxylesterase to produce the active metabolite SN-38.⁹²

TOP1 inhibitors work by binding to TOP1 cleavage complexes (TOP1cc), represented in Figure 11. The selective binding at this enzyme-DNA interface, leads to their classification of 'interfacial inhibitors'. Such inhibitors bind to the interface of macromolecules, thus the TOP1cc provides the topoisomerase I and DNA, and when in conjunction with one another, provide the binding site for camptothecins.⁹³ It was said that camptothecin and its derivatives are very selective for such macromolecular complexes, leading to the fact that TOP1cc is the only target. Therefore, camptothecins are argued to be ideal pharmacological agents as their selectivity, rather than potency, can be exploited for precise control contributing to therapeutic effect.⁹¹

Of the several camptothecin derivatives poised to become the pharmacological agent in many cancer treatments, exatecan shows great promise. In a study published in *Cancer Chemotherapy and Pharmacology*, the therapeutic effects of exatecan mesylate, in its salt form (DX-8951f), was compared to that of other FDA approved camptothecin derivatives such as irinotecan (CPT-11) and topotecan (SK&F104864) using human tumor xenografts in nude mice. A total of 16 human cancer lines were examined: 6 colon cancers, 5 lung cancers, 2 breast cancers, 1 renal cancer, and 2 gastric cancers. The two gastric cancer cell lines included a CPT-11 sensitive tumor, gastric adenocarcinoma SC-6, and its CPT-11 resistant variant, SC-6/CPT-11. After a total of four injections four days apart, exatecan revealed a tumor growth inhibition rate (IR) of $\geq 58\%$ on 15 of the 16 cancer cell lines and an IR $\geq 80\%$ on 14 of the cell lines. Alternatively, CPT-11 displayed an IR $\geq 58\%$ on 11 of the cell lines and an IR $\geq 80\%$ on only 8 of the 16 cell lines. In

addition, exatecan was considered effective against the gastric line SC-6/CPT-11, where CPT-11 obviously was not effective. A summarization of the study results suggest exatecan revealed superior antitumor activity when applied to different cancer cell lines and over a broader range of doses than other camptothecin derivatives such as irinotecan (CPT-11) and topotecan (SK&F104864).⁹⁴ It has also been suggested that the inhibitory effects of exatecan are 3 and 10 times more than that of the CPT-11 active metabolite (SN-38) and topotecan respectively.⁹⁵ In addition, many chemotherapeutics are rendered noneffective due to P-glycoprotein (P-gp) mediated multidrug resistance via cellular efflux. Exatecan, however, is suggested to overcome P-gp mediated drug resistance, as it most likely is an incompatible substrate to the membrane drug transporter.^{96,97}

As previously mentioned, deruxtecan (Dxd), an exatecan derivative, was able to inhibit the growth of T-DM1 resistant cell line N87-TDMR due to the hypothesis that maytansinoids, such as DM1, among other cytotoxic compounds are substrates for MDR1 or ABC transporters. In addition, cancer patients can soon acquire DM1 resistance due to mechanisms previously discussed involving the reduced expression of lysosomal transporters for maytansinoids and mitosis exiting events. These findings strengthen the objectives of producing an ADC with a payload containing a novel camptothecin TOP1 inhibitor. A study published within Clinical Cancer Research suggests that DS-8201a with the Dxd payload is a superior therapy for T-DM1 insensitive carcinoma. T-DM1 carries an average of 3.5 MCC-DM1 drug linker complexes, or in other words is said to have a drug-antibody ratio (DAR) of 3.5,⁹⁸ where as DS-8201a has a DAR of approximately 8.⁸⁶ Despite the impressive DAR of DS-8201A, other publications suggest that reduced drug loading per antibody can increase the therapeutic index by contributing to increased stability, slower *in vivo* drug clearance, and reduced toxicity due to off-target

cytotoxicity from liberated chemotherapeutic agents in the plasma.^{86,99} The tetrapeptide within the DS-8201a ADC crosslinker is cleaved by lysosomal proteases, such as cathepsin B and L, which are highly expressed in rapidly proliferating tumor cells.^{100,101} As previously alluded to earlier, carcinomas exhibit tumor-environments which can potentially deviate from non-diseased systemic conditions. Rapid cell proliferation and apoptosis may contribute to the variation of proteases within the tumor-environment. Multi-cancer states may add complexity to the magnitude and distribution of such tumor-environments, promoting extracellular drug release that may result in the ‘bystander effect’ and other off-target effects.

Proposal of HER2 Selective ADC Conjugated to Exatecan

Given the potential of a trastuzumab-containing ADC in the treatment of HER2 positive NSCLC, one may find it apparent that the evidence has been put forth to support the production of an ADC including HER2 selective trastuzumab as the monoclonal antibody. Considering the provided cellular mechanisms of T-DM1 resistance, novel camptothecin molecules appear more appealing. Of the camptothecin derivatives, an overwhelming number of publications suggest exatecan is more potent across a broader range of concentrations and is less susceptible to resistivity by various carcinoma. In order to maximize stability and specificity, a non-cleavable crosslinker such as SMCC would be ideal. This requires nearly the entire immunoglobulin structure of trastuzumab to be degraded before release of an active metabolite. This is virtually exclusively a lysosomal process after internalization. A proposed mechanism of the conjugation reaction involves the succinimide group of sulfo-SMCC reacting with a primary amine from exatecan. Next, a thiol provided from trastuzumab must react with the maleimide group of sulfo-SMCC. Please refer to Figure 12 for a visual representation of the proposed trastuzumab-SMCC-exatecan ADC.

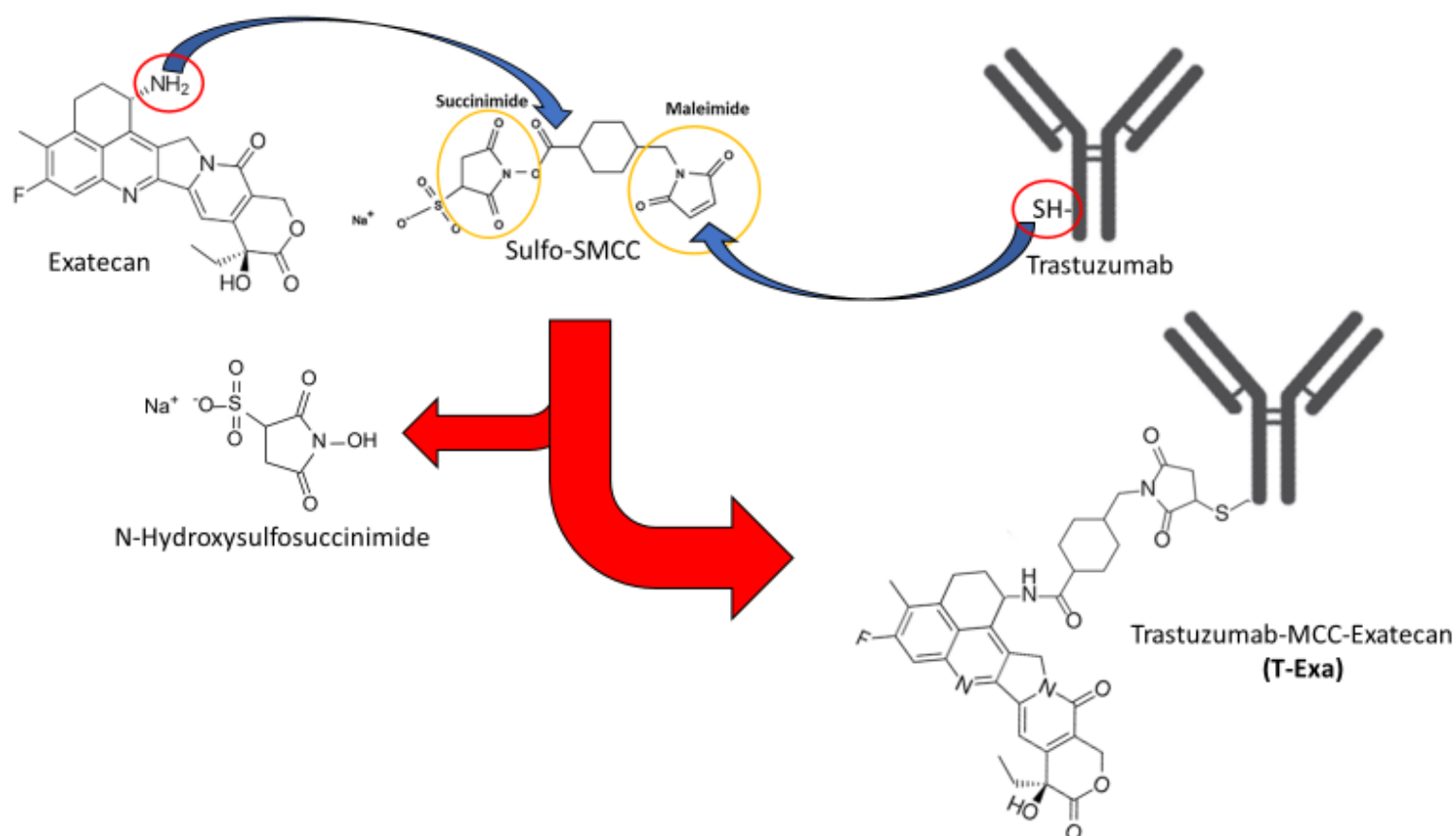


Figure 12. Proposed Constituents of a Synthesized Trastuzumab-MCC-Exatecan ADC:

Trastuzumab-MCC-Exatecan ADC (T-Exa) is produced by the primary amine group of exatecan attacking the electrophilic carbon of the ester bound to the succinimide group of sulfo-SMCC, producing an amide linkage. N-hydroxysulfosuccinimide is lost as a proposed byproduct. A thiol group from reduced inter-chains of trastuzumab initiates the thiol-maleimide reaction, completing the conjugation.^{102,103} Components reproduced with permission.

Trastuzumab, and other IgG1 antibodies contain a total of 32 cysteine residues resulting in 16 disulfide bonds: 12 pairs of intra-chain disulfide bonds and 4 pairs of inter-chain disulfide bonds, with very low observed free thiol variants.¹⁰⁴ Of the 4 pairs of inter-chain disulfide bonds, a disulfide bond exists between each of the two associated light and heavy chain connections, as well as 2 disulfide bonds between the two heavy chains near the hinge region.¹⁰⁵ It is well published that the 4 inter-chain disulfide bonds are more susceptible to reducing agents than the intra-chain disulfide bonds. More specifically, the inter-chain disulfide bonds between light and

heavy chains are slightly more susceptible to reduction than the 2 disulfide bonds between the 2 heavy chains.¹⁰⁶ The proposed mechanism involves the exploitation of the reduced inter-chain disulfide bonds to provide the thiol groups required for the maleimide-thiol reaction. Only after verification of each stage of conjugation is confirmed, the final proposed T-Exa ADC must be analyzed in terms of selectivity and cytotoxicity. Comparison of such a synthesized ADC to another approved selective cancer therapy will be crucial in order to fulfill the project objectives.

CHAPTER 2: OBJECTIVES, HYPOTHESIS, AND AIMS

The main objective of this project is to synthesize an ADC specific for HER2 positive NSCLC that displays even greater cytotoxicity than other approved ADCs with maytansinoid-derived payloads, such as TDM-1. Literature reviews suggest this can be achieved by the utilization of a novel camptothecin-based payload, and of the published derivatives, exatecan appears to be the most effective. In order to maximize stability and minimize the ‘bystander effect’ it was decided to incorporate a non-cleavable crosslinker attributed to SMCC, unlike DS-8201a which relies on a cleavable peptide linker. With all constituents strategically selected for and highlighted in Figure 12, the cytotoxicity of a synthesized trastuzumab-SMCC-exatecan (T-Exa) ADC will be tested *in vitro* on HER2 positive and HER2 negative cancer cell lines. In addition, the products of the intermediate phases of the conjugation process, such as unreduced and reduced trastuzumab will also partake in the cytotoxic analysis as controls. In order to determine that the camptothecin exatecan is more effective than DM1, the cytotoxicities of the two drugs will be independently compared for this measure. Finally, the synthesized T-Exa will be compared to commercially available T-DM1, in terms of which ADC offers greater effectiveness in decreasing both HER2 positive and negative tumor cell viability.

Project Aims

Aim 1. Produce the anti-HER2 antibody-drug conjugate associated with exatecan

- Develop a protocol to effectively conjugate the HER2 selective monoclonal antibody trastuzumab to the cytotoxic agent exatecan via the non-cleavable crosslinker SMCC.
- A protocol for trastuzumab disulfide bond reduction must also be produced.
- **Hypothesis:** Because the proposed mechanism for this conjugation reaction requires the presence of free thiol (-SH) groups, then conjugation can occur if antibody inter-chain disulfide bond reduction is achieved.

Aim 2. Test the effectiveness of the conjugation reaction

- Verify trastuzumab reduction and recovery via Ellman's thiol detection assay and BCA protein quantification assay.
- Use mass spectrometry to identify ADC products after the conjugation protocol.
- **Hypothesis:** If antibody reduction and conjugation are successful, then ADCs should be synthesized with varying DARs, which can be quantified using mass spectrometry. In addition, the extent of reduction should limit the final DAR.

Aim 3. Verify the function of the anti-HER2 antibody-drug conjugate

- After verification of production, the synthesized T-Exa ADC will undergo *in vitro* cytotoxic analysis on HER2 positive and negative cancer cell lines using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay.
- Compare the effectiveness of exatecan and DM1 in terms of *in vitro* cancer cell growth inhibition on both HER2 positive and negative cancer cell lines.
- Compare the effectiveness of synthesized T-Exa and commercially purchased T-DM1 in terms of *in vitro* cancer cell cytotoxicity on both HER2 positive and negative cell lines.
- **Hypothesis:** If it can be demonstrated that exatecan is more cytotoxic on HER2 positive cancer cell lines when compared to DM1, then synthesized T-Exa should be more cytotoxic than T-DM1 and in a cell selective manner based on HER2 expression levels.

CHAPTER 3: MATERIALS AND METHODS

Trastuzumab Reduction

For the monoclonal antibody reduction, Trastuzumab (5 to 10mg) was mixed with reducing agent Dithiothreitol, or DTT (Sigma Aldrich, D9779) at 50x to 100x nanomolar (nmol) concentration. DTT is a dithiol enantiomeric reducing agent, effective for reducing disulfide bonds, such as those within the trastuzumab structure, to free thiol groups. Ideally, DTT would preferentially target disulfide bonds participating in inter-chain interactions, as sterics make intra-chain reduction less favorable. As a consequence of forming thiol groups from disulfide bonds, DTT itself becomes oxidized as its two thiol groups combine to form a cyclic disulfide 6-membered ring. Please refer to figure 13 for the DTT chemical reaction.

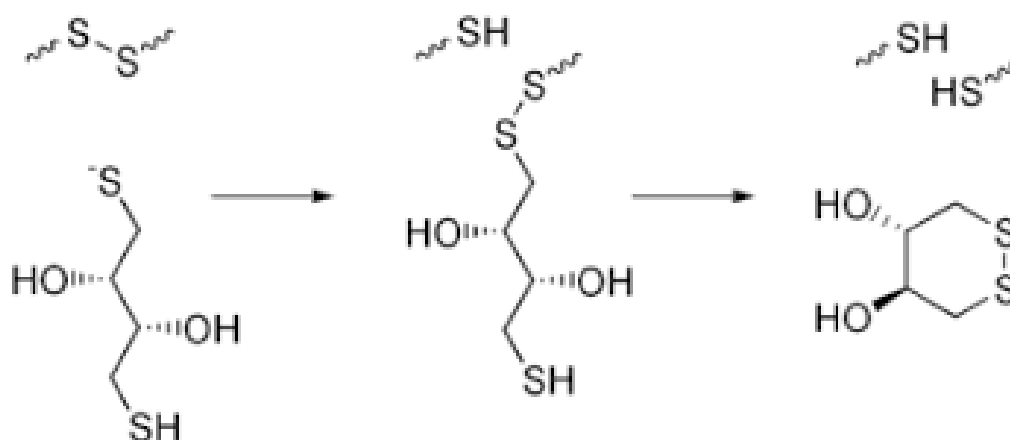


Figure 13. DTT Disulfide Reduction Chemical Reaction: DTT initiates a reduction-oxidation (redox) reaction in which the native antibody disulfide bonds become reduced while the DTT molecule itself becomes oxidized due to the formation of a cyclic disulfide bond. Note: DTT is considerably unstable in its reduced form, however, the free thiolate anion (-S⁻) of DTT is reactive, rather than the free thiol group (-SH). Therefore, the reaction must be carried out in an alkalotic environment.^{107,108} Reproduced with permission.

For smaller volume reductions, it is recommended that the reaction be brought up to at least 500 uL with Ellman's reaction buffer; 1mM EDTA in 10x Dulbecco's phosphate-buffered saline (DPBS) to act as a buffer and contribute to a more alkalotic environment. The reaction was

conducted at 37°C while stirring for 0.5 to 1 hour. After the incubated stirring stage, the reaction underwent a wash step which involved spinning the reduction reaction in a 100,000 molecular weight cut-off (MWCO) centrifugal column filter (Sartorius, VS0642) at a spin force of 4,000 Relative Centrifugal Force (RCF) for 10 minutes. It is known that trastuzumab has a molecular weight of 148 kDa, with the light and heavy chains weighing approximately 23.4 kDa and 49.3 kDa respectively. It is also known that excessive reduction of the monoclonal antibodies can result in inter-chain dissociation due to the reduction of 4 inter-chain disulfide bonds connecting the heavy and light chains or adjacent heavy chains that significantly contribute to the tetrameric quaternary structure. If this were to occur in any degree of severity, the various molecular fragments would theoretically be washed through the column filter while the relatively standard-sized antibodies weighing more than 100 kDa would be retained. It is crucial to extract only the full antibodies that still retain an intact variable region (VL and VH), as they retain binding specificity for the HER2 antigen. The chain fragments offer no specific antigen binding selectivity, thus it is of best interest that they be excluded for further testing. This filtration step was conducted 3 times, with 2 mL of 1x DPBS added to the sample for each spin. The final filtration spin resulted in approximately 100-200uL of reduced trastuzumab solution.

To verify reduction occurred, an Ellman's Reagent Assay was conducted to quantify the number of reduced thiols per antibody. This assay involves the mixture and incubation of three components; 100 µL of Ellman's Reaction Buffer (1mM EDTA in 10x Dulbecco's phosphate-buffered saline (DPBS)), 2 µL Ellman's Reagent, and 10 uL of the standards and samples to be tested. The mixtures were then incubated at room temperature for 15 minutes in the absence of light. The Ellman's Reagent (Thermofisher Scientific, 225852) includes compound 5,5'-dithio-bis-(2-nitrobenzoic acid), or also known as DTNB, which reacts with thiol groups to yield a

disulfide group and 2-nitro-5-thiobenzoic acid (TNB).¹⁰⁹ Production of TNB exhibits a yellow color in which the intensity correlates with the concentration of thiols from the sample.¹¹⁰ Absorbances of standards with known cysteine concentrations are used to produce a scatter plot featuring absorbance at 412 nm vs. nmol/uL. The optical density (OD) of the sample at an absorbance of 412 nm was read using the BioTek Synergy 4 Multi-Detection Microplate Reader. A trendline can be found to produce a linear regression equation. The absorbance of the reduction reaction sample can be integrated in this equation for the y-variable to solve for the x-variable, or concentration of thiol groups in nmol/uL within the tested sample.

The overall amount of antibody also must be quantified. This can be accomplished using a bicinchoninic acid assay, or BCA assay (Thermofisher scientific, 23250). The basis of this colorimetric protein assay relies on the ability of peptide bonds to reduce divalent calcium cations (Ca^{2+}) to monovalent calcium cations (Ca^+) via a biuret reaction in an alkaline environment. Two bicinchoninic acid sodium salt molecules then form a complex with a single monovalent calcium cation that manifests in an intense purple color that exhibits a strong linear absorbance at 562 nm.¹¹¹ Because the assay measures the amount of calcium reduction, and considering calcium reduction is dependent on peptide bonds, the intensity of the purple coloration is in proportion to the amount of total protein in the tested sample. A set of 9 standards with known concentrations of bovine serum albumin were prepared. A ratio of 50:1, BCA Assay reagents A and B were mixed respectively. Then, 200 μL of this working reagent is added to 20 μL of each standard and the sample from the reduction reaction. The peptide-initiated chelation of Ca^{2+} is temperature specific. Therefore, the reaction was carried out at 37°C for 30 min. Much like the Ellman's Reagent Assay, the reactions were scanned and absorbance was read, but at a wavelength of 562 nm. A scatter plot was then generated displaying

absorbance at 562nm vs ug/mL of the standards, where a trendline was found to produce a linear regression equation. Again, the absorbance of the reduction reaction sample was treated as the y-variable of the regression equation, to solve for the concentration of total protein in the sample in ug/mL, which can be converted to nmol. Knowing the nmol of protein and nmol of thiols in the sample, the ratio of thiols to antibody was found.

Exatecan-SMCC Complex Formation

Sulfo-SMCC, or sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, was the crosslinker utilized. It is known to be an effective amine-to-sulfhydryl linker, particularly for antibody conjugation.¹¹² It exhibits two main functional groups useful for such an association: a sulfo-N-Hydroxysuccinimide (NHS) functional group which reacts with primary amine groups to form stable amide bonds, and a maleimide functional group that reacts with sulfhydryl (thiol) groups to form stable thioether bonds.¹¹³ Considering the molecular structure of exatecan reveals a primary amine group, and the assumption of the presence of free thiol groups on the antibody after trastuzumab reduction using DTT, sulfo-SMCC would be the linker of choice for this conjugation. Yet there exists a caveat; primary amine groups exist throughout the immunoglobulin structure of trastuzumab. In order to better the chances of the sulfo-NHS functional group of sulfo-SMCC solely reacting with the primary amine of exatecan and not that of trastuzumab, it was elected to conduct the drug-linker association reaction in the absence of reduced trastuzumab initially.

Exatecan, sourced from (Medchem, DX8951f), was stored at -20°C at a stock solution concentration of 5mg/mL. Sulfo-SMCC sourced from (Sigma Aldrich, M6035) was stored at 4°C at a stock solution concentration of 1mg/mL. After identifying the nmol of thiol from the trastuzumab reduction reaction that is desired to undergo conjugation, 4x nmol exatecan was

used. A subset of the reduced trastuzumab was collected and saved for a control. Approximately 2x more sulf-SMCC was used in excess of exatecan. The proper amounts of exatecan and sulfo-SMCC were added together, as well as 1x DPBS to bring the reaction volume up to 500 μ L. The reaction was incubated for 4 hours at room temperature while stirring in the absence of light.

Reduced Trastuzumab-SMCC-Exatecan (T-Exa) ADC Reaction

After the 4-hour drug-linker reaction had elapsed, the proper amount of reduced trastuzumab in order to supply the desired amount of free thiol groups was added to the drug-linker reaction to carry out the antibody-drug conjugate (ADC) reaction. This solution then stirred at 4°C in the absence of light overnight, or for ideally at least 8 hours. The sample was then allowed to stir for 1 hour at room temperature, again in the absence of light to allow the solution to gradually warm. Similarly to the reduction reaction, a wash step was conducted while utilizing a 10,000 MWCO centrifugal column filter (Millipore Sigma, UFC901024) in order to retain the ADCs and allow unreacted exatecan and sulfo-SMCC to be eliminated. Centrifugation was initiated with a spin force of 4,000 RCF for 10 minutes. This filtration step was conducted 3 times, with 2 mL of 1x DBPS added to the sample for each spin. The final filtration spin resulted in approximately 100-200 μ L of retained ADC product. Another BCA assay was necessary to identify the amount of antibody recovered after the conjugation reaction. A subset of the final T-Exa ADC product as well as the saved trastuzumab reduction product were submitted for mass spectrometry analysis.

Mass Spectrometry Analysis

Samples collected after both the trastuzumab reduction protocol and trastuzumab-SMCC-exatecan ADC conjugation process were submitted to Analytical and Biological Mass

Spectroscopy at the University of Arizona, with a minimum amount of 50 µg per sample. Mass spectroscopy results were obtained using electrospray ionization mass spectroscopy (ESI/MS).

Mammalian Cell Culturing

The two cell lines utilized for the MTT assay later to be discussed are Calu3 and MDA-MB-231. Calu3 is a human non-small-cell lung cancer cell line commonly used for epithelial respiratory models. It was originally isolated from the pleural effusion of a male patient with a lung adenocarcinoma.¹¹⁴ Calu3 cells have a mutated *CDKN2A* gene that codes for cyclin-dependent kinase inhibitor 2A (CDKI2A) protein which normally act as a negative regulator of cellular proliferation. Thus, a mutation in this gene translates to accelerated cellular division. This cell line also exhibits a mutated *TP53* gene, which codes for a tumor suppressive protein tumor protein p53, or p53.¹¹⁵ The p53 protein normally binds DNA to increase the expression of protein p21, which interacts with cell division-stimulating protein (cdk2). The p21-cdk2 complex normally prevents initiation of the subsequent stages of cell division.¹¹⁶ However, if p21 is made in insufficient amounts due to mutated p53, the next stages of cell division will then ensue as well as the unsuppressed expression of other genes; one being the erythroblastic (erb)-b2 receptor tyrosine kinase (*ERBB2*) gene. *ERBB2* gene codes for members of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, including the human epidermal growth factor receptor 2 (HER2) protein.¹¹⁷ The over expression of HER2 is a common biomarker for carcinoma, as it contributes to the hormone stimulation of cell growth.

MDA-MB-231 is an epithelial human breast cancer cell line originally isolated from a female with metastatic mammary adenocarcinoma.¹¹⁸ Despite being a cancer cell line with mutated p53 protein, it is considered a triple negative breast cancer (TNBC) cell line. More specifically, negative estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor

receptor 2 (HER2).¹¹⁹ Though the genomic regulatory mechanisms are not fully characterized, it is published that TNBCs exhibits increased expression of the ERBB receptor feedback inhibitor 1 (*ERRFI1*) gene.¹²⁰ *ERRFI1* is a cytoplasmic protein that is upregulated during rapid cellular proliferation, which can manifest as consequence of mutated *TP53*. It acts as a negative regulator for many epidermal growth factor receptor family members, including ERBB2, ERBB3, and ERBB4.¹²¹ With this correlation, it could be logical to assume that if an increase in *ERRFI1* leads to a decrease in ERBB2 expression, then this could be a potential mechanism for the reduction in HER2 expression in specifically TNBC cell lines.

Both Calu3 and MDA-MB-231 (ATCC[®], HTB-55 and HTB-26, respectively) grow in an adherent monolayer. The cells were cultured in RPMI media (Gibco, 11875085) supplemented with 10% fetal bovine serum (FBS) (Gibco, 10082147), and 1% penicillin and streptomycin (Gibco, 15140122). Culturing included T175 600mL graduated gamma irradiation sterilized tissue culturing flasks (Genesee Scientific). Incubation took place at 37°C under humidified conditions at 5% CO₂. With a total of 30 mL media in each flask, cell proliferation commenced until cellular adherence could be observed over 90-100% of the flask surface area via light microscopy. Once cultures achieved this state of being packed, they were either counted to be used for assays or passaged for continual growth in new media. Cells were dislodged from the flask surface using TrypLE (Gibco, 12604013), a trypsin replacement used to cleave peptide bonds that participate in cell adhesion.¹²² With all culture manipulations taking place within a ventilated fume hood, the nutrient-exhausted media was removed and approximately 2-4 mL of TrypLE was added to each flask depending on flask size and cell strain. The flasks were again incubated at 37°C for 5 minutes with occasional mechanical agitation. Full-culture dislodgment was verified by light microscopy. With each cell line segregated, 16mL of RPMI media was

added to each flask, then all cell solutions were summated into centrifugal tubes to be spun at 220 RCF for 6 minutes. After centrifugation, the supernatant was removed, and the pellet was resuspended in 10 mL of the same supplemented RPMI media previously described. Cells were then counted with the utilization of a hemocytometer and glass coverslip; 10 μ L of the cellular resuspension was added to 10 μ L of trypan blue (Gibco, 15250061) to make a 1/2 dilution. A total of 10 μ L of this mixture was applied to the hemocytometer, then analyzed in a light microscope for cell counting. This predicted number of cells/mL can be applied to the sample volume to calculate a cellular concentration for the resuspension solution.

MTT Assay for Cytotoxicity Verification

Not only was the production of the T-Exa ADC confirmed via mass spectrometry, but cytotoxicity also was analyzed via MTT assays. The basis of the colorimetric MTT assay involves incubating adherent mammalian cells in a culturing plate. After a post-inoculation settlement duration period has elapsed to allow cellular adhesion, the agent to be analyzed is added to the cultures in varying concentration for a determined length of time. Next, the MTT assay reagent (Sigma Aldrich, M5655) including 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is added to the culture for a 2-hour incubation. MTT is yellow in color, but it is enzymatically reduced by mitochondrial succinate dehydrogenase.¹²³ The reduction of the tetrazolium MTT component by the mitochondrial oxidoreductase enzymes yields an insoluble formazan product that is purple in color.¹²⁴ Because the purple formazan is an insoluble precipitate, DMSO was added to act as an organic solvent to resolubilize the purple formazan precipitate back into solution.¹²⁵ Colorimetric analysis of this purple transformation is scanned at a wavelength of 570 nm.¹²⁶ Please refer to Figure 14 for a depiction of the colorimetric MTT assay reaction.

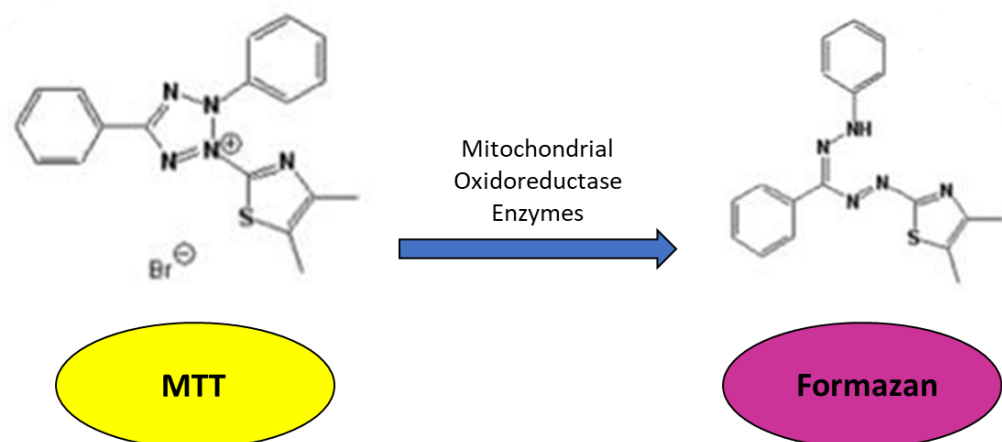


Figure 14. MTT Conversion to Formazan: The MTT assay relies on the presence of mitochondrial oxidoreductases to enzymatically convert yellow MTT to purple Formazan.

For the purposes of this study, agents subjected to cytotoxic analysis via MTT assays included the unmanipulated trastuzumab monoclonal antibody, reduced trastuzumab, solitary DM1 drug, solitary exatecan drug, synthesized trastuzumab-MCC-exatecan (T-Exa), and commercially available trastuzumab-MCC-DM1 (T-DM1). Cytotoxicities of these six agents were tested on HER2 positive and HER2 negative cancer cells lines; Calu3 and MDA-MB-231 respectively. Please refer to the ‘Mammalian Cell Culturing’ section within *Chapter 3 Materials and Methods* of this report for further explanation regarding cellular phenotype and culturing techniques.

For each MTT assay, 5×10^3 cells in 100 μL of RPMI media were added to each well of a 96-well plate. The plates were then incubated at 37°C with 5% CO_2 under humidified conditions for 24 hours to allow cellular adhesion on the plate surface. Considering the 96-well plates are 12 wells wide, 11 different concentrations of each agent to be tested were made with the last well receiving no agent to provide a control. Antibody and ADC samples were prepared with a starting concentration of 100 $\mu\text{g}/\text{mL}$, with consecutive (1/10) serial dilutions out to 10^{-8} $\mu\text{g}/\text{mL}$. Trials involving individual drugs had initial preparations starting at 10 $\mu\text{g}/\text{mL}$, with consecutive

(1/10) serial dilutions out to 10^{-9} $\mu\text{g/mL}$. Dilutions of each agent were made using serum-free RPMI media. The corresponding dilutions of each agent were then added to plates containing Calu3 and MDA-MB-231 cancer cell lines. Each plate containing the agent dilutions were incubated for either 1, 2, or 3 days. After the designated incubation period, 10 μL of MTT reagent was added to each well and allowed to incubate back in the same conditions for 2 more hours. This period allows the conversion of the yellow MTT tetrazolium compound to the purple insoluble formazan by the mitochondrial oxidoreductases. The media was then removed via suction, and 100 μL of the organic solvent DMSO (Sigma Aldrich, 276855) was added to resolubilize the purple formazan back into solution. The plates were then scanned and absorbance of the color change was read at a wavelength of 570 nm. Each agent underwent MTT analysis at least four times for each cell line. Because the amount of mitochondrial oxidoreductase present in the culture is directly proportional to the amount of living cells, increasing purple coloration intensity is directly correlative to increasing amounts of living cells and ultimately decreasing cytotoxic effects of the added agent of analysis. Average absorbance values of each dilution for all agents were found and divided by the average absorbance of the control wells that were void of agent to create a ratio or percentage of cell viability. Plots of percent cell viability vs. agent concentration ($\mu\text{g/mL}$) were made, while IC₅₀ (Graphpad Prism8) values were found in order to compare the cytotoxicities of each agent tested on both HER2 positive and negative cancer cell lines.

CHAPTER 4: RESULTS

Trastuzumab Reduction Verification

As previously emphasized, the production of the T-Exa ADC depicted in Figure 12 relies on the presence of free thiol groups provided by the monoclonal trastuzumab antibody. Considering there are virtually no free thiol groups on IgG1 antibodies, it was crucial to reduce the inter-chain disulfide bonds with the use of DTT as the reducing agent. After performing the trastuzumab reduction protocol provided within *Chapter 3 Materials and Methods* of this report, the filtered and collected samples were analyzed using the Ellman's Assay in order to not only verify reduction occurred, but also provide a means of quantifying the free thiols obtained. Figure 15 reveals the trastuzumab reduction findings after the Ellman's Assay.

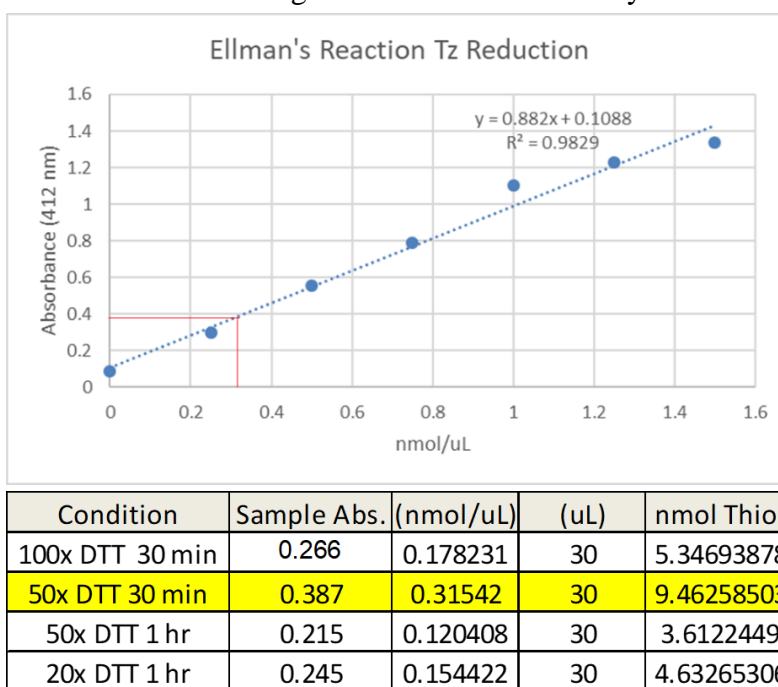


Figure 15. Ellman's Reaction Results: The absorbance (412 nm) vs concentration (nmol/μL) plot produced by reading the absorbance of known standards provided the regression line equation used to associate the absorbance of collected samples to a given concentration. Condition 50x DTT, with respect to total trastuzumab (Tz) subject to reduction, for a 30-minute reaction proved to be the most efficacious. All reactions were conducted at 37°C.

Though many trials and conditions were tested, Figure 15 features an absorbance vs concentration plot and the associated regression line equation, all produced by reading the absorbance of a series of standards with known thiol concentrations. By reading the absorbance of the collected reduction samples that have undergone the Ellman's Assay, their absorbance values could then be applied to the regression line equation to algebraically solve for the concentration of thiols. Furthermore, because the volume of each sample was known, the total nmol of thiol per sample was then calculated. This data reveals that reacting trastuzumab with 50x DTT for a 30-minutes stirred reaction at 37°C yielded the most nmol of thiol when compared to the other conditions. This same strategy was applied in order to determine the final amount of antibody recovered after the reduction protocol and filtration process. In this instance, the BCA assay was utilized to quantify the amount of protein, or antibody, recovered. Figure 16 reveals the BCA assay results of the samples that underwent reduction.

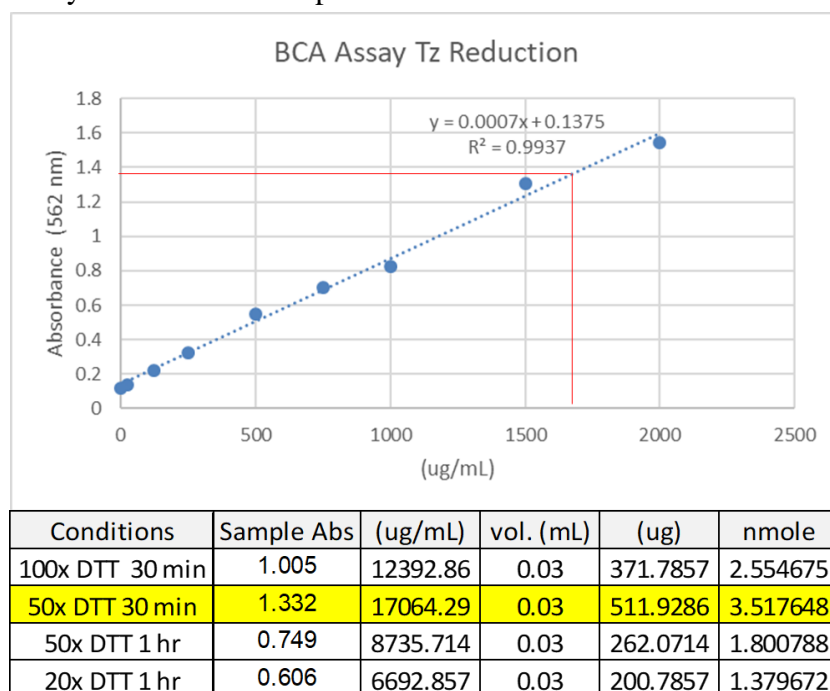


Figure 16. BCA Assay Results: The absorbance (562 nm) vs concentration ($\mu\text{g/mL}$) plot produced by reading the absorbance of known standards provided the regression line equation used to associate the absorbance of collected samples to a given concentration. Condition 50x

DTT, with respect to total trastuzumab (Tz) subject to reduction, for a 30-minute reaction proved to be the most efficacious. All reactions were conducted at 37°C.

Much like the Ellman's assay analysis, the BCA assay involves making a series of standards with known protein concentrations. These standards were read at an absorbance of 562 nm, providing the regression line equation when plotted on an absorbance vs concentration graph. A more in-depth explanation of the BCA assay can be found within *Chapter 3 Materials and Methods* of this report. The absorbance of the reduced samples that have undergone the BCA assays were read and applied to the regression equation to algebraically solve for the concentration of protein in each sample. Similar to the results found in that of the Ellman's assay, the condition with 50x DTT to trastuzumab antibody for a 30-minute stirring duration at 37°C revealed the greatest amount of antibody recovery when compared to the other conditions. By knowing the amount of thiols (9.46 nmol) and amount of recovered antibody (3.52 nmol), simple division is used to determine that the optima reduction sample contains 2.7 thiols per antibody. This method of testing reduction conditions permitted the ability to optimize the free thiol yield, antibody recovery, and efficacy of the reduction protocol.

T-Exa Synthesis Verification

After the verification of successful trastuzumab reduction, antibody conjugation was the following step. As previously stated, the exatecan-SMCC complex had to first be formed in the absence of trastuzumab due to the reactivity of sulfo-SMCC with primary amine groups. Then the reduced trastuzumab antibody was added to the reaction for conjugation, leading to the synthesis of the trastuzumab-MCC-exatecan (T-Exa) ADC. Please refer to *Chapter 3 Materials and Methods* of this report for a detailed description regarding the conjugation protocol in its entirety. After the conjugation reaction occurred, the sample was filtered via centrifugal spin

column filtration to rid of unconjugated drug-linker complexes. Another BCA assay was conducted to determine the final concentration of the sample for mass spectrometry submission. Please refer to Figure 17 for a representation of the mass spectrometry findings.

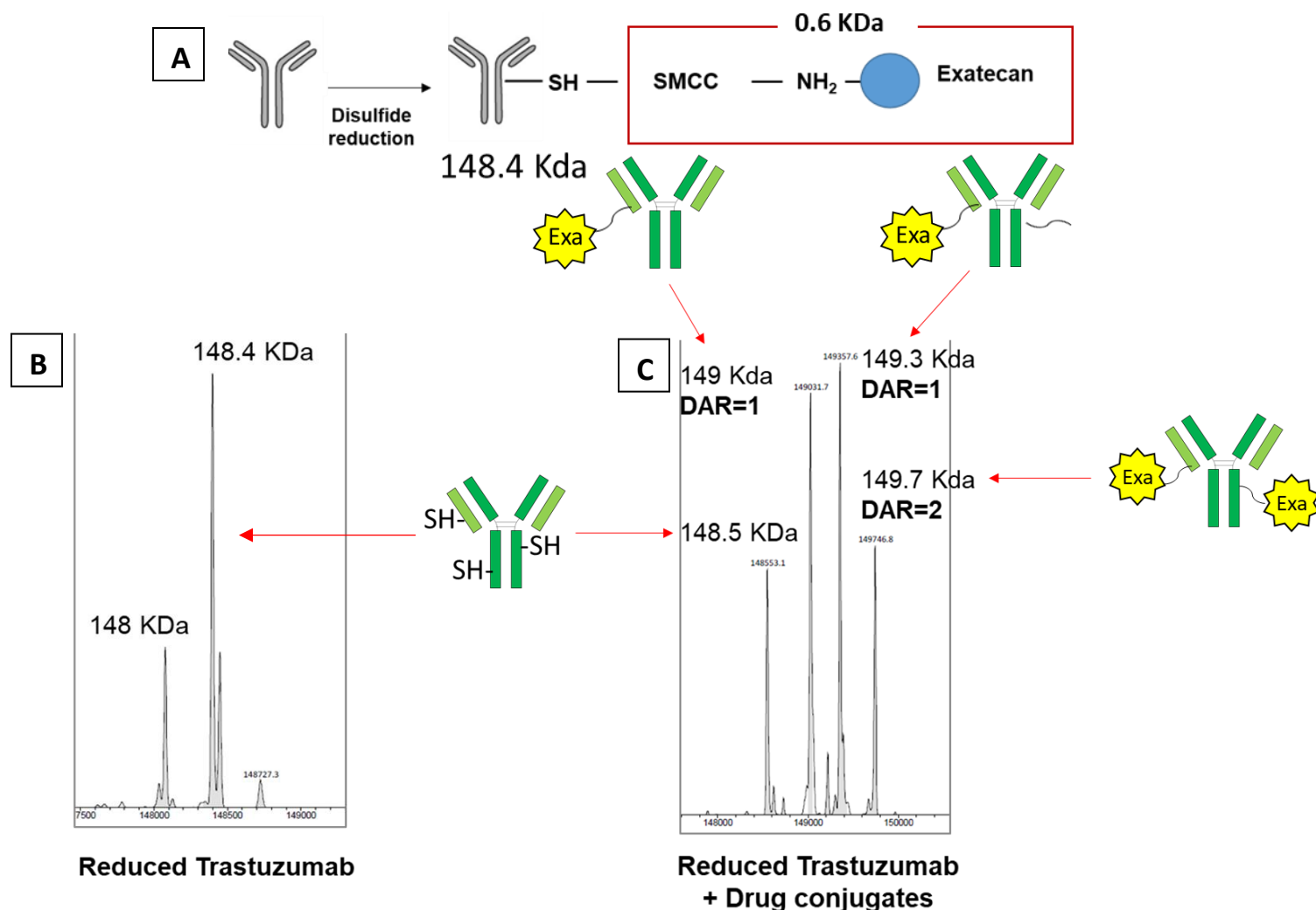


Figure 17. Mass Spectrometry Results: Interpretation of mass spectrometry submissions of both reduced trastuzumab and conjugated trastuzumab. **A)** Schematic suggesting the reduced antibody weighs approximately 148.4 KDa, while the exatecan-SMCC complex weighs 0.6 KDa. **B)** Mass spectrometry results of the reduced trastuzumab antibody. **C)** Mass spectrometry results of the conjugated trastuzumab antibody and associated drug-antibody ratios (DAR) for each predominant peak. The conjugated sample reveals DARs ranging from 1 to 2, as well as a species presumed to contain an SMCC crosslinker not associated with a drug.

As Figure 17 portrays from the mass spectrometry analysis, the conjugated sample does contain some unreacted reduced antibodies, as well as conjugated species with DARs of 1 to 2.

T-Exa Cytotoxicity Analysis

With the mass spectrometry data supporting the successful synthesis of the desired T-Exa ADC, the cytotoxicity of the ADC was then analyzed. This was accomplished but utilizing the colorimetric MTT assay as previously described in *Chapter 3 Materials and Methods*. The first MMT assay was conducted with the intent of analyzing the cytotoxicity of three agents; unmanipulated trastuzumab antibody, reduced trastuzumab antibody, and the synthesized T-Exa ADC on both HER2 negative MDA-MB-231 and HER 2 positive Calu3 cancer cells. The MTT assay plots depicting cytotoxicity can be found in Figure 18.

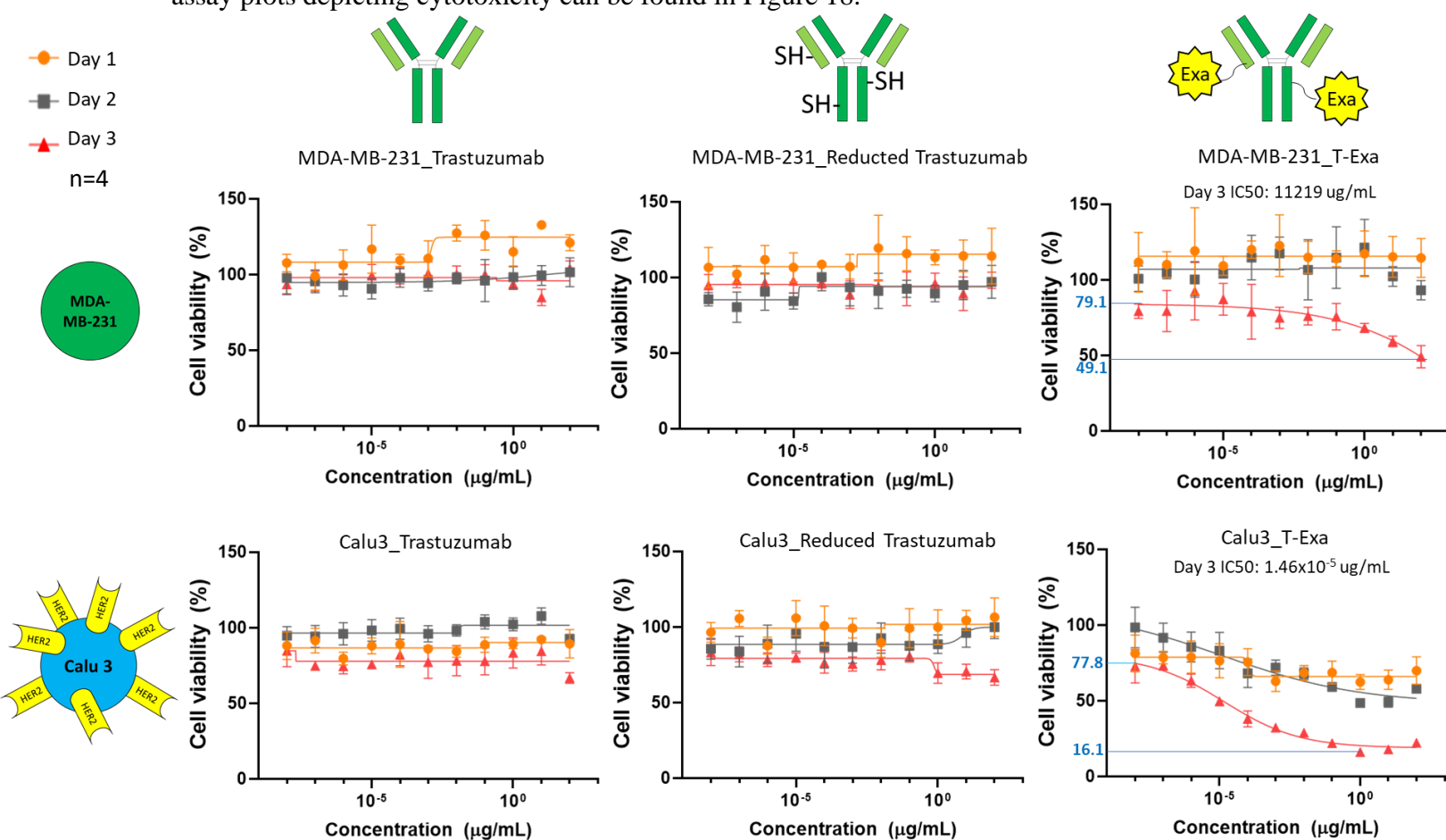


Figure 18. MTT Assay Results for Trastuzumab, Reduced Trastuzumab, and T-Exa: The cytotoxicities of the three agents trastuzumab, reduced trastuzumab, and synthesized T-Exa were analyzed using HER2 negative MDA-MB-231 and HER2 positive Calu3 cancer cells via the MTT assay. Cell Viability Percentage vs Concentration ($\mu\text{g/mL}$) plots were made to compare cell viability as agent concentration increased for days 1, 2, and 3. Both trastuzumab and reduced trastuzumab displayed virtually no cytotoxic effect on either cell line. The synthesized T-Exa ADC displayed minor cytotoxic effects on MDA-MB-231 on day 3, but only at higher concentrations. T-Exa started to reveal pronounced cytotoxicity on Calu3 beginning on day 2. Each trial was repeated a minimum of 4 times ($n=4$).

Understandably, agents absent of drug did not elicit cell death in any capacity, as the plots remained linear and horizontal near 100% cell viability for all concentrations for days 1, 2, and 3 on both cancer cell lines. T-Exa was largely noncytotoxic on HER2 negative MDA-MB-231 for days 1 and 2, however did begin to elicit cytotoxic effects on day 3 at T-Exa concentrations greater than $1 \mu\text{g/mL}$ while reaching a cell viability as low as 49.1%. The significant finding of this trial was the extent in which T-Exa revealed notable cytotoxic effects on HER2 positive Calu3 cells. Though day 1 was largely insignificant, cell death became apparent on day 2 and even more so pronounced on day 3 as cell viability started at 77.8% for $10^{-8} \mu\text{g/mL}$ concentration and decreased to 16.1% for $100 \mu\text{g/mL}$ concentration. The Calu3_T-Exa plot for day 3 produced an IC_{50} of $1.46 \times 10^{-5} \mu\text{g/mL}$.

Comparing Cytotoxicities of DM1 and Exatecan

In order to test the original hypothesis that exatecan is more cytotoxic than DM1, the cytotoxicities of both lone chemotherapeutic agents unassociated with an antibody were analyzed on both MDA-MB-231 and Calu3 cell lines via an MTT assay. Though speculation of exatecan being a superior chemotherapeutic agent compared to DM1 existed, the objective of this trial was to provide evidence that such a speculation was warranted. The MTT assay results and associated plots comparing DM1 and exatecan cytotoxicities can be found in Figure 19.

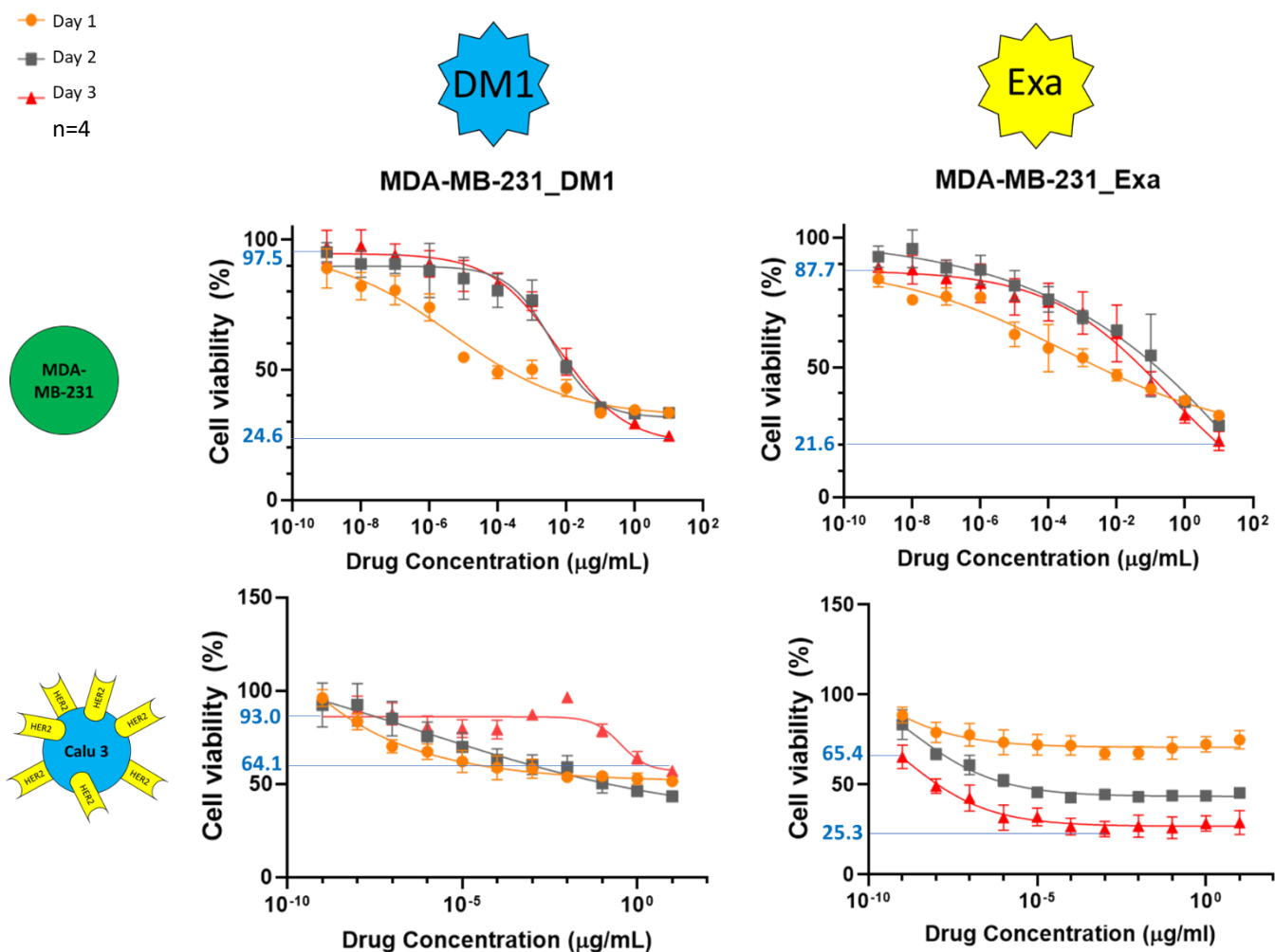


Figure 19. MTT Assay Results for DM1 and Exatecan: The cytotoxicities of DM1 and exatecan were compared on both HER2 negative MDA-MB-231 and HER2 positive Calu3 cancer cells using the MTT assay. The cytotoxicities of both agents were very similar on MDA-MB-231 cells, while exatecan appears to exhibit greater cytotoxicity on Calu3 cells. Each trial was repeated a minimum of 4 times (n=4).

As depicted in Figure 19, DM1 and exatecan displayed very similar effects on the triple negative MDA-MB-231 cells. However, deviations were apparent on Calu3 cells. More specifically, the day 3 plot for exatecan on Calu3 cells reveals 65.4% cell viability at 10⁻⁹ µg/mL concentration, which drops to as low as 25.3% with increasing concentrations. A much milder decline in cell

viability was observed for DM1 on Calu3 cells on day 3, with a maximum of 93.0% cell viability which only regressed to 64.1% at a maximum concentration of 10 $\mu\text{g/mL}$.

Comparing Cytotoxicities of T-DM1 and T-Exa:

Having mentioned the potential flaws of DM1 due to cellular resistance mechanisms, as well as produced data projecting exatecan to be more cytotoxic than DM1 on HER2 positive cells, it was logical to compare the cytotoxicities of ADCs yielding DM1 and exatecan payloads. More specifically, comparing the effects of T-DM1 and T-Exa on both HER2 negative MDA-MB-231 and HER2 positive Calu3 cancer cell lines via an MTT assay. The assay was carried out in the same manner as the other MTT assays previously discussed, including similar concentration ranges and durations of agent exposure. The results for this ADC comparative trial are provided in Figure 20.

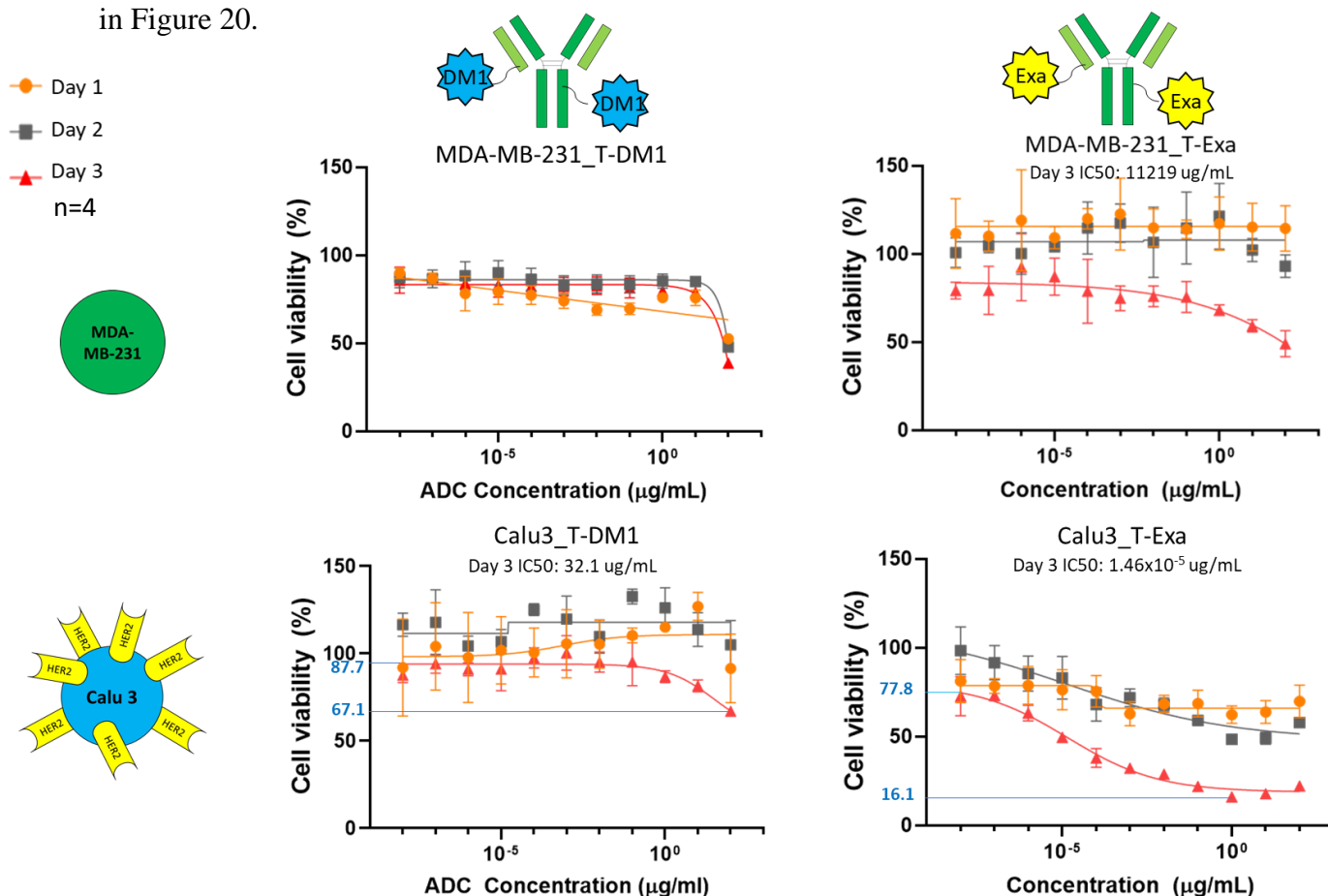


Figure 20. MTT Assay Results for T-DM1 and T-Exa: The cytotoxicities of DM1 and exatecan were compared on both HER2 negative MDA-MB-231 and HER2 positive Calu3 cancer cells using the MTT assay. T-DM1 provides virtually no cytotoxicity on MDA-MB-231 cells and displayed minimal cytotoxicity on day 3 at higher concentrations on Calu3 cells. T-Exa on day 3 displayed notable cell death on MDA-MB-231 cells at higher concentrations, and even more so for days 3 on Calu3 cells. Each trial was repeated a minimum of 4 times (n=4).

The MTT assay results provided in Figure 20 suggest T-DM1 offered no cytotoxicity on MDA-MB-231, with the exception of a sharp decline in cell viability around 100 $\mu\text{g/mL}$ which is presumed to be a manifestation of human pipetting error or an absorbance plating effect.

Likewise, T-Exa displays virtually no cytotoxicity on MDA-MB-231 cells for days 1 and 2, but does reveal a decline in cell viability on day 3 in wells with a concentration of 1 $\mu\text{g/mL}$ or greater. Regarding the response of the Calu3 cells on day 3, maximum and minimum cell viabilities ranged from 87.7 to 67.1% and 77.8 to 16.1% for the T-DM1 and T-Exa treatments respectively. These results suggest that the synthesized T-Exa ADC is potentially more cytotoxic on HER2 positive Calu3 cells than clinically approved and commercially available T-DM1.

Please refer to Table 1 for a summary of the MTT assay results.

Table 1. Day 3 MTT Assay Results Summary

Day 3 MTT Assay Results	MDA-MB-231		Calu3	
	Cell Viability % Range	IC50 ($\mu\text{g/mL}$)	Cell Viability % Range	IC50 ($\mu\text{g/mL}$)
Exatecan	87.7% - 21.6%	n/a	65.4% - 25.3%	n/a
DM1 (Mertansine)	97.5% - 24.6%	n/a	93.0% - 64.1%	n/a
T-Exa	79.1% - 49.1%	11219	77.8% - 16.1%	1.46×10^{-5}
T-DM1	n/a	n/a	87.7% - 67.1%	32.1

CHAPTER 5: DISCUSSION

In order to synthesize the T-Exa ADC with the constituents provided in Figure 12, a free thiol to interact with the sulfo-SMCC crosslinker is crucial. Unlike DM1, exatecan does not acquire a thiol group, but rather a primary amine. Therefore, the thiol group must be provided by the antibody to be conjugated. Considering IgG1 antibodies are virtually absent of free cysteine residues, the reduction of the inter-chain disulfide bonds of trastuzumab is imperative for ADC synthesis. Not only was this reduction step arguably the most vital component of this project, but it was also the most difficult to accomplish. Too mild reduction conditions resulted in no thiol production, and too aggressive reduction conditions severely decreased antibody recovery due to extensive fragmentation of the tetrameric quaternary structure of the immunoglobulin molecules. Many reduction trials over many conditions were tested in order to produce a protocol that yielded acceptable thiol production on IgG1 trastuzumab antibodies, and is most importantly reproducible. Verification of not only optimal thiol production but also antibody recovery using 50x DTT with reference to nmol of trastuzumab for a 30-minute stirred reaction at 37°C was apparent by the Ellman's assay and BCA assay results from Figures 15 and 16 respectively. The optimal reduction condition yielded approximately 2.7 thiol groups per antibody, which was conceivably enough to undergo conjugation.

Now that the antibody can provide the thiols to act on the maleimide groups of sulfo-SMCC, the exatecan drug molecule naturally contains the primary amine group to act as nucleophile to attack the electrophilic carbon of the succinimide group of SMCC. After considering T-DM1 synthesis relies on primary amines offered by lysine residues on trastuzumab, it was recognized that the exatecan-MCC amide linkage must occur first, in the absence of trastuzumab to ensure exatecan was the only species within the reaction to offer the primary amine. If the succinimide

group of the sulfo-SMCC crosslinker were to react with lysine residues of the antibody first, then the only reactive group on the crosslinker would be the maleimide group. Because exatecan does not contain thiol groups to interact with the maleimide group, no successful antibody-drug conjugation would occur and thus no cytotoxicity would be offered. Therefore, forming the exatecan-MCC complex prior to reduced antibody addition was crucial.

Mass spectrometry was integral for not only providing evidence that the T-Exa ADC was synthesized, but to also identify the variations of conjugated species produced as well as the corresponding DARs. As made apparent by the mass spectrometry results provided in Figure 17, the conjugated sample appeared to have ADC species yielding 1 to 2 drug-linker complexes per antibody. Higher DAR's may be preferred, however, T-Exa synthesis was proven none the less.

Though evidence was put forth to conclude T-Exa was ultimately synthesized, an ADC is considered worthless if it does not exhibit selectivity or cytotoxicity. The MTT assays provided essential results to suggest that trastuzumab and reduced trastuzumab offered no cytotoxicity on either MDA-MB-231 or Calu3 cells, all the while T-Exa was able to display cytotoxicity due to its associated payloads. More specifically, T-Exa was considerably more cytotoxic on HER2 positive Calu3 cells, while exhibiting mild cytotoxicity on HER2 negative MDA-MB-231 cells with prolonged incubation durations and at higher concentration. This can allow one to discern that T-Exa offers selectivity in terms of the cells that are affected based on HER2 expression. Furthermore, the MTT assays used for the individual agent comparison of DM1 and exatecan provided more insight. Though a significant difference between the two drugs was not observed on MDA-MB-231 cells, a notable difference in cytotoxicity was apparent when Calu3 cells were treated with the agents. Exatecan achieved the lowest percent of cell viability for day 3 trials when compared to DM1 as provided in Figure 19, further bolstering the argument that an

exatecan-based payload would contribute to greater efficacy of an ADC. However, the most revealing MTT assay was the comparison of the clinically approved and commercially available T-DM1 with the synthesized T-Exa ADC, more specifically on Calu3 cells. The day 3 data provided in Figure 20 proposes that T-Exa, with a DAR of 1-2, is considerably more cytotoxic than T-DM1, which features a DAR of 3-4. In essence, despite T-Exa having roughly half of the amount of drug conjugated per antibody compared to T-DM1, it still provides greater cytotoxicity and in a cell-selective manner. This was an interesting discovery and warrants promise for HER2 selective therapies yielding exatecan-based payloads.

CHAPTER 6: FUTURE DIRECTIONS

Though the objectives of this study have been achieved, there are numerous areas of improvement still yet to be addressed. An overwhelming portion of time towards this project was devoted to optimizing the antibody reduction protocol, and an efficacious and reproducible reduction protocol was made possible because of these efforts. However, the ADC conjugation protocol divulges room for improvement. For example, commercially available ADCs commonly feature DARs of 3 to 4 or higher. It may be of benefit to explore the possibility of not only improving the thiol yield after antibody reduction, but also increasing the number of drug-linker complexes that conjugate each antibody in efforts to increase the DAR, greater than 1 to 2. This may be done by attempting versions of the existing conjugation protocol, but with varying conditions such as adjusting the amount of reducing agent, modifying the drug-to-linker ratio when forming the drug-linker complex, and even altering the reaction temperature and duration. This would understandably require a great deal of time over numerous trials, but could prove beneficial in optimize the synthesis protocol to maximize ADC efficacy. However, this concept

should not go without scrutiny. Though the cytotoxicity of an ADC *in vitro* undoubtedly increases as the DAR increases, the same trend is not observed in a physiological setting. There is a growing appreciation for how the DAR contributes to the biodistribution and pharmacokinetics of an ADC. In fact, the cytotoxicity of an ADC is known to decrease *in vivo* due to enhanced plasma clearance of heavily loaded antibodies.⁹⁹ ADC hydrophobicity is affected by the DAR as well as the drug-linker design. With respect to ADCs synthesized by conjugating drug-linker complexes to native cysteine or lysine residues on the immunoglobulin structure, most investigators attempt to limit the DAR to a range of 2 to 4 to maximize biological activity.^{127,128} A study published in *Nature Biotechnology* analyzed the pharmacokinetics of an anti-CD70 ADC with a DAR of 8, conjugated to a cytotoxic payload of monomethyl auristatin F (MMAF) using the protease cleavable valine-citrulline-*p*-aminobenzylcarbamate (val-cit-PABC) linker. The conclusion of the immunohistochemistry (IHC)-based biodistribution study revealed that a DAR of 8 contributed to enhanced clearance *in vivo*. More specifically, staining for this anti-CD70 ADC in rat models was profound within the entire hepatic sinusoidal endothelium as well as Kupffer cells. Minimal staining was observed in animals dosed with the unconjugated anti-CD70 antibody.¹²⁹ It is also known that ADC stability and aggregation are both affected by environmental pH, the buffer system, and the DAR.¹³⁰ Considering the cytotoxicity, efficacy, and rate of plasma clearance of an ADC *in vivo* may be considered sub-optimal as the DAR increases towards extreme conjugations, the suggestion to improve the conjugation protocol in order to increase the DAR of T-Exa should be interpreted not so much as a crucial improvement, but rather as an avenue for exploration. Furthermore, aggregation assays can also be used to establish a threshold in order to determine at what DAR an ADC could potentially begin to lose

efficacy in a physiological setting. This concept may prove more advantageous than simply declaring that the cytotoxicity of an ADC increases *in vitro* as the DAR increases.

In addition, a method of isolating only associated drug-linker complexes prior to reduced antibody addition would help limit the binding of crosslinkers to the antibody, absent of drug. The binding of a solitary linker molecule to the reduced antibody contributes no benefit and would preferably be avoided as this can lead to misinterpretation of mass spectrometry data. Lastly, *in vitro* analysis of the efficacy, cytotoxicity, and selectivity of a biological therapy is a stark comparison to similar analysis but in an *in vivo* setting. It is conceivable that not all trends and results found within an assay plate are exactly translatable in a physiological setting. Perhaps the best form of ADC analysis would be through the utilization of *in vivo* studies. However, this is beyond the scope of this project, as verifying the ADC synthesis chemistry was the primary objective.

CHAPTER 7: CONCLUSION

Considering the data and evidence put forth in this report, one can surmise that the objectives of this project have been realized. Aim 1, or production of an anti-HER2 ADC associated with exatecan, was achieved through the production and revision of a T-Exa synthesis protocol. Aim 2, or verification of the effectiveness of the conjugation reaction, was provided by the reliance on Ellman's assays, BCA assays, and mass spectrometry analysis to conclude T-Exa was in fact synthesized and that the logic behind the proposed chemistry was sound. The data provided in this report supports acceptance of the hypothesis that antibody reduction is crucial for the proposed conjugation mechanism, as the reduced antibody disulfide bonds must provide free thiol groups. In addition, the extent of antibody reduction was integrally associated with the DAR. In the context of the trials discussed in this report, the Ellman's and BCA assays provided

the metrics required to determine that each recovered trastuzumab antibody after reduction yielded an average of 2.7 free thiol groups. The mass spectrometry analysis of the conjugated sample from Figure 17 suggests that a DAR of 2 was the extent of conjugation. This finding is logical considering the average 2.7 thiols per antibody set the conjugation limit. Aim 3, or analyzing the function of the anti-HER2 ADC, was completed via the utilization of MTT assays. Through this capacity, T-Exa proved to elicit cell-specific cytotoxic effects, more specifically on HER2 positive cancer cells, with reduced cytotoxic effects on HER2 negative cancer cells. In addition, T-Exa also revealed greater cytotoxicity on HER2 positive cells when compared to commercially available T-DM1. This finding supports the other hypothesis that T-Exa would be more efficacious than T-DM1 in eliciting cancer cell death if exatecan was in fact more cytotoxic than DM1 on HER2 positive cancer cell lines.

Though there are no currently approved target-based therapies for HER2 positive non-small cell lung cancer, an anti-HER ADC yielding exatecan or exatecan-derived payloads does not seem to be an unrealistic therapeutic option in the very near future. Though this project and associated analysis is not void of flaws, there appears to be a substantial promise in targeted-based therapies with exatecan payload that could be more efficacious than currently available therapies. Perhaps the most significant achievement of this work was not the ability to produce T-Exa, but developing the protocol to reduce trastuzumab and potentially other IgG1 antibodies of the like. It would be with great gratification if this technology could one day be used to synthesized exponential combinations of antibodies and cytotoxic agents in efforts to provide life-saving therapy for numerous types of cancers that were otherwise unimaginable.

REFERENCES

1. Chabner BA WW. Pharmacology and toxicity of antineoplastic drugs. In: Beutler E, Lichtman MA, Coller BS, et al., editors. Hematology. New York: McGraw Hill, 1995: 143–55. <https://medtextfree.wordpress.com/2011/12/26/chapter-16-pharmacology-and-toxicity-of-antineoplastic-drugs/>. Accessed January 30, 2020.
2. Carey PJ. Drug-induced myelosuppression: Diagnosis and management. *Drug Saf.* 2003;26(10):691-706. doi:10.2165/00002018-200326100-00003
3. Evans WE, Horner M, Chu YQ et al. Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase deficient child with acute lymphocytic leukemia. *J Pediatr* 1991; 119: 985–9. <https://books.google.com/books?id=29ylqDZRH9MC&pg=PA471&lpg=PA471&dq=Evans+WE,+Horner+M,+Chu+YQ,+et+al.+Altered+mercaptopurine+metabolism,+toxic+effects,+and+dosage+requirement+in+a+thiopurine+methyltransferase+deficient+child+with+acute+lymphocytic+leuke>. Accessed January 30, 2020.
4. Lennard L, Van Loon JA, Weinshilboum RM. Pharmacogenetics of acute azathioprine toxicity: Relationship to thiopurine methyltransferase genetic polymorphism. *Clin Pharmacol Ther.* 1989;46(2):149-154. doi:10.1038/clpt.1989.119
5. Lung Cancer - Non-Small Cell: Statistics | Cancer.Net. American Society of Clinical Oncology. <https://www.cancer.net/cancer-types/lung-cancer-non-small-cell/statistics>. Published 2019. Accessed January 30, 2020.
6. What Is Lung Cancer? | Types of Lung Cancer. The American Cancer Society. <https://www.cancer.org/content/cancer/en/cancer/lung-cancer/about/what-is.html>. Published 2019. Accessed January 30, 2020.
7. PDQ Adult Treatment Editorial Board. *Non-Small Cell Lung Cancer Treatment (PDQ®): Health Professional Version.*; 2002. <http://www.ncbi.nlm.nih.gov/pubmed/26389304>. Accessed January 30, 2020.
8. Non-small cell lung cancer: MedlinePlus Medical Encyclopedia. A.D.A.M. Inc. <https://medlineplus.gov/ency/article/007194.htm>. Published 2020. Accessed January 30, 2020.
9. Subramanian MP, Puri V. Neoadjuvant vs. adjuvant chemotherapy in locally advanced non-small cell lung cancer-is timing everything? *J Thorac Dis.* 2019;11(12):5674-5676. doi:10.21037/jtd.2019.12.40
10. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. The distribution and functions of immunoglobulin isotypes. 2001.
11. M. D. [The Fc portion of antibodies]. - PubMed - NCBI. 1992. <https://www.ncbi.nlm.nih.gov/pubmed/1566003>. Accessed January 31, 2020.
12. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. The structure of a typical antibody molecule. 2001.

13. Peng HP, Lee KH, Jian JW, Yang AS. Origins of specificity and affinity in antibody-protein interactions. *Proc Natl Acad Sci U S A*. 2014;111(26). doi:10.1073/pnas.1401131111
14. Kim EK, Kim KA, Lee CY, Shim HS. The frequency and clinical impact of HER2 alterations in lung adenocarcinoma. *PLoS One*. 2017;12(2). doi:10.1371/journal.pone.0171280
15. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001;2(2):127-137. doi:10.1038/35052073
16. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: Current status and future perspectives. *Nat Rev Clin Oncol*. 2012;9(1):16-32. doi:10.1038/nrclinonc.2011.177
17. Pillai RN, Behera M, Berry LD, et al. HER2 mutations in lung adenocarcinomas: A report from the Lung Cancer Mutation Consortium. *Cancer*. 2017;123(21):4099-4105. doi:10.1002/cncr.30869
18. Rubin I, Yarden Y. The basic biology of HER2. *Ann Oncol*. 2001;12(SUPPL. 1). doi:10.1093/annonc/12.suppl_1.S3
19. Tai W, Mahato R, Cheng K. The role of HER2 in cancer therapy and targeted drug delivery. *J Control Release*. 2010;146(3):264-275. doi:10.1016/j.jconrel.2010.04.009
20. Lax I, Burgess WH, Bellot F, Ullrich A, Schlessinger J, Givol D. Localization of a major receptor-binding domain for epidermal growth factor by affinity labeling. *Mol Cell Biol*. 1988;8(4):1831-1834. doi:10.1128/mcb.8.4.1831
21. Pietras RJ, Arboleda J, Reese DM, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene*. 1995;10(12):2435-2446.
22. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. ErbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science (80-)*. 1987;237(4811):178-182. doi:10.1126/science.2885917
23. Telesco SE, Radhakrishnan R. Atomistic insights into regulatory mechanisms of the HER2 tyrosine kinase domain: A molecular dynamics study. *Biophys J*. 2009;96(6):2321-2334. doi:10.1016/j.bpj.2008.12.3912
24. Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol*. 2005;1(1). doi:10.1038/msb4100012
25. Schaeffer L, Duclert N, Huchet-Dymanus M, Changeux JP. Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. *EMBO J*. 1998;17(11):3078-3090. doi:10.1093/emboj/17.11.3078
26. Wei Z, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res*. 2002;12(1):9-18. doi:10.1038/sj.cr.7290105
27. Yenush L, Zanella C, Uchida T, Bernal D, White MF. The Pleckstrin Homology and

- Phosphotyrosine Binding Domains of Insulin Receptor Substrate 1 Mediate Inhibition of Apoptosis by Insulin. *Mol Cell Biol*. 1998;18(11):6784-6794. doi:10.1128/mcb.18.11.6784
28. Metz HE, Houghton AMG. Insulin receptor substrate regulation of phosphoinositide 3-kinase. *Clin Cancer Res*. 2011;17(2):206-211. doi:10.1158/1078-0432.CCR-10-0434
 29. Fruman DA, Meyers RE, Cantley LC. PHOSPHOINOSITIDE KINASES. *Annu Rev Biochem*. 1998;67(1):481-507. doi:10.1146/annurev.biochem.67.1.481
 30. White MF. The IRS-Signaling system: A Network of Docking Proteins That Mediate Insulin and Cytokine Action. *Recent Prog Horm Res*. 1998;53:119-138. doi:10.1007/978-3-642-80481-6_8
 31. Dieterle AM, Böhler P, Keppeler H, et al. PDK1 controls upstream PI3K expression and PIP 3 generation. *Oncogene*. 2014;33(23):3043-3053. doi:10.1038/onc.2013.266
 32. Fresno Vara JÁ, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev*. 2004;30(2):193-204. doi:10.1016/j.ctrv.2003.07.007
 33. Alimandi M, Romano A, Curia MC, et al. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*. 1995;10(9):1813-1821.
 34. Pinkas-Kramarski R, Soussan L, Waterman H, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J*. 1996;15(10):2452-2467. doi:10.1002/j.1460-2075.1996.tb00603.x
 35. Lenferink AEG, Pinkas-Kramarski R, Van De Poll MLM, et al. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J*. 1998;17(12):3385-3397. doi:10.1093/emboj/17.12.3385
 36. Cohen BD, Kiener PA, Green JM, Foy L, Perry Fell H, Zhang K. The relationship between human epidermal growth-like factor receptor expression and cellular transformation in NIH3T3 cells. *J Biol Chem*. 1996;271(48):30897-30903. doi:10.1074/jbc.271.48.30897
 37. Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. *J Biol Chem*. 1998;273(22):13819-13827. doi:10.1074/jbc.273.22.13819
 38. Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem*. 1996;271(9):5251-5257. doi:10.1074/jbc.271.9.5251
 39. Lonardo F, Di Marco E, King CR, et al. The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. *New Biol*. 1990;2(11):992-1003. <http://www.ncbi.nlm.nih.gov/pubmed/1983208>. Accessed February 14, 2020.

40. Ben-Levy R, Paterson HF, Marshall CJ, Yarden Y. A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO J.* 1994;13(14):3302-3311. doi:10.1002/j.1460-2075.1994.tb06632.x
41. Karunagaran D, Tzahar E, Beerli RR, et al. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* 1996;15(2):254-264. doi:10.1002/j.1460-2075.1996.tb00356.x
42. Kern JA, Slebos RJC, Top B, et al. C-erbB-2 expression and codon 12 K-ras mutations both predict shortened survival for patients with pulmonary adenocarcinomas. *J Clin Invest.* 1994;93(2):516-520. doi:10.1172/JCI117001
43. Takenaka M, Hanagiri T, Shinohara S, et al. The prognostic significance of HER2 overexpression in non-small cell lung cancer. *Anticancer Res.* 2011;31(12):4631-4636. <http://www.ncbi.nlm.nih.gov/pubmed/22199341>. Accessed February 17, 2020.
44. Hirsch FR, Varella-Garcia M, Franklin WA, et al. Evaluation of HER-2/neu gene amplification and protein expression in non-small cell lung carcinomas. *Br J Cancer.* 2002;86(9):1449-1456. doi:10.1038/sj.bjc.6600286
45. Harpole DH, Richards WG, Sugarbaker DJ, Herndon JE, Marks JR. Localized Adenocarcinoma of the Lung: Oncogene Expression of Erbb-2 and P53 in 150 Patients. *Clin Cancer Res.* 1995;1(6):659-664. doi:10.1016/0169-5002(96)84256-5
46. Dean L. *Trastuzumab (Herceptin) Therapy and ERBB2 (HER2) Genotype*. National Center for Biotechnology Information (US); 2012. <http://www.ncbi.nlm.nih.gov/pubmed/28520362>. Accessed February 20, 2020.
47. Spring L, Niemierko A, Comander AH, et al. Tolerability and effectiveness of pertuzumab-containing neoadjuvant (NA) regimens vs. AC-TH for HER2-positive (+) localized breast cancer (BC). *J Clin Oncol.* 2016;34(15_suppl):586-586. doi:10.1200/jco.2016.34.15_suppl.586
48. Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): A randomised multicentre, open-label, phase 2 trial. *Lancet Oncol.* 2012;13(1):25-32. doi:10.1016/S1470-2045(11)70336-9
49. Oh DY, Bang YJ. HER2-targeted therapies — a role beyond breast cancer. *Nat Rev Clin Oncol.* 2020;17(1):33-48. doi:10.1038/s41571-019-0268-3
50. Park J, Neve R, Szollosi J, Benz C. Unraveling the biologic and clinical complexities of HER2. *Clin Breast Cancer.* 2008;8(5):392-401. doi:10.3816/CBC.2008.n.047
51. Khoury T, Mojica W, Hicks D, et al. ERBB2 juxtamembrane domain (trastuzumab binding site) gene mutation is a rare event in invasive breast cancers overexpressing the ERBB2 gene. *Mod Pathol.* 2011;24(8):1055-1059. doi:10.1038/modpathol.2011.64
52. Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. *Exp Hematol.* 1999;27(10):1533-1541. doi:10.1016/S0301-472X(99)00089-2

53. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin Oncol.* 1999;26(4 SUPPL. 12):60-70.
54. Baselga J, Albanell J. Mechanism of action of anti-HER2 monoclonal antibodies. *Ann Oncol.* 2001;12(SUPPL. 1). doi:10.1093/annonc/12.suppl_1.S35
55. Lane HA, Motoyama AB, Beuvink I, Hynes NE. Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. *Ann Oncol Off J Eur Soc Med Oncol.* 2001;12 Suppl 1:S21-2. doi:10.1093/annonc/12.suppl_1.s21
56. DailyMed - HERCEPTIN- trastuzumab kit HERCEPTIN- trastuzumab injection, powder, lyophilized, for solution. National Institutes of Health. <https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=492dbdb2-077e-4064-bff3-372d6af0a7a2>. Accessed February 20, 2020.
57. Beck A, Goetsch L, Dumontet C, Corvaia N. Strategies and challenges for the next generation of antibody-drug conjugates. *Nat Rev Drug Discov.* 2017;16(5):315-337. doi:10.1038/nrd.2016.268
58. Erickson HK, Park PU, Widdison WC, et al. Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res.* 2006;66(8):4426-4433. doi:10.1158/0008-5472.CAN-05-4489
59. Kovtun Y V., Audette CA, Ye Y, et al. Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. *Cancer Res.* 2006;66(6):3214-3221. doi:10.1158/0008-5472.CAN-05-3973
60. Staudacher AH, Brown MP. Antibody drug conjugates and bystander killing: is antigen-dependent internalisation required. *Br J Cancer.* 2017;117(12):1736-1742. doi:10.1038/bjc.2017.367
61. Bernardes GJL, Casi G, Trüssel S, et al. A traceless vascular-targeting antibody-drug conjugate for cancer therapy. *Angew Chemie - Int Ed.* 2012;51(4):941-944. doi:10.1002/anie.201106527
62. Singh AP, Sharma S, Shah DK. Quantitative characterization of in vitro bystander effect of antibody-drug conjugates. *J Pharmacokinet Pharmacodyn.* 2016;43(6):567-582. doi:10.1007/s10928-016-9495-8
63. Rinnerthaler G, Gampenrieder SP, Greil R. HER2 directed antibody-drug-conjugates beyond T-DM1 in breast cancer. *Int J Mol Sci.* 2019;20(5). doi:10.3390/ijms20051115
64. Oroudjev E, Lopus M, Wilson L, et al. Maytansinoid-antibody conjugates induce mitotic arrest by suppressing microtubule dynamic instability. *Mol Cancer Ther.* 2010;9(10):2700-2713. doi:10.1158/1535-7163.MCT-10-0645
65. Barok M, Tanner M, Köninki K, Isola J. Trastuzumab-DM1 is highly effective in preclinical models of HER2-positive gastric cancer. *Cancer Lett.* 2011;306(2):171-179. doi:10.1016/j.canlet.2011.03.002

66. Hunter FW, Barker HR, Lipert B, et al. F. W. Hunter et al., “Mechanisms of resistance to trastuzumab emtansine (T-DM1) in HER2-positive breast cancer,” *British Journal of Cancer*. Springer Nature, pp. 1–10, 16-Dec-2019. Mechanisms of resistance to trastuzumab emtansine (T-DM1) in HER2-positi. *Br J Cancer*. December 2019:1-10. doi:10.1038/s41416-019-0635-y
67. File:Figure 10 02 03.jpg - Wikimedia Commons. <https://commons.wikimedia.org/w/index.php?curid=49926271>. Accessed February 25, 2020.
68. File:Spindle chromosomes-en.png - Wikimedia Commons. https://commons.wikimedia.org/wiki/File:Spindle_chromosomes-en.png. Accessed February 25, 2020.
69. File:Mertansine skeletal.svg - Wikimedia Commons. https://commons.wikimedia.org/wiki/File:Mertansine_skeletal.svg. Accessed February 25, 2020.
70. SMCC [4-(N-Maleimidomethyl)cyclohexanecarboxylic acid N-hydroxysuccinimide ester] *CAS 64987-85-5* | AAT Bioquest. <https://www.aatbio.com/products/smcc-4-n-maleimidomethyl-cyclohexanecarboxylic-acid-n-hydroxysuccinimide-ester-cas-64987-85-5>. Accessed February 25, 2020.
71. Junttila TT, Li G, Parsons K, Phillips GL, Sliwkowski MX. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat*. 2011;128(2):347-356. doi:10.1007/s10549-010-1090-x
72. Barok M, Joensuu H, Isola J. Trastuzumab emtansine: Mechanisms of action and drug resistance. *Breast Cancer Res*. 2014;16(2):209. doi:10.1186/bcr3621
73. Lambert JM, Chari RVJ. Ado-trastuzumab emtansine (T-DM1): An antibody-drug conjugate (ADC) for HER2-positive breast cancer. *J Med Chem*. 2014;57(16):6949-6964. doi:10.1021/jm500766w
74. Ogitani Y, Hagihara K, Oitate M, Naito H, Agatsuma T. Bystander killing effect of DS-8201a, a novel anti-human epidermal growth factor receptor 2 antibody–drug conjugate, in tumors with human epidermal growth factor receptor 2 heterogeneity. *Cancer Sci*. 2016;107(7):1039-1046. doi:10.1111/cas.12966
75. Barok M, Tanner M, Könink K, Isola J. Trastuzumab-DM1 causes tumour growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells in vivo. *Breast Cancer Res*. 2011;13(2):R46. doi:10.1186/bcr2868
76. Peters S, Stahel R, Bubendorf L, et al. Trastuzumab emtansine (T-DM1) in patients with previously treated HER2-overexpressing metastatic non–small cell lung cancer: Efficacy, safety, and biomarkers. *Clin Cancer Res*. 2019;25(1):64-72. doi:10.1158/1078-0432.CCR-18-1590
77. Phillips GDL, Fields CT, Li G, et al. Dual targeting of HER2-positive cancer with trastuzumab emtansine and pertuzumab: Critical role for neuregulin blockade in antitumor

- response to combination therapy. *Clin Cancer Res.* 2014;20(2):456-468. doi:10.1158/1078-0432.CCR-13-0358
78. Takegawa N, Nonagase Y, Yonesaka K, et al. DS-8201a, a new HER2-targeting antibody–drug conjugate incorporating a novel DNA topoisomerase I inhibitor, overcomes HER2-positive gastric cancer T-DM1 resistance. *Int J Cancer.* 2017;141(8):1682-1689. doi:10.1002/ijc.30870
 79. Kovtun Y V., Audette CA, Mayo MF, et al. Antibody-maytansinoid conjugates designed to bypass multidrug resistance. *Cancer Res.* 2010;70(6):2528-2537. doi:10.1158/0008-5472.CAN-09-3546
 80. Kovtun Y V., Goldmacher VS. Cell killing by antibody-drug conjugates. *Cancer Lett.* 2007;255(2):232-240. doi:10.1016/j.canlet.2007.04.010
 81. Hamblett KJ, Jacob AP, Gurgel JL, et al. SLC46A3 is required to transport catabolites of noncleavable antibody maytansine conjugates from the lysosome to the cytoplasm. *Cancer Res.* 2015;75(24):5329-5340. doi:10.1158/0008-5472.CAN-15-1610
 82. Kinneer K, Meekin J, Tiberghien AC, et al. SLC46A3 as a potential predictive biomarker for antibody–drug conjugates bearing noncleavable linked maytansinoid and pyrrolobenzodiazepine warheads. *Clin Cancer Res.* 2018;24(24):6570-6582. doi:10.1158/1078-0432.CCR-18-1300
 83. Gavet O, Pines J. Progressive Activation of CyclinB1-Cdk1 Coordinates Entry to Mitosis. *Dev Cell.* 2010;18(4):533-543. doi:10.1016/j.devcel.2010.02.013
 84. Kavallaris M. Microtubules and resistance to tubulin-binding agents. *Nat Rev Cancer.* 2010;10(3):194-204. doi:10.1038/nrc2803
 85. Visconti R, Della Monica R, Grieco D. Cell cycle checkpoint in cancer: A therapeutically targetable double-edged sword. *J Exp Clin Cancer Res.* 2016;35(1):153. doi:10.1186/s13046-016-0433-9
 86. Ogitani Y, Aida T, Hagihara K, et al. DS-8201a, a novel HER2-targeting ADC with a novel DNA topoisomerase I inhibitor, demonstrates a promising antitumor efficacy with differentiation from T-DM1. *Clin Cancer Res.* 2016;22(20):5097-5108. doi:10.1158/1078-0432.CCR-15-2822
 87. Nakada T, Sugihara K, Jikoh T, Abe Y, Agatsuma T. The Latest Research and Development into the Antibody–Drug Conjugate, [fam-] Trastuzumab Deruxtecan (DS-8201a), for HER2 Cancer Therapy. *Chem Pharm Bull.* 2019;67(3):173-185. doi:10.1248/cpb.c18-00744
 88. Shitara K, Iwata H, Takahashi S, et al. Trastuzumab deruxtecan (DS-8201a) in patients with advanced HER2-positive gastric cancer: a dose-expansion, phase 1 study. *Lancet Oncol.* 2019;20(6):827-836. doi:10.1016/S1470-2045(19)30088-9
 89. Wang JC. Cellular roles of DNA topoisomerases: A molecular perspective. *Nat Rev Mol Cell Biol.* 2002;3(6):430-440. doi:10.1038/nrm831
 90. Aguilera A, García-Muse T. R Loops: From Transcription Byproducts to Threats to

- Genome Stability. *Mol Cell*. 2012;46(2):115-124. doi:10.1016/j.molcel.2012.04.009
91. Pommier Y. Topoisomerase I inhibitors: Camptothecins and beyond. In: *Nature Reviews Cancer*. Vol 6. Nature Publishing Group; 2006:789-802. doi:10.1038/nrc1977
 92. Pommier Y, Barcelo JM, Rao VA, et al. Repair of Topoisomerase I-Mediated DNA Damage. *Prog Nucleic Acid Res Mol Biol*. 2006;81:179-229. doi:10.1016/S0079-6603(06)81005-6
 93. Pommier Y, Kiselev E, Marchand C. Interfacial inhibitors. *Bioorganic Med Chem Lett*. 2015;25(18):3961-3965. doi:10.1016/j.bmcl.2015.07.032
 94. Kumazawa E, Jimbo T, Ochi Y, Tohgo A. Potent and broad antitumor effects of DX-8951f, a water-soluble camptothecin derivative, against various human tumors xenografted in nude mice. *Cancer Chemother Pharmacol*. 1998;42(3):210-220. doi:10.1007/s002800050807
 95. Minami H, Fujii H, Igarashi T, et al. Phase I and pharmacological study of a new camptothecin derivative, exatecan mesylate (DX-8951f), infused over 30 minutes every three weeks. *Clin Cancer Res*. 2001;7(10):3056-3064. <http://www.ncbi.nlm.nih.gov/pubmed/11595695>. Accessed March 10, 2020.
 96. Zhang Q, Li F. Combating P-glycoprotein-Mediated Multidrug Resistance Using Therapeutic Nanoparticles. *Curr Pharm Des*. 2013;19(37):6655-6666. doi:10.2174/1381612811319370009
 97. Mitsui I, Kumazawa E, Hirota Y, et al. A New Water-soluble Camptothecin Derivative, DX-8951f, Exhibits Potent Antitumor Activity against Human Tumors in vitro and in vivo. *Japanese J Cancer Res*. 1995;86(8):776-782. doi:10.1111/j.1349-7006.1995.tb02468.x
 98. Lewis Phillips GD, Li G, Dugger DL, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res*. 2008;68(22):9280-9290. doi:10.1158/0008-5472.CAN-08-1776
 99. Hamblett KJ, Senter PD, Chace DF, et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res*. 2004;10(20):7063-7070. doi:10.1158/1078-0432.CCR-04-0789
 100. Aggarwal N, Sloane BF. Cathepsin B: Multiple roles in cancer. *Proteomics - Clin Appl*. 2014;8(5-6):427-437. doi:10.1002/prca.201300105
 101. Ruan J, Zheng H, Fu W, Zhao P, Su N, Luo R. Increased Expression of Cathepsin L: A Novel Independent Prognostic Marker of Worse Outcome in Hepatocellular Carcinoma Patients. Coleman WB, ed. *PLoS One*. 2014;9(11):e112136. doi:10.1371/journal.pone.0112136
 102. File:Exatecan.svg - Wikimedia Commons. <https://commons.wikimedia.org/wiki/File:Exatecan.svg>. Accessed March 10, 2020.
 103. Sulfo-SMCC [4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester, sodium salt] *CAS#: 92921-24-9* | AAT Bioquest.

- <https://www.aatbio.com/products/sulfo-smcc-4-n-maleimidomethyl-cyclohexane-1-carboxylic-acid-3-sulfo-n-hydroxysuccinimide-ester-sodium-salt-cas-92921-24-9>. Accessed March 10, 2020.
104. Cheng Y, Chen MT, Patterson LC, et al. Domain-specific free thiol variant characterization of an IgG1 by reversed-phase high-performance liquid chromatography mass spectrometry. *Anal Biochem.* 2017;519:8-14. doi:10.1016/j.ab.2016.12.003
 105. Kim W, Kang KH, Suh J-K. Characterization of Biopharmaceuticals Focusing on Antibody Therapeutics. In: *Biopharmaceuticals*. InTech; 2018. doi:10.5772/intechopen.79107
 106. Liu H, Chumsae C, Gaza-Bulseco G, Hurkmans K, Radziejewski CH. Ranking the susceptibility of disulfide bonds in human IgG1 antibodies by reduction, differential alkylation, and LC-MS analysis. *Anal Chem.* 2010;82(12):5219-5226. doi:10.1021/ac100575n
 107. File:Disulfide reduction by DTT-2.png - Wikimedia Commons. https://commons.wikimedia.org/wiki/File:Disulfide_reduction_by_DTT-2.png. Accessed March 13, 2020.
 108. Seo A, Jackson JL, Schuster J V., Vardar-Ulu D. Using UV-absorbance of intrinsic dithiothreitol (DTT) during RP-HPLC as a measure of experimental redox potential in vitro. *Anal Bioanal Chem.* 2013;405(19):6379-6384. doi:10.1007/s00216-013-7063-2
 109. DTNB (Ellman's Reagent) (5,5-dithio-bis-(2-nitrobenzoic acid). <https://www.thermofisher.com/order/catalog/product/22582#/22582>. Accessed March 13, 2020.
 110. Shetab-Boushehri SV. Ellman's method is still an appropriate method for measurement of cholinesterases activities. *EXCLI J.* 2018;17:798-799. doi:10.17179/excli2018-1536
 111. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985;150(1):76-85. doi:10.1016/0003-2697(85)90442-7
 112. Sulfhydryl-Reactive Crosslinker Chemistry | Thermo Fisher Scientific - US. <https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/sulfhydryl-reactive-crosslinker-chemistry.html>. Accessed March 13, 2020.
 113. Fontaine SD, Reid R, Robinson L, Ashley GW, Santi D V. Long-term stabilization of maleimide-thiol conjugates. *Bioconjug Chem.* 2015;26(1):145-152. doi:10.1021/bc5005262
 114. Fogh J, Fogh JM, Orfeo T. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst.* 1977;59(1):221-226. doi:10.1093/jnci/59.1.221
 115. Blanco R, Iwakawa R, Tang M, et al. A gene-alteration profile of human lung cancer cell lines. *Hum Mutat.* 2009;30(8):1199-1206. doi:10.1002/humu.21028
 116. The p53 tumor suppressor protein - Genes and Disease - NCBI Bookshelf.

- <https://www.ncbi.nlm.nih.gov/books/NBK22268/>. Accessed March 13, 2020.
117. Cavazzoni A, Alfieri RR, Cretella D, et al. Combined use of anti-ErbB monoclonal antibodies and erlotinib enhances antibody-dependent cellular cytotoxicity of wild-type erlotinib-sensitive NSCLC cell lines. *Mol Cancer*. 2012;11:91. doi:10.1186/1476-4598-11-91
 118. Cailleau R, Olivé M, Cruciger QVJ. Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. *In Vitro*. 1978;14(11):911-915. doi:10.1007/BF02616120
 119. Subik K, Lee JF, Baxter L, et al. The expression patterns of ER, PR, HER2, CK5/6, EGFR, KI-67 and AR by immunohistochemical analysis in breast cancer cell lines. *Breast Cancer Basic Clin Res*. 2010;4(1):35-41. doi:10.1177/117822341000400004
 120. Mudvari P, Ohshiro K, Nair V, Horvath A, Kumar R. Genomic Insights into Triple-Negative and HER2-Positive Breast Cancers Using Isogenic Model Systems. *PLoS One*. 2013;8(9). doi:10.1371/journal.pone.0074993
 121. ERFFI1 - ERBB receptor feedback inhibitor 1 - Homo sapiens (Human) - ERFFI1 gene & protein. <https://www.uniprot.org/uniprot/Q9UJM3#function>. Accessed March 13, 2020.
 122. TrypLE Express & TrypLE Select | Thermo Fisher Scientific - US. <https://www.thermofisher.com/us/en/home/life-science/cell-culture/mammalian-cell-culture/reagents/trypsin/trypse-express.html>. Accessed March 13, 2020.
 123. CHACON E, ACOSTA D, LEMASTERS JJ. Primary Cultures of Cardiac Myocytes as In Vitro Models for Pharmacological and Toxicological Assessments. In: *In Vitro Methods in Pharmaceutical Research*. Elsevier; 1997:209-223. doi:10.1016/b978-012163390-5.50010-7
 124. Lim SW, Loh HS, Ting KN, Bradshaw TD, Allaudin ZN. Reduction of MTT to purple formazan by vitamin E isomers in the absence of cells. *Trop Life Sci Res*. 2015;26(1):111-120.
 125. Patravale V, Dandekar P, Jain R. Nanotoxicology: evaluating toxicity potential of drug-nanoparticles. In: *Nanoparticulate Drug Delivery*. Elsevier; 2012:123-155. doi:10.1533/9781908818195.123
 126. Mahajan SD, Law WC, Aalinkeel R, et al. Nanoparticle-mediated targeted delivery of antiretrovirals to the brain. In: *Methods in Enzymology*. Vol 509. Academic Press Inc.; 2012:41-60. doi:10.1016/B978-0-12-391858-1.00003-4
 127. Lyon RP, Meyer DL, Setter JR, Senter PD. Conjugation of anticancer drugs through endogenous monoclonal antibody cysteine residues. In: *Methods in Enzymology*. Vol 502. Academic Press Inc.; 2012:123-138. doi:10.1016/B978-0-12-416039-2.00006-9
 128. Wang L, Amphlett G, Blättler WA, Lambert JM, Zhang W. Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. *Protein Sci*. 2005;14(9):2436-2446. doi:10.1110/ps.051478705
 129. Lyon RP, Bovee TD, Doronina SO, et al. Reducing hydrophobicity of homogeneous

- antibody-drug conjugates improves pharmacokinetics and therapeutic index. *Nat Biotechnol.* 2015;33(7):733-735. doi:10.1038/nbt.3212
130. Frka-Petesic B, Zanchi D, Martin N, Carayon S, Huille S, Tribet C. Aggregation of Antibody Drug Conjugates at Room Temperature: SAXS and Light Scattering Evidence for Colloidal Instability of a Specific Subpopulation. *Langmuir.* 2016;32(19):4848-4861. doi:10.1021/acs.langmuir.6b00653