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Design, Synthesis, and Evaluation of a Novel Cytotoxic Peptide-Doxorubicin Conjugate Targeting Triple-Negative Breast Cancer

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ABSTRACT

Design, Synthesis, and Evaluation of a Novel Cytotoxic Peptide-Doxorubicin Conjugate Targeting Triple-Negative Breast Cancer

by Elmira Ziaei

The aim of this thesis was to design, synthesize, and characterize a novel peptide-doxorubicin conjugate, evaluate in vitro stability and cytotoxicity properties of the conjugate and study its mechanism for uptake into the triple-negative breast cancer (TNBC) cells. For the synthesis of the conjugate, first, doxorubicin (Dox) was conjugated to a highly efficient established cross-linker, MCC, to give an intermediate product, MCC-Dox. MCC-Dox was characterized using NMR spectroscopy, mass spectrometry, and RP-HPLC and purified for subsequent reaction. Second, the 11-mer peptide 18-4 (NH_2-CWxEAAYQrFL-$CONH_2$) with high proteolytic stability and specificity for breast cancer cells was reacted with MCC-Dox to synthesize the final product peptide-Dox conjugate which was also purified and characterized. The evaluation of the stability in water, media, and human serum showed that conjugate possesses decent stability. The results from the evaluation of stability of conjugate in human serum up to 48 h showed that half-life for the conjugate was 18 h. Furthermore, when the conjugate was incubated in aqueous conditions with pH 7, more than 80% of it was still intact after 48 h. However, the half-life of the conjugate in the aqueous conditions with pH 5, or cell culture medium was 24 h and ~ 48 h, respectively. The cytotoxicity studies showed that the conjugate was as toxic as free Dox toward the TNBC cells, MDA-MB-231 and MDA-MB-468, and was 30 times less toxic toward the non-cancerous breast cells, MCF-10A, compared to the free doxorubicin. Furthermore, results from evaluating surface expression levels of keratin 1 (K1) in

different breast cancer cell lines and a normal breast cell line using immunohistochemistry staining suggested that cell-surface expression of K1 in normal breast MCF-12A cell line is much less than that of TNBC MDA-MB-231 and MDA-MB-468 cell lines. The results presented here highlight the potential of a targeted peptide-Dox conjugate as a new modality for TNBC treatment.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADC	Antibody Drug Conjugate
APN	Aminopeptidase N
AUC	Area Under the Curve
BSA	Bovine Serum Albumin
Boc	t-Butoxycarbonyl
CHCA	α-Cyano-4-hydroxycinnamic acid
DAPI	4′,6-Diamidino-2-phenylindole
DCM	Dichloromethane
DHC	Docosahexaenoic Acid
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
Dox	Doxorubicin
Et_2O	Diethylether
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HBSS	Hank's Balanced Salt Solution
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate
HUVEC	Human Umbilical Vein Endothelial Cells
IHC	Immunohistochemistry
K1	Keratin 1

PBS	Phosphate-buffered Saline
PDC	Peptide Drug Conjugate
PTX	Paclitaxel
MALDI-TOF MS	Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry
NHS	N- hydroxysuccinimide
NMM	N-methyl- morpholine
NMR	Nuclear Magnetic Resonance
NOD-SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
RP-HPLC	Reversed-phase High Pressure Liquid chromatography
r.t.	Room temperature
SMCC	succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate
tBu	*tert*-butyl
TFA	Trifluoroacetic acid
TIS	triisoproylsilane
TNBC	Triple-Negative Breast Cancer
Trp	Tryptophan

CHAPTER 1: Targeted Drug Delivery to Triple-Negative Breast Cancer Cells

1.1 INTRODUCTION

1.1.1 Targeted Breast Cancer Therapy

Cancer treatment is hampered by the non-specific uptake of the chemotherapeutic agents by the peripheral tissues causing toxicities and side effects such as myelosuppression, a dose-limiting cardiotoxicity, nausea, vomiting and diarrhea.[1] Several approaches have been used to enhance the site-specific delivery of chemotherapeutic agents to cancer cells in tumors sparing peripheral healthy cells and tissues.[2] In this regard, targeting ligands like engineered antibodies and tumor homing peptides have gained attention as these target specific receptors on cancer cells.[3-6] Targeted antibodies or peptides are covalently conjugated to chemotherapeutic agents via a linker to provide antibody-drug conjugates (ADCs) or peptide-drug conjugates (PDCs), respectively, that deliver chemotherapeutic specifically to cancer cells (**Figure 1.1**).[2, 3, 5] To date, eight ADCs have been approved by FDA for the treatment of various cancers.[2] Ado-trastuzumab emtansine (Kadcyla) and brentuximab vedotin (Adcetris) are two ADCs, that are used clinically for the treatment of metastatic breast cancer and refractory Hodgkin's lymphoma, respectively.[2, 7, 8] Kadcyla consists of a monoclonal antibody (Herceptin) linked to the cytotoxic agent (DM1) through a nonreducible thioether linker called N-maleimidomethyl-cyclohexane-1-carboxylic acid (MCC) linker.[9, 10] This drug pairs a tubulin inhibitor with a HER2 blocker to treat HER2-positive metastatic breast cancer.[9, 10] Although ADCs have shown to be effective, PDCs have few advantages over ADCs, such as lower chance of immunogenicity, easier chemistry of conjugation of peptides to drugs, and better control over peptide-drug ratio.[9] Another advantage of peptides over

antibodies is the small size of peptides which allows them to diffuse through the vasculature and penetrate deep into the interstitial space of tumors.[9] In addition, some PDCs have the ability to bypass p-glycoprotein efflux and lower the chance of drug resistance.[9] Many ADCs have shown poor activity in drug resistant cells which overexpress p-glycoprotein efflux pumps.[9] Furthermore, PDCs can be easily synthesized to homogeneity, are economical and can penetrate deep into the tumors. Design of PDCs involves selection of a proper targeting peptide, linker that allows sufficient circulation time and is mainly cleaved at the tumor site, and a highly toxic chemotherapeutic drug.

A significant challenge with chemotherapeutic drugs, is their toxicity to non-cancerous (normal) cells and tissues.[11] As a result, the clinical application of these drugs is limited by side effects such as nausea, myelosuppression and cardiotoxicity.[1] In addition; the efficacy of the drugs is reduced because they do not specifically accumulate at tumor sites.[11] Examination of the biochemistry of tumor tissue and its microenvironment has revealed that cancerous cells express different types of biomarkers or overexpress same biomarkers as normal cells.[9] In order to target chemotherapeutic agents specifically to cancerous cells, various methods such as targeted delivery of therapeutic molecules have been explored.[9] The use of targeting ligands such as peptides, antibodies and prodrugs with high affinity for receptors or markers expressed on tumors can specifically deliver chemotherapeutic agents to tumor.[9, 11] Different methods such as direct conjugation of drugs to targeting ligands, or conjugation of ligands to the surface of a drug carrier like liposomes containing the drug have been explored.[9] Over the last two decades, the pharmaceutical industry's interest in peptide based therapeutics has highly increased, due to physicians and patients' preference for protein therapeutics, and the convenience of these therapeutics in synthesis, stability and manufacturing viewpoints.[11] Peptide based therapeutics could also potentially resolve unmet medical needs in vast range of therapeutic areas, such as cancer therapy, and treatments for

cardiovascular disease and metabolic disorders.[11] The advantages of peptide-based therapeutics include, easy, rapid and cost effective synthesis of peptides using automated solid phase peptide synthesis methodology, easy penetration of peptides into deep tissues due to their small size, and lower possibility of causing immunogenicity.[9]

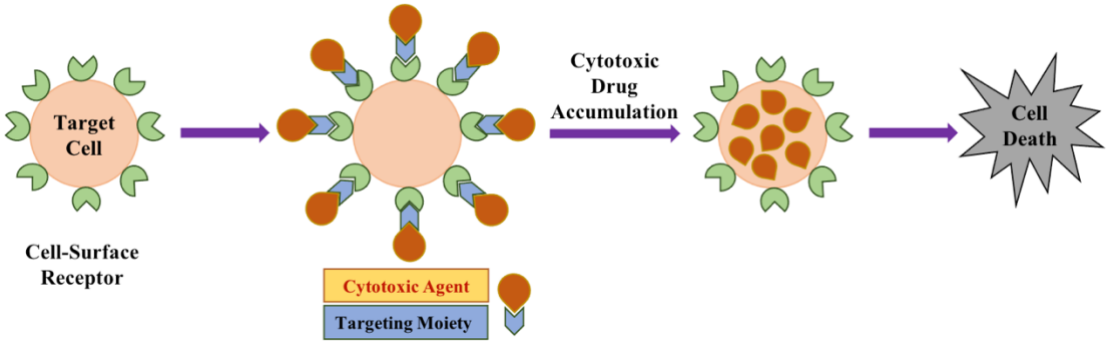


Figure 1.1. Schematic illustration of targeted anti-cancer therapy using a tumor targeting ligand that delivers the cytotoxic agent to which it is covalently linked to the cancer cell with specific over-expressed surface biomarkers.

Despite promising results with peptides as targeting ligands, incorporating peptides in targeted drug delivery for clinical applications is hindered due to their fast-proteolytic degradation in vitro and in vivo.[1] Other disadvantages include fast renal clearance and difficulty maintaining secondary structure of peptides.[9]

1.1.2 Triple-Negative Breast Cancer (TNBC)

Breast cancer is a malignant disease, and one the most common types of cancer and second leading cause of death among women worldwide.[12] Breast cancer is divided into four subtypes based on the level of expression of three types of receptors including, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor (HER2).[12] The fourth subtype is triple negative breast cancer (TNBC) in which the poorly differentiated breast

cancer cells lack all three receptors.[12] TNBC represents ~15% to 20% of all breast cancers with lower overall survival rate in metastatic diseases.[13] Clinically, TNBC cells are characterized as highly metastatic to central nervous system and visceral organs at early stages, with fairly aggressive local growth and rapid progression.[14] Gene analysis of different TNBC samples showed that most TNBCs are basal type epithelial cells which can be stained with antibodies to some keratins (e.g. Keratin 5) using immunohistochemistry.[15]

Until recently, the standard of care treatment for patients with TNBC tumor is a combination of surgery ± radiotherapy ± immunotherapy ± chemotherapy.[15] Some of the major cytotoxic chemotherapeutics used for the treatment of metastatic TNBC include taxanes, anthracyclines, ixabepilone and capecitabine.[15] The absence of a targetable over-expressed biomarker in TNBC tumors has made the development of effective targeted therapies extra challenging.[13] Therefore, more research studies in design and development of effective targeted chemotherapeutics for the treatment of TNBC is necessary.

Furthermore, TNBC is beginning to be better understood by its molecular characteristics and clinical response to therapeutics.[16] And, the use of chemotherapy as the backbone of therapy against TNBC is changing based on molecular subtyping, as “one size fits all” approach hasn’t shown promising results.[16] Currently, many research investigations and clinical studies are in progress exploring various potential targets in TNBC including antibody-drug conjugates (ADCs), peptide-drug conjugates (PDCs), PARP inhibition and immune-directed therapy with checkpoint inhibition.[16] Recent molecularly targeted approaches have shown encouraging clinical activity against TNBC.[16] It has been reported that three new targeted therapies for TNBC have been recently approved, including atezolizumab which is used in combination with nabpaclitaxel, talazoparib, and olaparib.[16] In near future, the term “TNBC” will no longer be appropriate, as both genome sequencing and molecular classification of TNBC are advancing to identify potential molecular targets in this breast cancer subtype.[16]

1.1.3 Different Targeting Ligands and Tumor-Specific Biomarkers in Breast Cancer

Identification of biomarkers or proteins expressed on tumor cells and designing the targeting ligands which bind to biomarkers with high affinity are essential for diagnosis and treatment of any type of cancer.[17]. Integrins, human epidermal growth factor, and aminopeptidase N are a few of tumor-specific biomarkers that are overexpressed in tumors (**Figure 1.2)**.[9] In this section, I will discuss these molecular markers and the ligands that target them.

1.1.3.1 Integrins

One type of tumor-specific biomarkers identified by researchers are integrins. Integrins, membrane glycoprotein receptors, facilitate cell adhesion for cytokines, extracellular matrix proteins, growth hormones and immunoglobulins.[9] Binding of specific extracellular ligands to integrins ultimately results in regulation of cell proliferation, differentiation, migration and apoptosis.[9] Tumor growth, invasion, angiogenesis, cancer cell transformation, and metastasis are different stages of cancer in which certain subtypes of integrins with diverse roles are overexpressed in cancer cells.[9] It has been reported that RGD (Arg-Gly-Asp) motif present in extracellular proteins, such as fibronectin, plasminogen, laminin and etc., target integrins.[9] Therefore, peptides with RGD motif can target integrins and inhibit the interaction between integrins and their native ligands, and as a result can be applied for cancer-targeted therapeutics.[9]

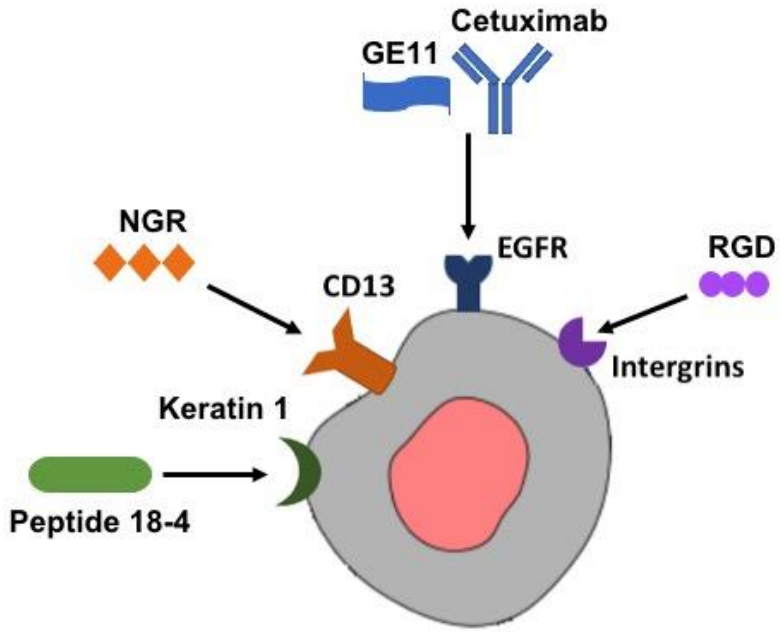


Figure 1.2. Schematic illustration of different targeting ligands and their specific biomarkers. For instance, RGD peptide binds integrins, GE11 peptide and Cetuximab antibody bind EGFR receptor, and NGR peptide bind CD13 receptor. [9]

1.1.3.2 Epidermal Growth Factor Receptor

Another glycoprotein that is overexpressed in different types of human cancers is epidermal growth factor receptor (EGFR).[9] EGFR consists of three parts, an extracellular N-terminal binding domain, a transmembrane region which is hydrophobic, and an intracellular C-terminal tyrosine kinase domain.[9] Epidermal growth factor (EGF) is one of the ligands that binds to and activates EGFR receptor.[9] Following binding, series of signaling pathways are activated which promote different cell processes necessary for the proliferation, survival and spread of cancer cells.[9] As a result, inhibition of EGFR using ligands specific to this receptor is one of major approaches in treating cancer.[9] Cetuximab, a clinically approved anti-EGFR monoclonal antibody, is one of many EGFR blockers developed against cancer.[9] Other types of therapies investigated to inhibit EGFR receptor include antibody drug conjugates, and

antisense oligonucleotides.[9] A 12-mer peptide GE11 (YHWYGYTPQNVI) has been identified that binds EGFR on cell surface and is being used as a targeting ligand for chemotherapeutics and diagnostics.[9]

1.1.3.3 Aminopeptidase N

CD13 or Aminopeptidase N (APN), found in many different human cell types, such as leukocytes, fibroblasts, and epithelial cells, is an integral membrane enzyme which is Zn^{2+} dependent.[9] APN has many different functions including playing a role in cell cycle control, angiogenesis, tumor cell invasion, metastasis, degradation of peptides, and protein maturation.[9] It has been reported that specific isoforms of APN are overexpressed in different cancers, such as prostate cancer, acute myeloid leukemia and colon cancer.[9] A tripeptide (Asn-Gly-Arg) called NGR has been engineered that specifically targets and binds APN.[9] As a result NGR can be used as a ligand in peptide tumor-targeted therapies.[9]

1.1.3.4 HER2

Nearly 20% of breast tumors are caused by mutation or amplification of the oncogene human epidermal growth factor receptor (HER2). HER2-positive breast cancer is identified if immunohistochemistry testing shows overexpression of HER2 protein, or there is evidence of show HER2 gene amplification on florescence in situ hybridization testing.[18]

1.1.3.5 Keratin 1

Keratin proteins are intermediate filament (IF)-forming heteropolymers that are expressed in all epithelial cells.[19] Keratin filaments provide critical structural framework within the cellular cytoplasm to provide support against mechanical and non-mechanical stressors.[19, 20] Keratin proteins are found in the epithelial cells of many different organs

such as skin, breast, liver, eyes, etc.[19] Mammalian epithelial cells consist of 54 keratin genes, 28 type I and 26 type II.[19, 20] Since not all keratins are expressed in a given cell, epithelial cells are classified based on their profile of keratin expression.[20] For instance, K1/K10, K15, K9 and K2 are all expressed in keratinocytes, whereas K6 and K16 are keratins of epidermis.[19] Keratins not only support epithelial cell integrity, they also regulate variety of cellular functions such as protein translation control, apico-basal polarization, membrane protein targeting and organelle positioning.[19]

Keratins are largely used as tumor markers in cancer diagnosis and prognosis.[19] Several studies suggested higher cell-surface expression of keratin 1 (K1) receptor in breast cancerous cells including breast and neuroblastoma cancer cells compared to non-cancerous cells. Soudy R. *et al*. used affinity chromatography followed by liquid chromatography-tandem mass spectrometry to identify Keratin 1 as a receptor for the p160 peptide(s) on the surface of breast cancer cells.[17] The authors showed the presence of K1 on MCF7 cell surface using confocal microscopy. In addition, higher expression (total) of K1 was observed in MCF7 breast cancer cells compared to non-cancerous MCF10A cells using Western blot analysis. Chuang *et al.* found K1 present on the plasma membrane of neuroblastoma (NMB7) cells.[21] In addition, the surface keratin 8 was found in colon cancer cells using a monoclonal antibody.[22]

1.1.4 Peptide-Drug Conjugates for Tumor Targeting

One of the antitumor drugs that is widely used in treatment of breast cancer is Doxorubicin (Dox).[1] By inhibiting the topoisomerase II and causing DNA damage, Dox induces apoptosis and kills the cancerous cells.[1] However, due to lack of tumor targeting specificity of Dox and the emergence of tumor multidrug resistance, when applied systematically to patients, it causes detrimental side effects such as myelosuppression and a

dose-limiting cardiotoxicity.[1] One way to overcome nonspecific toxicity and other limitations of Dox is to prepare a peptide-Dox conjugate by attaching a targeting peptide ligand to Dox through a linker which can deliver and release Dox inside cancerous cells efficiently.[1]

Selective drug delivery using peptide-based tumor targeting ligands can be achieved in two ways.[9] One way is to conjugate tumor specific peptides directly to an anti-tumor drug such as chemotherapeutics, toxins, antisense oligonucleotides and pro-apoptotic peptides in order to design a molecule called peptide-drug conjugate (PDC).[9] Remarkable successful studies have been reported in targeting tumors using PDCs.[9] For example, in one study doxorubicin (Dox), an antineoplastic drug, is conjugated to RGD4C peptide to target a specific subtype of integrins overexpressed in angiogenic blood vessels and tumor cells of a breast tumor model.[9] The Dox-RGD4C conjugate proved to be more effective at inhibiting the growth and spread of metastatic cancerous cells in mice model.[9] This PDC also displayed less toxicity to liver and heart, despite showing equal efficacy to free Dox *in vitro*.[9] In another study, conjugating Dox to Luteinizing-hormone-releasing hormone LHRH peptide ligands showed similar ability in inhibition of tumor growth compared to that of free Dox.[9] Few PDCs have reached clinical trials including a PDC, GRN1005, which targets a receptor-related protein called LRP-1 that is overexpressed on the surface of the blood brain barrier.[9] Here, PDC is composed of a 19-mer angiopep2 peptide (TFFYGGSRGK- RNNFKTEEY) covalently conjugated to three units of paclitaxel, GRN1005 has shown to be highly efficacious in treating solid brain tumors.[9]

Another way to design a PDC is to conjugate tumor specific peptides to an anti-tumor drug via a linker.[2] The primary purpose in using a linker in design of a drug-conjugate is the role it mainly plays in the circulation time of the conjugate and release of the cytotoxic drug such as doxorubicin at the target site for improved efficacy.[2] When the chemical reactions

between a peptide, linker and a drug happens to synthesize a PDC molecule, two chemical bonds are formed.[2] One bond forms between the peptide and the linker and the other between the linker and the drug.[2] After administration of PDC in systemic circulation, the two bonds and any other functional group that is present in the linker become susceptible to cleavage by the enzymes or physiological conditions present in blood and serum which would lead to the separation of peptide from the drug.[2] Hence, the chemistry of a linker is the determining factor for its potency in release of the drug only after intracellular uptake of the PDC by the tumor cells for selective cytotoxicity.[2] Based on the functional groups that are present in a linker and how they behave under in vitro and in vivo conditions, linkers are classified in to four groups of acid cleavable (carbonate and hydrazone), enzyme cleavable (amide, ester and carbamate), non-cleavable (oxime, thioether, and triazole), and reducible disulfide.[2] Herein, some of the PDCs categorized by the bond between the linker and the drug are discussed.

1.1.4.1 Peptide-Doxorubicin Conjugates with Amide/Ester Linker

As mentioned above (section 1.1.3.5), p160 is a linear 12-mer peptide that targets and binds breast cancer cells (**Figure 1.3**).[23] Peptide p160 was discovered using *in vitro* phage display peptide library which was screened against WAC2 neuroblastoma cells.[23, 24] Zhang *et al.* found that p160 binds to and internalizes into breast cancer cells such as MDA-MB-435 cells with high affinity and specificity.[23, 24] Later, in order to find improved analogues of p160, Ahmed *et al*. used p160 as a lead peptide to make a synthetic peptide array of 70 linear peptides.[25] As a result, peptide **18**, a 10-mer p160 analogue was identified which showed higher binding affinity to MDA-MB-435 and MCF-7 cancer cells by 3 folds compared to p160 was identified.[9, 25] However, since peptides p160 and 18 had low proteolytic stability in presence of human serum and liver homogenate, Soudy R. *et al* further optimized peptide 18

for higher binding affinity and improved proteolytic stability to yield a 10-mer peptide **18-4** (**Figure 1.3**).[9, 11] The optimization involved two D-amino acid substitutions which improved the proteolytic stability of peptide 18-4 in human serum and liver homogenate.[11, 25] As a result, it was found that 18-4 facilitated high tumor uptake, and caused minimal toxicity in healthy tissues compared to peptide 18.[11, 25]

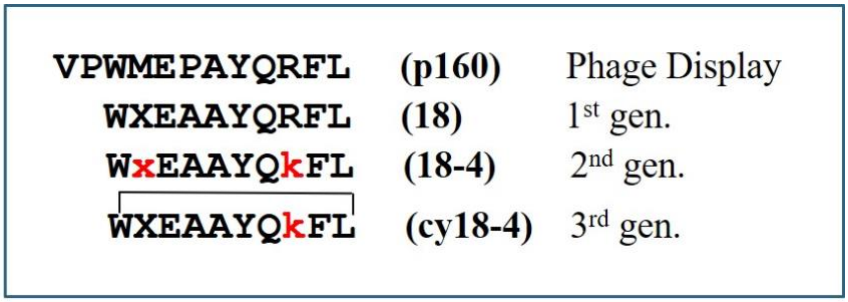


Figure 1.3. Sequence of peptide p160 and its analogues. Residues in red lower case are D-amino acids (x is D-norleucine and k is D-Lysine).

Later, Raghuwanshi Y. *et al.* synthesized peptide cy18-4 (**Figure 1.3)**, a novel cyclic peptide analogue of linear peptide 18-4, and demonstrated through *in vivo* animal studies using mice carrying orthotopic breast tumors that the cyclic peptide preferentially accumulates in tumor.[26] In vivo imaging studies in live animals showed that 2 h after injection of Cy5.5 labeled peptide cy18-4, the peptide was mainly observed in breast tumor, and its potential elimination sites liver and kidney.[26]

Soudy R. *et al*. conjugated peptide 18-4 to Doxorubicin (Dox) through a hydrolysable linker, glutaric anhydride, at two different sites, to form peptide-Dox ester and peptide-Dox amide conjugates (**Figure 1.4**).[1] Cytotoxicity studies showed that the toxicity of one of the

conjugates toward breast cancerous cells was similar to that of free Dox, while it was considerably less toxic toward noncancerous cells compared to free Dox.[1] Although the data from their study suggested that peptide-Dox ester conjugate could be used as a potential drug conjugate to improve the therapeutic index of Dox, the in vitro studies of the two conjugates showed limitations such as peptide 18-4-Dox ester conjugate had short life of 2 hours and the peptide 18-4-Dox amide had low rate of Dox release.[1] `As a result, it could be concluded that glutaric anhydride is not an efficient linker in improving the efficacy of peptide-Dox conjugate.[1]

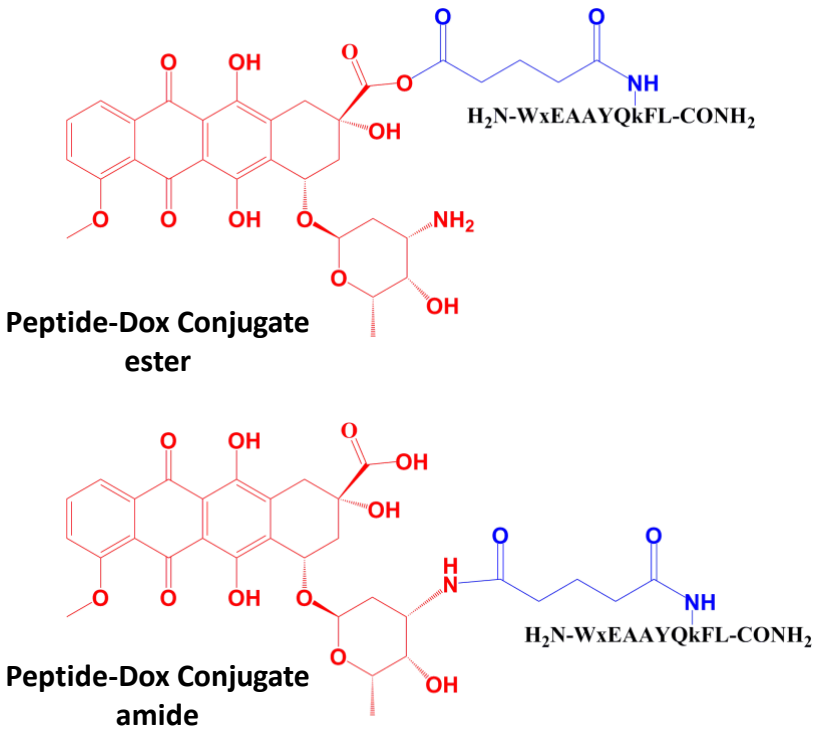


Figure 1.4. Peptide –Doxorubicin conjugates reported previously, where Dox (red) is conjugated to the linker via an ester or an amide bond.[1]

1.1.4.2 Peptide-Doxorubicin Conjugates with Peptide Linker

Liang et al. designed a peptide-Dox conjugate using cRGDfC as a targeting peptide ligand, Dox as a chemotherapeutic agent, and Valine-Citrulline dipeptide (VC) as a linker that was intended to be cleaved specifically by a carboxypeptidase, cathepsin B, that is present in excess in the lysosomes of cancer cells.[2, 27] The authors conjugated the tumor homing cyclic RGD peptide to the dipeptide linker via a maleimide thioether bond which is non-cleavable, and the linker to Dox via a para-aminobenzyl carbamate (PABC) spacer which is an enzyme cleavable bond that undergoes spontaneous 1,6-elimination (**Figure 1.5**).[2, 27] The excess amount of cathepsin B in Lysosomes of cancer cells facilitates the cleavage of dipeptide (VC) and further removal of PABC spacer which results in site-specific release of Dox.[2, 27]

The linker chemistry used in cRGD-VC-Dox containing dipeptide and thioether linkage resembles the linker chemistry used in FDA approved ADC drug brentuximab vedotin (Adcetris).[2, 27] cRGD-VC-Dox conjugate was found to have superior in vitro and in vivo efficacies compared to another PDC with a reducible disulfide linker emphasizing the potency of dipeptide (VC) linker in releasing the drug at the target site.[2, 27]

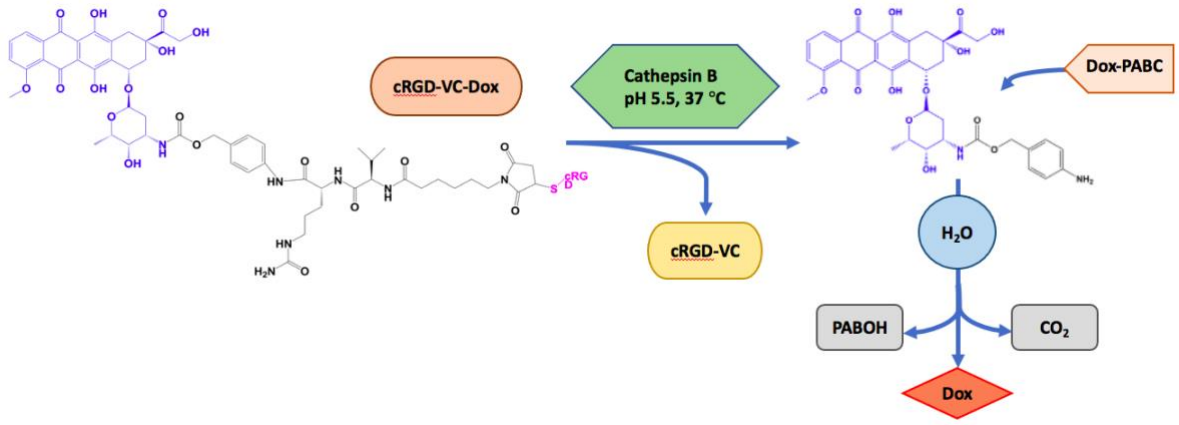


Figure 1.5. cRGD-VC-Dox conjugate undergoing spontaneous 1,6-elimination after the cleavage of the C-terminal amide of citrulline by cathepsin B enzyme.[27] (VC is Valine-Citrulline dipeptide, PABC is para-aminobenzyl carbamate spacer, and PABOH is para-aminobenzyl alcohol.)

1.1.4.3 Peptide-Doxorubicin Conjugates with Hydrazone Linker

Researchers have employed acid-sensitive linkers such as hydrazone in design of several PDCs.[2] After being administered in to systemic circulation, acid-sensitive linkers maintain stability in blood circulation (pH 7.4), and when exposed to acidic conditions in the tumor microenvironment (pH 6.5−6.9) or cellular compartments such as endosomes (pH 5.5-6.2) and lysosomes (pH 4.5-5.0), the acid labile bond in these linkers get cleaved leading to release of unmodified drug in cancer cells.[2] Despite of these, predicting the clinical outcome of conjugates with acid-sensitive linkers have been difficult due to the variability of the stability of these linkers in plasma.[2] Hydrazone, a popular acid-sensitive linker, have been employed in development of several PDCs such as T10-ERK-Dox and ABD-Dox.[2, 28, 29]

Sheng et. al. designed a novel PDC (T10-ERK-Dox) consisting of both the hyrdazone and the thioether linkages using aldoxorubicin (Doxo-EMCH), a derivative of Dox of which the 13-

carbonyl group is substituted with a hydrazone linker, conjugated to a dual targeting hybrid peptide (T10-ERK) (**Figure 1.6)**.[2, 28] In vitro studies of T10-ERK-Dox conjugate showed that not only Dox is released from the rest of conjugate inside cells (most likely in the acidic compartments such as lysosomes or endosomes), but also, the amount of Dox found inside the whole cells was significantly higher for the conjugate than that of free Dox.[2, 28] In vivo studies in MCF-7/ADR tumor bearing mice supported the superior efficacy of T10-ERK-Dox conjugate (72.2 ± 4.6% inhibition) in inhibiting tumor growth compared to free Dox (45.7 ± 2.8% inhibition).[2, 28]

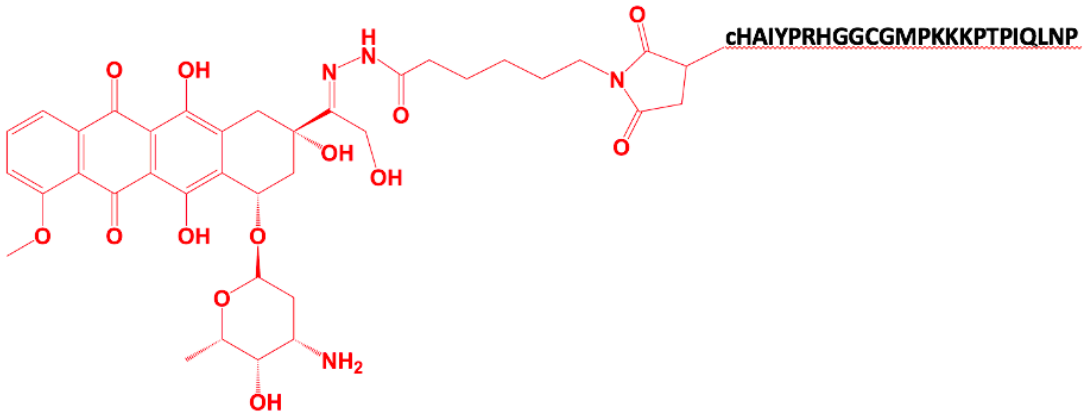


Figure 1.6. Structure of T10-ERK-Dox conjugate**.[28]**

Another popular approach for the targeted delivery of chemo drugs is the use of albumin binding chemistry.[2] Yousefpour et. al. designed a PDC using aldoxorubicin that covalently binds to albumin through a thioether bond, and an albumin-binding protein domain (ABD) with 47 amino acid residues that binds albumin through non-covalent interactions.[2, 29] The advantage of non-covalent interactions of ABD with albumin would be the easier dissociation of ABD (or the ABD conjugate) for releasing the drug into cancer cells.[2, 29] The in vivo PK studies of ABD-Dox conjugate in BALB/c mice demonstrated that the plasma elimination half-life of ABD-Dox conjugate was significantly higher (29.4 ± 0.8 h) compared to free Dox (only in the order of minutes).[2, 29] Furthermore, results from biodistribution studies done in mice

treated with ABD-Dox conjugate showed that the level of Dox in the tumor over 72 h was much higher than that in mice treated with free Dox.[2, 29] At last, Yousefpour et. al. concluded that ABD-Dox conjugate had superior therapeutic efficacy over free Dox in treatment of tumor in both pancreatic and colon mice models.[2, 29]

1.1.5 Rationale, Hypothesis, and Objectives

Phillips L. *et al* used disulfide and thioether linkers to link a monoclonal antibody, trastuzumab (Herceptin), to DM1 which is cytotoxic agent in order to determine pharmacokinetics, in vitro and in vivo efficacy, and toxicity of the designed antibody-drug conjugates.[10, 26] Cell culture results from treatment of HER2-amplified breast cancer lines with various trastuzumab ADCs for 3 days showed no significant difference in activity among the ADCs tested.[10] However, through *in vivo* studies, it was found that the ADC with the non-reducible MCC linker (trastuzumab-MCC-DM1 or Kadcyla) displayed higher activity compared with the free drug or ADCs linked through disulfide linkers.[10] Determining the effect of different linkers on serum concentration of various ADCs, pharmacokinetic analysis of trastuzumab-maytansinoid conjugates in nude mice shows increased serum concentrations of conjugates with disulfide linkers.[10] The cleavage of the disulfide linker was shown to be inefficient due to oxidizing nature of endocytic pathway.[10] On the other hand, measuring serum concentrations of trastuzumab-MCC-DM1 for 1 week indicated that the MCC linker had superior efficiency in keeping the ADC stable in presence of serum over the 7 days compared to the other conjugates.[10] Furthermore, data from *In vivo* toxicity studies suggested that trastuzumab-MCC-DM1 showed the best safety and efficacy profile since increased linker stability in vivo correlated with increased antitumor activity of ADC.[10] Phillips L. *et al* proposed that after internalization of trastuzumab-MCC-DM1 in $HER2^+$ tumor cells, the conjugate undergoes intracellular proteolytic degradation in the lysosome to release active

drug.[10] They also showed that lysine-MCC-DM1 was the primary active metabolite.[10] It was concluded that trastuzumab-MCC-DM1 had superior pharmacokinetics and safety profile compared with conjugates linked with disulfide linkers.[10] Trastuzumab-MCC-DM1 was selected for clinical development and later approved by FDA in 2013. [10, 30] The clinical success of Kadcyla also drew our attention. The antibody is conjugated to the drug here using sulfo-SMCC linker (**Figure 1.7 B**).[10] Sulfo-SMCC (**Figure 1.7 A**) is heterobifunctional cross-linker that allows covalent conjugation of molecules containing amines and sulfhydryls through its *N*- hydroxysuccinimide (NHS) ester and maleimide groups respectively.[10, 31]

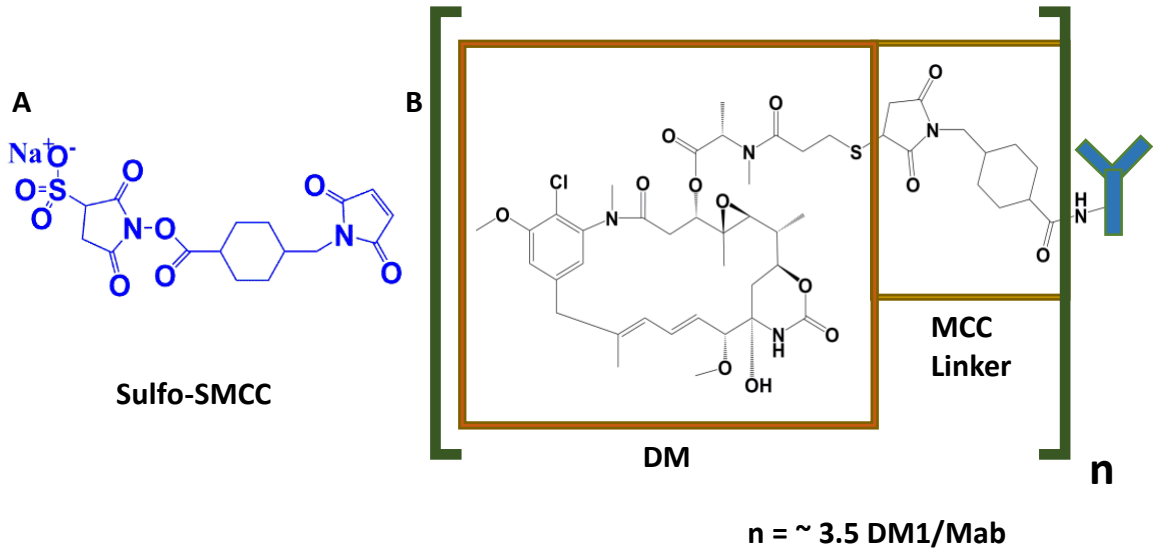


Figure 1.7. (A) The chemical structure of sulfo-SMCC linker.[10] **(B)** The chemical structure of ADC drug Kadcyla.[10](Rxlist.com, 2017)

Therefore, we decided to employ MCC linker in design of our novel peptide-drug conjugate where the drug, doxorubicin, is linked to the peptide 18-4 via a succinimidyl thioether bond. Breast cancer cell targeting peptide 18-4 (WxEAAYQrFL) is an engineered peptide for targeting breast cancer cells that is proteolytically stable and binds specifically

overexpressed receptor keratin 1 on the surface of breast cancer cells.[1, 9] Peptide 18-4 was conjugated to Dox to give peptide-Dox thioether conjugate.

The **objective of my study** was to synthesize peptide-drug conjugate (Conjugate 1) using SMCC linker, evaluate in vitro stability and cytotoxicity properties of conjugate 1 and study its mechanism for uptake into the breast cancer cells.

In order for the anti-tumor drug to work successfully, it should reach the tumor site and release Dox before degradation by the metabolic enzymes. Since an established cross-linker succinimidyl thioether is being used, which showed good in vivo pharmacokinetics in some of the FDA approved drugs currently used for cancer treatment.[23] Based on this, we **hypothesized** that the conjugation of a breast cancer cell targeting peptide to chemotherapeutic Dox through succinimidyl thioether linker can improve the therapeutic efficacy of the drug. In addition, we hypothesized that the expression of keratin 1 in breast cancer cells is higher than that in non-cancerous (normal) cells.[9]

Chapter 2 describes the design, synthesis, purification, and characterization of peptide18-4-doxorubicin conjugate. In addition, the conjugate was evaluated for its cytotoxicity using TNBC and non-cancerous breast tissue derived cells lines. In chapter 3, I evaluated the surface expression of K1 in two TNBC cell lines and one non-cancerous breast tissue derived cell line. Chapter 4 describes my attempts to synthesis and purify conjugate for the in vivo mice efficacy study to be done in the future.

CHAPTER 2: Design, Synthesis, and In Vitro Evaluation of a Novel Peptide 18-4-Doxorubicin Conjugate

2.2 INTRODUCTION

Breast cancer is the second leading cause of death among women worldwide.[17] Antineoplastic agents such as anthracyclins (e.g. doxorubicin, daunomycin, etc.) are widely used in cancer treatments of various types like breast cancer.[1] Although chemotherapy as well as other types of therapies such as hormonal therapies, and radiotherapies can possibly improve the survival of breast cancer patients, the systematic application of these therapies could also lead to more complications and ultimately death of the patients.[17]

Triple negative breast cancer (TNBC), an aggressive subtype of breast cancer, is difficult to target as this breast cancer subtype lacks expression of hormone receptors (estrogen or progesterone) as well as lacks overexpression of human epidermal growth factor receptor 2 (HER2).[32, 33] Chemotherapy is the mainstay treatment for TNBC.[32] Several cancer targeting peptides have been proposed for targeting overexpressed receptors in breast cancer.[17, 26, 34-38] For instance, peptide **18-4** binds keratin 1 (K1) and peptide GE11 binds epidermal growth factor receptor (EGFR or ErbB1) overexpressed on breast cancer cells, and both keratin 1 and EGFR are suggested to play key roles in TNBC.[17, 36] The linker is an important component of the PDC and allows timely release of the drug after it is internalized. Linkers like esters, amides, disulfides, and acid-labile hydrazones have been explored that get cleaved in the intracellular tumor environment.[1, 10, 38-41] In addition, thioether linker has been used where the drug is released from the peptide or antibody by proteolytic degradation (intracellular) of the peptide/antibody. The succinimidyl thioether linker is gaining attention as it is present in two clinically used ADCs, Kadcyla and Adcetris.[4] The succinimidyl thioether

linkage shows superior in vivo efficacy compared to disulfide bonded ADCs.[10] For the cytotoxic agent, it is recommended to use a highly toxic agent in the PDC with IC50 values in the subnanomolar range such as drugs like maytansine derivatives, auristatin and doxorubicin (Dox).[3]

In this chapter, the chemistry for covalently conjugating peptide **18-4** to Dox to obtain a novel peptide-Dox conjugate (**Figure 2.7**) for specific delivery of Dox to TNBC is explored. Different chemistries for conjugation were explored in previous studies.[42] Conjugation using SMCC linker, that gave best results with pure and high yield of conjugate, are described. The stability of the conjugate in different environments is studied, followed by in vitro cytotoxicity using TNBC and normal breast tissue-derived cells. The results show that conjugate is highly stable in human serum, and displays much higher cytotoxicity toward TNBC cells compared to normal breast cells.

2.3 MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride (Dox.HCl) salt and aldoxorubicin were bought from LC Laboratories (MA, USA) and MedChem express (NJ, USA), respectively. Fmoc-Leu TentaGel S RAM resin (loading 0.21 mmol/g) was bought from Rapp Polymere GmbH (Germany). The coupling agent 2-(6-chloro-1H-benzotriazole-1-yl)- 1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and the Fmoc-amino acids with the following side chain protections: *tert*-butyl in tyrosine, *tert*-butoxy in glutamic acid, trityl (Trt) in glutamine and cysteine, *tert*-butoxycarbonyl (Boc) in lysine and tryptophan, and pentamethyl dihydrobenzofuran (Pbf) in D-arginine were purchased from Fischer Scientific (IL, USA). N, N-dimethylformamide (DMF), N-methylmorpholine (NMM), trifluoroacetic acid (TFA), triisoproylsilane (TIS), piperidine, and all other reagents were bought from Sigma-Aldrich. Sulfo SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate) was purchased from Thermofisher Scientific (NY, USA). α-Cyano-4-hydroxycinnamic acid (CHCA) matrix was bought from Fluka. Ultra-pure water was from Milli-Q system. MTT (98%) and IGEPAL®CA-630 (or NP40) were purchased from Sigma-Aldrich.

Human breast cancer cell lines (MDA-MB-231 and MDA-MB-468) and normal breast cell line (MCF-10A) were purchased from ATCC (USA). All cells were maintained in a 5% CO_2 incubator (37 °C). MDA-MB-231 was cultured in DMEM/F-12 (cat # 11330-032, Corning, Virginia, USA), 100 IU/mL penicillin, and 100 IU/mL streptomycin supplemented with FBS, whereas, MDA-MB-468 was cultured in DMEM (1x) + GlutaMAX^{TM}-l media (10567-014, Gibco) containing 100 IU/mL penicillin, and 100 IU/mL streptomycin supplemented with FBS. MCF-10A was cultured in DMEM/F-12 with HEPES (cat # 10-092-CV) and without HEPES (cat # 10-090-CV, Corning, Virginia, USA), 100 IU/mL penicillin,

and 100 IU/mL streptomycin supplemented with horse serum. Human serum was purchased from Sigma-Aldrich.

Peptide synthesis was done employing an automated peptide synthesizer Tribute from Protein Technologies (Protein Technology, Inc., Arizona, USA). Purification and HPLC analysis were conducted on a RP-HPLC system Prominence-i (Shimadzu Corp., Kyoto, Japan) using C18 semi-preparative (10 mm × 250 mm, 5 μm) and analytical (4.6 mm × 250 mm, 5 μm) columns. For mass spectra, either an autoflex speed MALDI-TOF mass spectrometer (Bruker, USA) or EVOQ Triple Quadrupole LC-TQ Mass Spectrometer (Bruker, USA) was used. UV-Vis spectrophotometer (UV-2600, Shimadzu) was used to obtain an absorption spectrum. NMR experiments were conducted on an Ascend 400 MHz NMR spectrometer (Bruker BioSpin Corporation, Billerica, Massachusetts, USA).

2.3.1 Peptide Synthesis

The 11-mer peptide (NH_2-CWxEAAYQrFL-$CONH_2$) was synthesized on Rink amide resin (0.1 mmol scale) pre-loaded with Fmoc-Leu residue (Fmoc-Leu Rink amide resin, loading 0.21 mmol/g) using automated SPPS.[11, 25, 26] The activation and coupling at each step were carried out for 2 hours using HCTU and NMM in DMF. After each coupling step, Fmoc deprotection was done using piperidine/DMF (2:8). After complete assembly of the peptide sequence, the peptide was released from the resin by treating the resin with a cleavage mixture of TFA/TIS/water (10 mL, 90:5:5) for 2 h.[11, 26] Crude peptide was precipitated by adding diethyl ether (20 mL, chilled) to the filtered TFA cocktail which was then collected by centrifugation (10 min). The crude peptide was characterized using MALDI-TOF and RP-HPLC. The purity of the peptide was assessed using an analytical C18 column. The peptide eluted with 35% acetonitrile at 31 min (t_R) (method used: 10-100% with 0.05% TFA, flow rate = 1 mL/min, 100 min run time at 220 nm). MALDI-TOF $[M+H]^+$ found 1399.2 (calc. 1399.7).

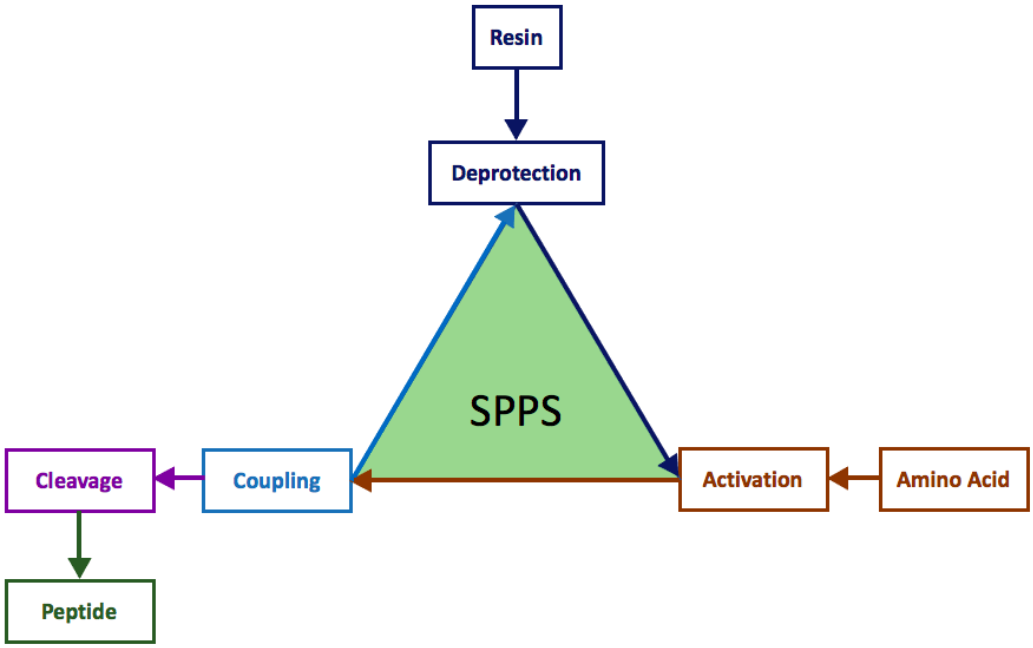


Figure 2.1. Schematic illustration of solid phase peptide synthesis.

2.3.2 Synthesis of MCC-Dox

To a solution of sulfo-SMCC (5 mg, 11.5 µmol) in 1.2 mL of DMF, water, and PBS (100 mM, pH 7.6) at ratio 1:1:0.4, Dox-HCl (4.3 mg, 7.7 µmol) in DMF/water (1 mL, 1:1) was added. The reaction mixture was stirred (r.t.) under nitrogen for 4 h.

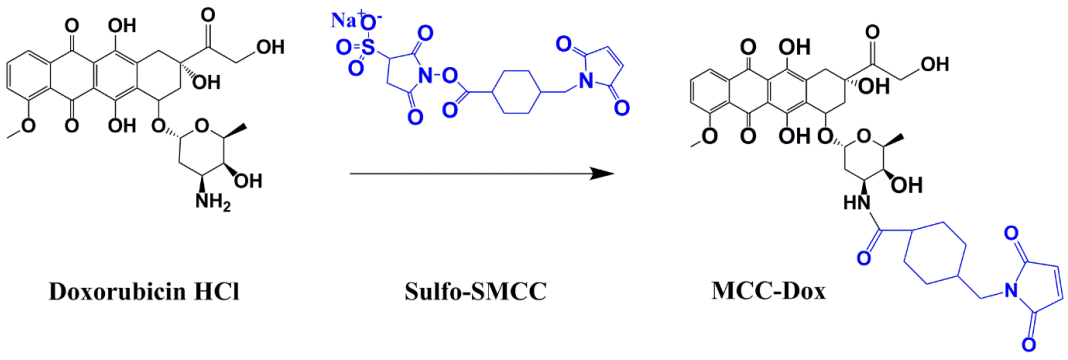


Figure 2.2. Conjugation of Doxorubicin to MCC linker.

The purification of the resulting crude product (MCC-Dox) was carried out using a semi-preparative RP-HPLC (method used: 10-70% acetonitrile/water with 0.05% TFA at 1 mL/min in100 min). MCC-Dox peak that eluted at 36 min (t_R) with 48% acetonitrile in RP-HPLC was collected and combined and dried under a rotary evaporator to obtain pure MCC-Dox as a red powder with 91% (5.3 mg, 98% purity) yield. Pure MCC-Dox was characterized using ^{1}H and ^{13}C NMR spectroscopy (**Figure 2.5 A**), MALDI-TOF mass spectrometry and analytical HPLC (**Figure 2.5 B**). For NMR, MCC-Dox was dissolved in 100% deuterium oxide (Cambridge Isotope Laboratories Inc., Massachusetts, USA). 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{13}C-HSQC and HMBC) NMR experiments were run at 25 °C. The spectra were analyzed using Topspin 3.7 software (Bruker BioSpin Corporation, Billerica, Massachusetts, USA). Complete chemical shift assignments (^{1}H and ^{13}C) were made using the HSQC and HMBC experiments. MALDI-TOF $[M+Na]^{+}$ calc. 785.2, found 785.4.

2.3.3 Concentration of Peptide and MCC-Dox Solutions

The concentration of peptide solution was determined using SpectraMax QuickDrop UV-Vis spectrophotometer (Molecular Devices, USA). After dissolving a small amount of peptide in acetonitrile and water (2:3, 100 μl), peptide in solution (5 μl) was transferred to an eppendorf tube to measure its concentration. First, the instrument was switched on, and from the main screen, Read type was selected by touching [Life Science], followed by touching [Protein], and then selecting [Protein A280] as method. Next, [Mode] was set to [Molar Extinction], and [AU 1/mol ×1000] was set to 7.090 ($cm^{-1}M^{-1}$) as molar extinction coefficient for the peptide. Then, [Pathlength] was set to 0.5 mm (drop), followed by setting [Dilution Factor] and [MW kDa] to 1.000, and 1.400, respectively. On the next screen, [Units] and [Integration Time] were set to mg/ml and 1 second, respectively. When finished setting all parameters, on the next screen, the reference solution containing acetonitrile and water (2:3) (2 μl) was loaded in the sample port

followed by taking the reference measurement by touching the reference icon (blue) on the touch screen. After removing the reference by wiping the sample port, peptide (2 μl) was loaded in the sample port followed by taking the sample measurement by touching the sample icon (red) on the touch screen. The sample was removed by wiping the sample port, and the sample measurement was repeated two more times to obtain three concentration measurements. To convert the concentration of peptide in mg/mL to mM, the following equation was used:

$$\text{Concentration (mM)} = \frac{\text{Concentration (mg/mL)} \times 1000\ (mmol/mol)}{1000\ (mg/g) \times 1400\ (g/mol) \times 1000\ (L/mL)}$$

To dissolve the MCC-Dox, acetonitrile and water (1:1, 100 μl) were added to the round bottom flask containing pure MCC-Dox powder. MCC-Dox in aqueous solution (5 μl) was transferred to an eppendorf tube to measure its concentration. First, the instrument was switched on, and from the main screen, [Read Type] was selected by followed by selecting [Single Wavelength] method. Next, [Mode] was set to [Absorbance], and [Wavelength] and [Integration Time] were set to 481 nm and 2 seconds respectively. After finishing method parameters, the reference solution containing water/acetonitrile (1:1) (2 μl) was loaded in the sample port followed by taking the reference measurement by touching the reference icon (blue) on the touch screen. After removing the reference by wiping the sample port, pure MCC-Dox (2 μl) was loaded in the sample port followed by taking the sample measurement by touching the sample icon (red) on the touch screen. The sample was removed by wiping the sample port, and the sample measurement was repeated two more times to obtain three absorbance measurements. To calculate the concentration of MCC-Dox from obtained absorbances, the following equation was used:

$$\text{Concentration} = \frac{\text{Avg absorbance}}{10410\left(\frac{L}{mol.cm}\right) \times 0.05\ cm}$$

2.3.4 Synthesis of Peptide-Dox Conjugate

A solution of peptide (1.1 mg, 0.78 μmol) in DMF (0.25 mL) was added to the MCC-Dox (0.8 mg, 1 μmol) in DMF (0.5 mL), and additional DMF (1.25 mL) was added. Finally, a catalytic amount of DIPEA (1 wt%) was added, and the reaction mixture was stirred at r.t. under nitrogen. The reaction progress was monitored using RP-HPLC, and after 4 hours the conjugate formation halted. The DMF was removed by rotary evaporator and the mixture was diluted with acetonitrile/water and injected into RP-HPLC for purification. The conjugate eluted at 27 min (38% acetonitrile) using RP-HPLC (30-45% acetonitrile/water containing 0.05% TFA, 1 mL/min flow rate, 65 min run time, t_R= 27 min) to give pure peptide-Dox conjugate as TFA salt with 63.5% yield and 98% purity (**Figure 2.8 B)**. Q-TOF found 1081.43 charge +2 therefore unprotonated mass found (1081.43−1)×2 = 2160.86, calc. mass = 2160.9; MALDI-TOF $[M+H]^+$ found 2161.5, calc. 2161.9. Until used, the conjugate was stored as a dry powder at -20 °C.

2.3.5 Stability of the Conjugate in Aqueous and Aqueous Acidic Conditions

The stability of the conjugates in aqueous solution was assessed by dissolving the pure conjugates (100 μM) in water and acetonitrile (9:1, v/v, pH 7.0) and keeping at 4 °C or -20 °C, respectively. At different time intervals aliquots were taken and injected into RP-HPLC. The stability of the conjugate was monitored using the peak (area under the curve) from HPLC and the MALDI-TOF mass analysis of the HPLC peak. The stability was also evaluated in acidic aqueous media at ~ pH 5. A solution of the conjugate in PBS pH 5 (100 μM) was incubated at 37 ºC, and aliquots removed over time were examined with MALDI-TOF mass spectrometry. Stock solution concentrations for the conjugate were obtained using Quick drop spectrophotometer at 481 nm (ε = 10410 L mol^{-1} cm^{-1})[43] and 495 nm (ε = 9250 L mol^{-1} cm^{-1})[44] wavelengths, respectively.

2.3.6 Stability of the Conjugate in Cell Culture Media

Conjugate was dissolved in DMEM/F12 (+ HEPES) media and the resulting solution (100 μM) was incubated at 37 ºC for up to 48 hours. Aliquots, removed at different time intervals, were assessed using mass spectrometry analysis.

2.3.7 Stability of the Conjugate in Human Serum

A stock solution of conjugate at 1 mM concentration was prepared in sterile water. Human serum was thawed at r.t. Human serum (250 μL) was diluted by adding sterile water (650 μL) and conjugate stock solution (100 μL) to give a sample with 25% human serum and 100 μM conjugate.[1] The sample was kept at 37˚C in an incubator to imitate human body temperature. At regular intervals, aliquots (100 μL) were taken and diluted with methanol (200 μL). After centrifugation (500 g) for 15 min, the supernatant was analyzed by HPLC at 495 nm, and the major HPLC peaks were analyzed by mass spectrometry. The mobile phase used was acetonitrile/water with 0.05% TFA with a gradient of 30−55% with a flow rate of 1 mL/min in 100 min. The intact conjugate (relative percentage) was plotted against incubation time. HPLC and MALDI-TOF mass analysis were used to confirm degradation products, and $t_{1/2}$ was calculated as the time needed to hydrolyze half of the initial conjugate in human serum.

2.3.8 Cell Culture

After removing cells from liquid nitrogen and thawing, under the hood, warm media (1 mL) was added to the cells and then transferred to a 15 ml centrifuge vial to centrifuge, spin down in 1500 rpm for 5 mins at 4 ℃ in order to remove previous media that contained DMSO. The supernatant was removed, and warm media (15 mL) was added to the cell pellet and re-suspend the cells by vigorously pipetting the media up and down. The cell suspension was transferred to a 100 mm tissue culture plate and placed in cell culture incubator (37°C, 5% CO2)

undisturbed overnight. Next day the old media was removed and fresh media was added to the plate. The cells were fed with fresh media every two days till they reach 60% confluence.

2.3.8.1 Cell Subculture.

Human TNBC cell lines, MDA-MB-231 and MDA-MB-468, and normal mammary tissue derived cells MCF-10A were maintained at 37 °C in a 5% CO_2 -95% O_2 incubator and growth media were replaced every 48 h. All cell lines were obtained from ATCC. The MDA-MB-231 cells were cultured in DMEM/F-12 (ham) (1:1) (1X) with L-glutamine medium and 15 mM HEPES (Gibco, USA), and MDA-MB-468 cells were cultured in DMEM (1X) + GlutaMax dulbecco's modified of eagle's medium with 1 g/L D-glucose and 110 mg/L sodium pyrovate (Gibco, USA) medium. Both media were supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. The MCF-10A cells were cultured in DMEM/F-12 50/50, 1X (dulbecco's modified of eagle's medium / ham's F-12 50/50 Mix) with L-glutamine (Corning, USA) medium. The media for MCF-10A cells was supplemented with 5% horse serum (Gibco, USA), 1% penicillin and streptomycin. The medium for MDA-MB-231 and MDA-MB-468 cells were supplemented with 10% FBS (Corning, USA), 1% penicillin and streptomycin (Gibco, USA). Hank's balanced salt solution (HBSS) (without calcium chloride, magnesium chloride and magnesium sulfate) (Gibco, USA) was used to wash the cells before trypsinization.

After removing cell culture plate from cell culture incubator, under the hood, the old media was discarded and cells were rinsed with fresh warm media (2 mL) three times. Next, 0.25% trypsin (3 mL) was added to the plate and the plate was placed into the cell culture incubator 37°C for 5 minutes. Then, the cells were checked under the microscope to ensure that most cells are detached from the plate and floating in the solution. Next, under the hood, neutralization media (6 mL) containing FBS or Horse serum was added and mixed with the

cells by pipetting the solution. The solution containing cells was pipetted into a 50 mL centrifuge tube with cap, and centrifuged at 1500 RPM for 5 minutes at 4°C. After aspirating the supernatant, media (2 mL) was added to pelleted cells, and mixed cells by pipetting the solution 20 times.

Next, the cells were counted using hemocytometer by adding 10 μL of the cells to the hemocytometer while holding the coverslip in position. The chamber was placed in the microscope under a 10× objective. Only the cells located in the large, central gridded square were counted. Lastly, the cell count was multiplied by 10^4 to estimate the number of cells/mL. After transferring desired amount of cells in media to new plates, the plates were placed in cell culture incubator overnight. After 24 hr, the media was replaced with fresh warm media. This protocol was repeated once cells were ~80% confluent.

2.3.9 In Vitro Cytotoxicity

The cellular toxicity of the conjugate and free Dox were assessed for three cell lines (MDA-MB-231, MDA-MB-468, and MCF-10A) using MTT assay. After seeding the cells in 96-well microtiter plates (5×10^3 cells/well) and growing in complete media, the next day the media was substituted with fresh serum-free media (200 μL) containing different concentrations of the Dox or conjugate (0-100 μM). To prepare the conjugate solution in media, sterile DMSO (10 μl) was added to the round bottom flask containing conjugate powder. The conjugate in DMSO (10 μl) was transferred to an eppendorf vial and diluted with sterile water (50 μl). Concentration of the conjugate solution was measured using Quickdrop and the concentration of this stock solution of the conjugate was 117 μM. In order to obtain the desired concentrations (10-100 μM), the conjugate was diluted in media. It was incubated for 48 h at 37 °C. The negative and positive controls used were cells in serum-free medium (without any drug) and cells with Dox, respectively. To prepare the Dox solution in media, first, small amount of

Doxorubicin.HCl was dissolved in sterile water (60 μl). Next, small amount of Dox solution (5 μl) was transferred to an eppendorf vial and its concentration was measured using Quickdrop. The concentration of this stock solution of the Dox was 3535 μM. Furthermore, to obtain the desired concentrations (10-100 μM) for MTT assay, Dox solution (3535 μM) was diluted in media.

Cell survival assay was performed by adding MTT solution (20 μL, 5 mg/mL) to each well and the plates were further incubated for 2 h. A sterile MTT solution was prepared by dissolving MTT (60 mg) in PBS 1X (12 mL) and the resulting solution was filtered using EZFlow syringe filter (Foxx Life Sciences, diameter 13 mm, pore size 0.22 μm) with Foxx CA membrane (Foxx Life Sciences, Salem, NH, USA). To solubilize the formazan pellets that formed after adding MTT solution to the wells, a solubilizing solution (4 mM HCl, 0.1% NP40, 2-propanol, 1:1:1, v/v/v) was added to each well (100 μL/well). The plate was placed on a shaker for 10 mins and was read using plate reader (SpectraMax M5 UV VIZ plate reader) at 570 nm. The percent cell viability was calculated for each well by taking the absorbance ratio of treated cells to untreated cells and the average of the triplicates was plotted. The percent cell viability was plotted against the log drug (Dox or conjugate) concentrations and fitted using a non-linear fit of normalized data to obtain IC50 values using GraphPad Prism 7.04.

2.3.10 Immunofluorescent Confocal Microscopy of Uptake of Conjugate in Triple Negative Breast Cancer Cell Line MDA-MB-468

Experiments were carried out with 1×10^5 cells/chamber on a 4-chamber Falcon cell culture slide (Corning™ Falcon™, USA) overnight. Next day, media DMEM (1X) + GlutaMax (Gibco, USA) was removed and cells were rinsed with sterile PBS. Lysozyme expression

(green) was evaluated with LysoView 488. Conjugate 1 expression (red) was evaluated using doxorubicin natural fluorescence, and nucleus (blue) was stained with DAPI.

To prepare the conjugate solution in media, sterile DMSO (5 μl) was added to the round bottom flask containing conjugate powder. The conjugate in DMSO (5 μl) was transferred to an Eppendorf vial and diluted with sterile water (95 μl). Concentration of the conjugate solution was measured using Quickdrop. In order to obtain the desired concentration 8 μM, the conjugate was diluted in media. Conjugate solution in media (8 μM, 600 μl) was added to each chamber and incubated for 4 hr at 37 °C in the dark. Next, conjugate solution was removed using a pipette and cells were washed with warm media (three times, each 10 min). After preparation of LysoView solution by mixing LysoView 488 (Biotium, USA) dye (1 μl) with media (2 mL), LysoView solution (500 ul/well) was added to each chamber and incubated for 20 min at 37 °C in the dark. At last, the cells were washed with 0.5 mL in each chamber HBSS (three times, each 10 min), coated with a drop of DAPI (abcam, USA), covered with coverslip (Thermo Scientific, Germany), and analyzed with a Nikon confocal microscope. For each chamber, 10 randomized fields were photographed and analyzed with NIS-Elements AR software. Three independent experiments were performed in duplicate.

2.4 RESULTS AND DISCUSSION

2.4.1 Synthesis and Characterization of Peptide 18-4 with an Additional Cysteine.

The peptide (CWxEAAYQrFL) was synthesized using standard Fmoc SPPS on Fmoc-Leu TentaGel S RAM resin as a polymeric support at a scale of 0.1 mmol to give a C-terminally amidated peptide.[1, 11] Since the resin was already preloaded with the first amino acid residue, Leu, after swelling the resin and removing Fmoc, the next amino acid residue, Phe, was activated and coupled to the resin, followed by stepwise coupling of all amino acid

residues. Next, all the protecting groups of the amino acids' side chains were removed and peptide was cleaved from the resin using the cleavage cocktail, followed by precipitation of peptide using ice-cold ethyl alcohol. Peptide was purified using reversed phase (RP) HPLC with an acetonitrile/water gradient method and was ~ 99% pure. It was obtained in a good yield of 97% (**Figure 2.3**).

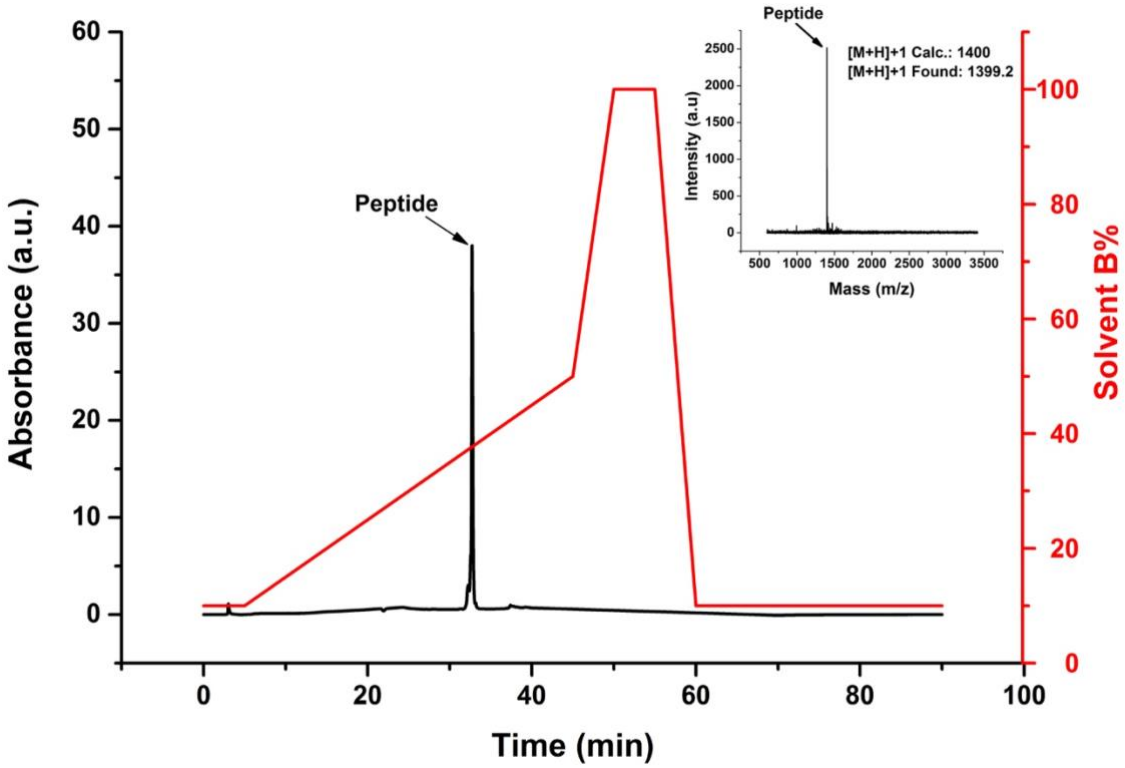


Figure 2.3. (A) RP-HPLC chromatogram of crude peptide. (B) MALDI-TOF (m/z) mass spec for pure peptide, $[M+H]^+$ calc. 1400 and found 1399.2.

2.4.2 Synthesis and Characterization of Dox-MCC

Dox was reacted with the sulfo-SMCC linker to obtain MCC-Dox. Sulfo-SMCC is a bifunctional crosslinker that allows covalent conjugation of molecules containing amines and sulfhydryls through its *N*- hydroxysuccinimide (NHS) ester and maleimide groups, respectively.[31] Dox has a primary amine which reacts with the NHS ester of sulfo-SMCC at ~ pH 7.6 to form an amide bond. The reaction was done in aqueous DMF (DMF/water; 1:1) at

r.t. and the pH was maintained by PBS. Sulfo-SMCC is a water-soluble molecule, whereas, Dox is highly lipophilic therefore a mix of DMF and water was used for solubilization. The reaction was monitored every hour using mass spectrometry and it was determined all free Dox was used up after 4 hours. MCC-Dox that formed, was purified using RP-HPLC. The MCC-Dox eluted at 52 minutes (t_R) and the main impurities were sulfo-SMCC and Dox that eluted at 32 and 41 minutes, respectively (**Figure 2.4**).

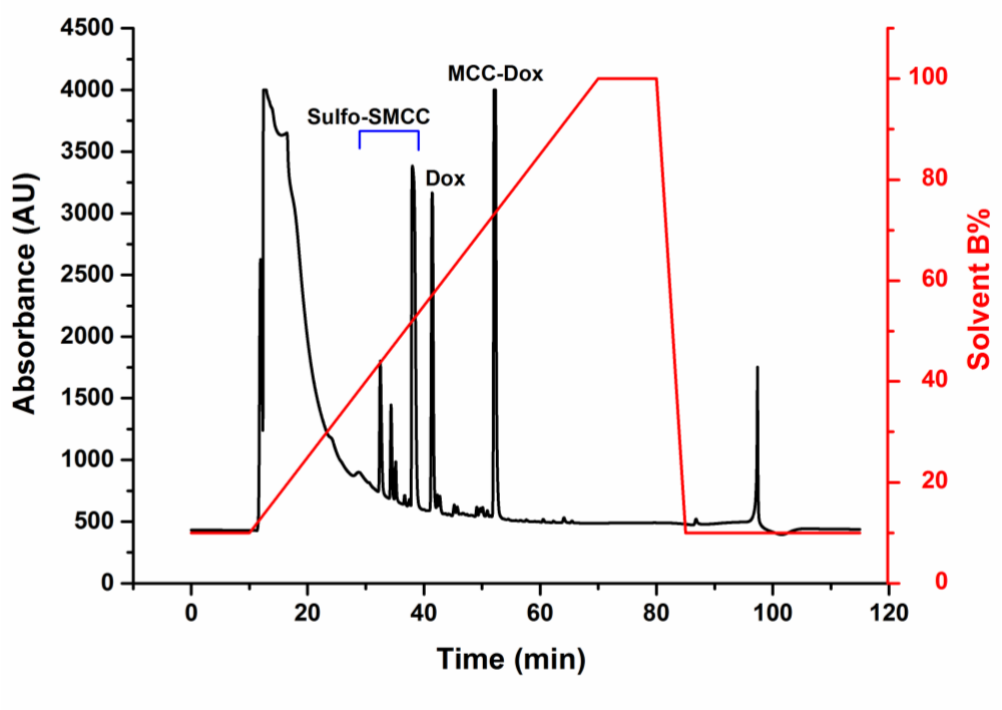


Figure 2.4. RP-HPLC chromatogram of crude MCC-Dox. Semi-prep column with a flow rate of 1 mL/min. Acetonitrile/water gradient that was used is shown.

Pure MCC-Dox that was collected was further characterized using analytical RP-HPLC that showed that MCC-Dox was >98% pure (**Figure 2.5 A**). The mass with MALDI-TOF analysis showed $[M+H]^{+1}$ as 784.7 (Calc. 784.7) (**Figure 2.5 B**). Overall, MCC-Dox was obtained in good yield (91%).

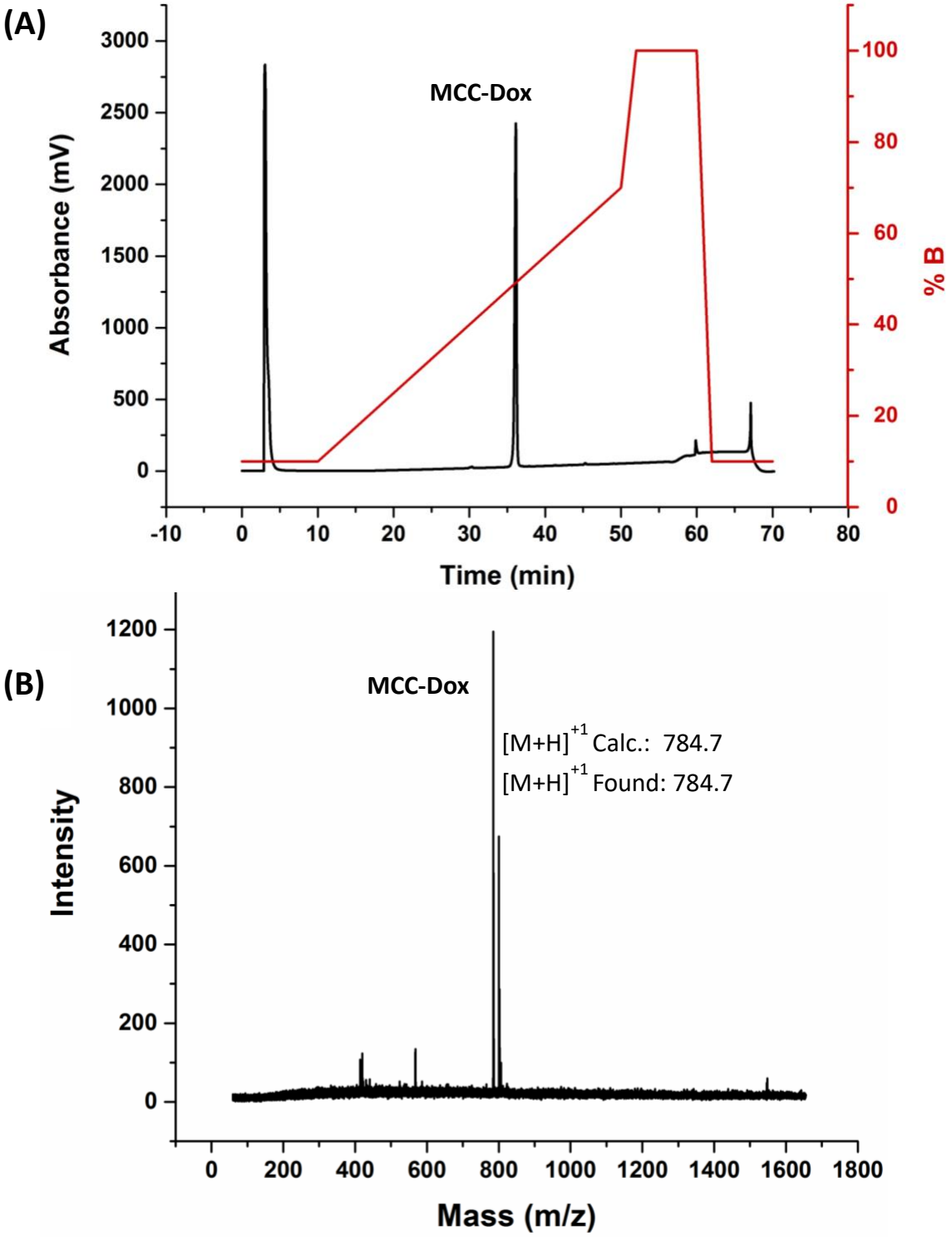
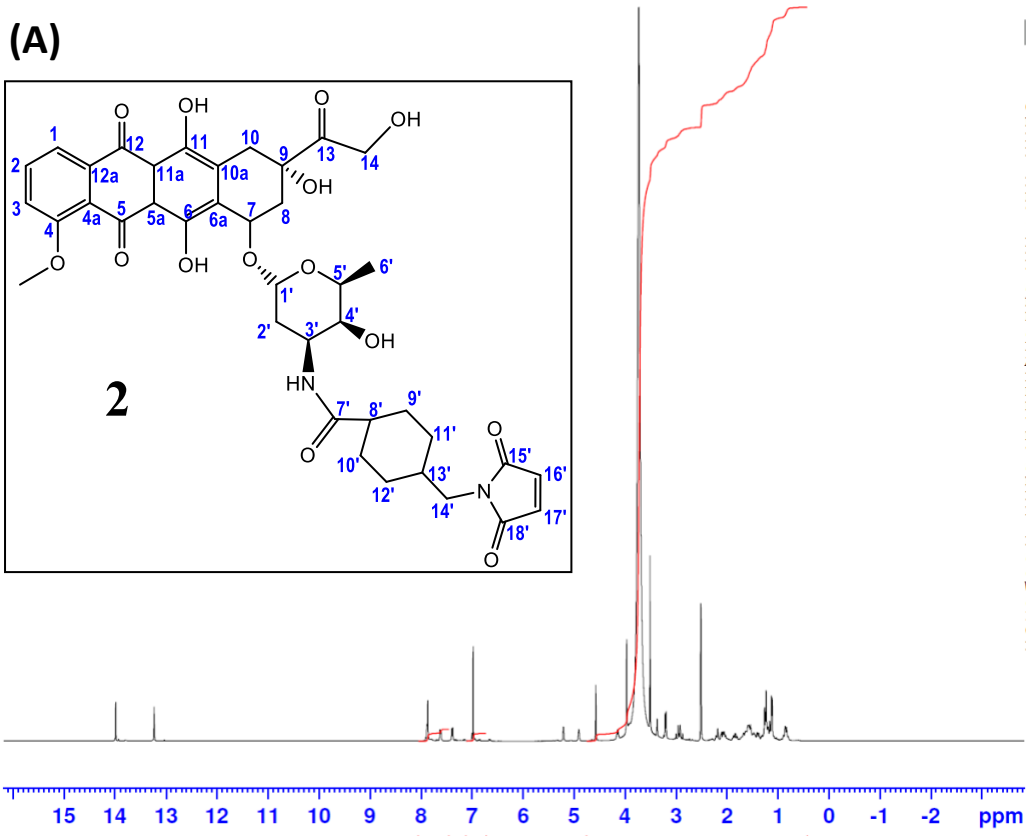
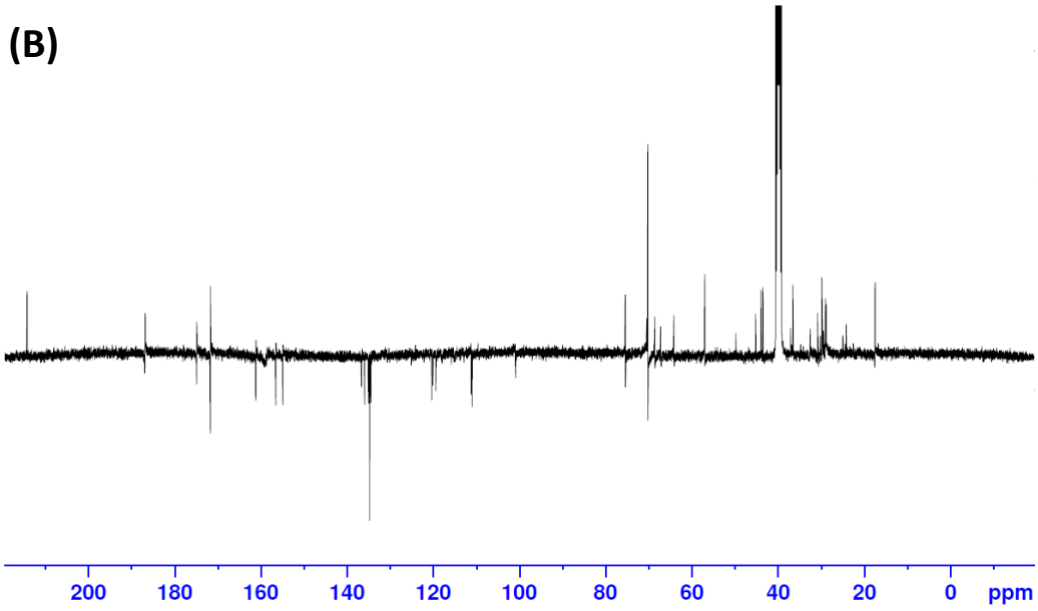


Figure 2.5. (A) RP-HPLC chromatogram of pure MCC-Dox. The purity of MCC-Dox was assessed using an analytical C18 column (Method used: 10-100% with 0.05% TFA, flow rate

= 1 mL/min, 70 min run time at 220 nm) **(B)** MALDI-TOF (m/z) mass spec for pure MCC-Dox, $[M+H]^+$ calc. 784.7 and found 784.7.

Pure MCC-Dox was characterized using NMR spectrometry (**Figure 2.6**). For ^{13}C-NMR experiment with ^{1}H decoupling, MCC-Dox was dissolved in 100% deuterium oxide (Cambridge Isotope Laboratories Inc., Massachusetts, USA). 1D (^{1}H and ^{13}C) and 2D (^{1}H–^{13}C-HSQC and HMBC) NMR experiments were conducted using 400 MHz Ascent NMR spectrometer (Bruker BioSpin Corporation, Billerica, Massachusetts, USA) at 25 °C. The spectra were analyzed using Topspin 3.7 software (Bruker BioSpin Corporation, Billerica, Massachusetts, USA). Complete chemical shift assignment (^{1}H and ^{13}C) for MCC-Dox was achieved using HSQC and HMBC experiments. The structure of MCC-Dox was confirmed by analysis of the chemical shifts in the initial compound (Dox) and in the conjugate compound 2.


(A)
2
15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 ppm


(B)
200 180 160 140 120 100 80 60 40 20 0 ppm

(C)

^{1}H NMR		
Number	**Chemical Shift**	**Proton**
11-OH	14.03	
6-OH	13.28	
1 and 2	7.92	2H, m
3	7.66	1H, m
NH	7.38	
16’ and 17’	6.99	2H, s
1’	5.22	1H, dd
7	4.94	1H, dd
14	4.57	2H, s
5’	4.14	1H, m
OMe	3.98	3H, s
3’	3.93	1H, m
4’	3.36	1H, m
14’	3.21	2H, d
10	2.97	2H, dd
DMSO	2.50	
8	2.14	2H, m
8’	2.06	1H, m
2’	1.83 and 1.39	2H, td and dd
9’ and 10’	1.66-1.55	4H, m
13’	1.48	1H, m
11’ and 12’	1.21 and 0.845	4H, m
6’	1.12	3H, d

^{13}C NMR	
Number	**Chemical Shift**
1	118.9
2	136.1
3	119.6
4	160.8
4a	119.1
5	186.5
5a	110.6
6	154.6
6a	134.0
7	70.0
8	36.5
9	75.0
10	32.1
10a	135.4
11	156.0
11a	110.8
12	186.5
12a	134.7
13 (C=O)	213.6
14	63.55
1’	100.0
2’	29.5
3’	44.6
4’	68.1
5’	66.7
6’	16.9
7’ (C=O)	174.1
8’	43.1
9’	28.3
10’	29.1
11’	28.1
12’	29.24
13’	35.8
14’	42.8
15’ and 18’	171.4
16’ and 17’	134.3

Figure 2.6. (A) Proton (^{1}H) decoupling and (B) Carbon (^{13}C) NMR spectra for compound **2** in DMSO-d6. (C) Chemical shift assignments for compound **2** for both the proton and carbon NMR spectra.

2.4.3 Synthesis and Characterization of Peptide-Dox Conjugate.

PDC was synthesized utilizing maleimide-thiol conjugation chemistry. First, Dox was conjugated to a sulfo-SMCC linker to give MCC-Dox. Next, the thiol group (from the cysteine side chain) of the peptide was reacted with the maleimide of MCC-Dox to obtain conjugate as shown in **Figure 2.7**. An extra cysteine residue was inserted at the N-terminal of the previously reported decapeptide **18-4**. It was hypothesized that the addition of cysteine in the N-terminal will not affect receptor binding and uptake. We have shown previously that a N- to C-terminal cyclized peptide shows increased uptake by the breast cancer cells.[26]

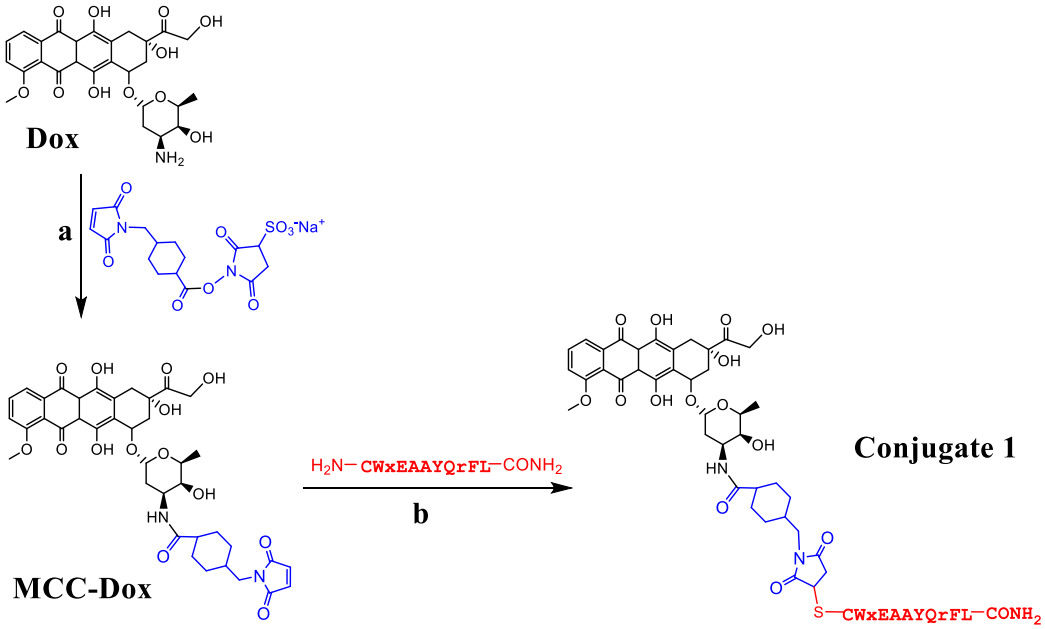
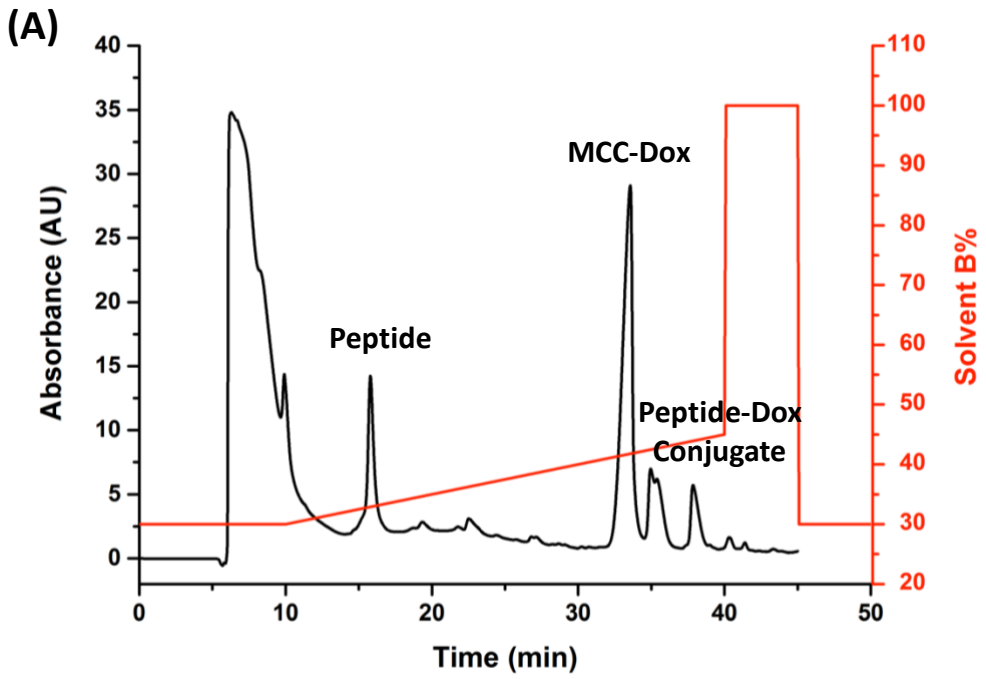


Figure 2.7. The chemical synthesis of PDC starting with Dox. The reagents used were (a) sulfo-SMCC, DMF/water and (b) peptide (H_2N-CWxEAAYQrFL-$CONH_2$), DMF/water. Lower case r equals D-arginine and x equals D-norleucine.

The crude conjugate was purified and characterized using RP-HPLC (**Figure 2.8 A**) and was obtained with 63.5% yield and 98% purity. The conjugate eluted at 42.5% using the semi-prep

column (flow rate 2 mL/min), and was found to be more hydrophobic compared to the peptide and MCC-Dox that eluted at 33% and 41 % ACN/water, respectively. The mass analysis of the pure conjugate using Q-TOF mass spectrometry showed a single peak. The peak observed was $[M+2H]^{+2}$ at 1081.4 (**Figure 2.8 C**). While the MALDI-TOF mass spectrometry showed three main peaks including the peak for the conjugate at 2161.5 ($[M+H]^{+}$ calc 2161.9) (**Figure 2.8 D**). The additional peak at 1765.1 is likely due to the fragmentation of the conjugate by the acidic matrix (α-cyano-4-hydroxycinnamic acid and trifluoroacetic acid) of MALDI-TOF. The mass found as 1765.1 (($[M+H]^{+}$ calc. 1764.7) is for the acetal hydrolysis fragment (Figure 2.9) , and the mass found as 1399 ($[M+H]^{+}$ calc. 1400) is for the peptide which are the product of fragmentation of the conjugate in aqueous acidic conditions. Additionally, the conjugate was also characterized using UV spectroscopy (**Figure 2.9**). Similar to doxorubicin, the conjugate showed two major picks, one at 325 nm, and another broad peak ranging from 481 nm - 495 nm .



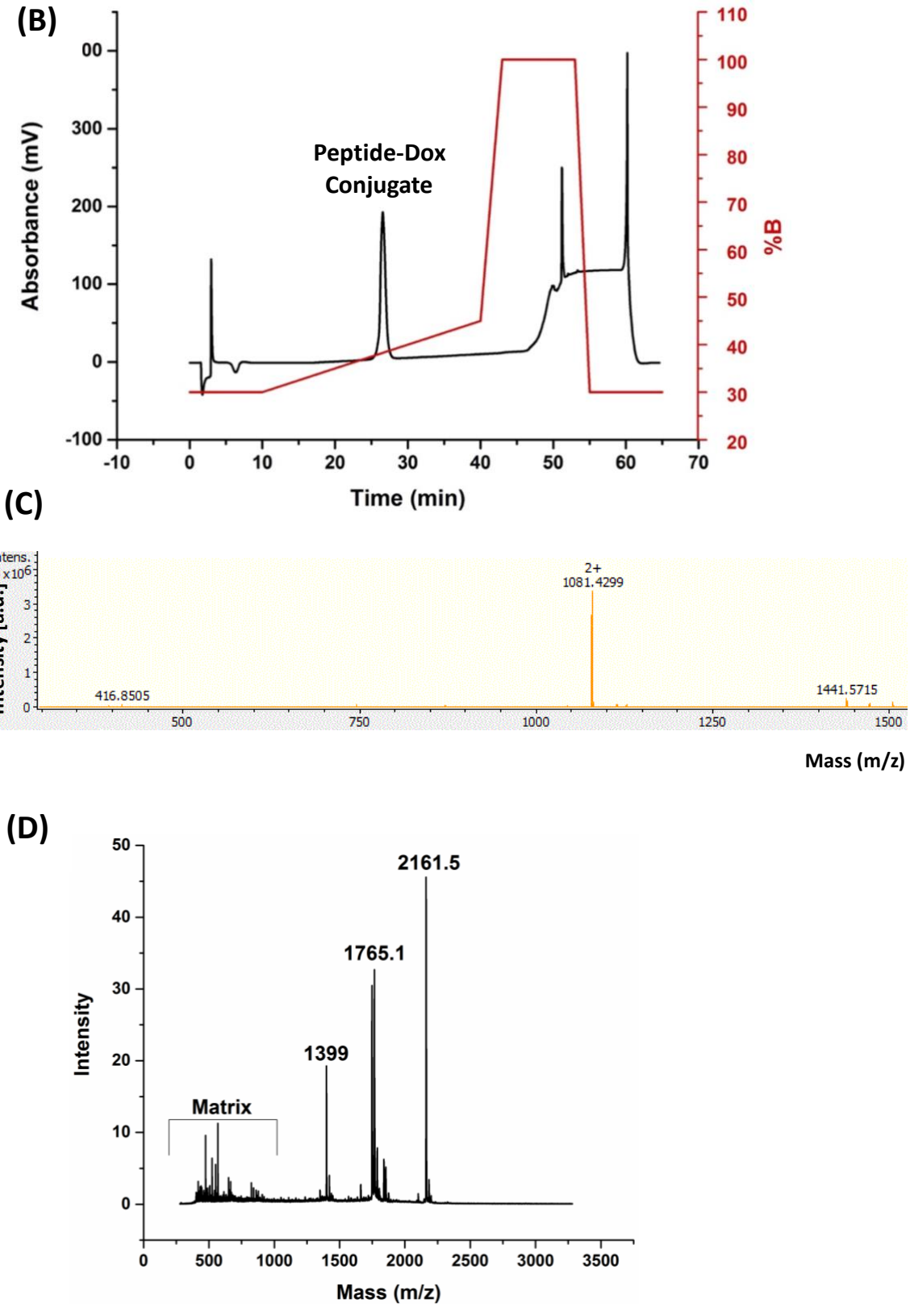


Figure 2.8. (A) RP-HPLC chromatogram of crude peptide-Dox conjugate. (B) RP-HPLC chromatogram of pure peptide-Dox conjugate. (C) Q-TOF mass spectrum for pure peptide-

Dox conjugate showing m/2, (M calc. 2161.9 and found 2161.8). (D) MALDI-TOF (m/z) mass spec for pure peptide-Dox conjugate, $[M+H]^+$ calc. 2161.9 and found 2161.5.

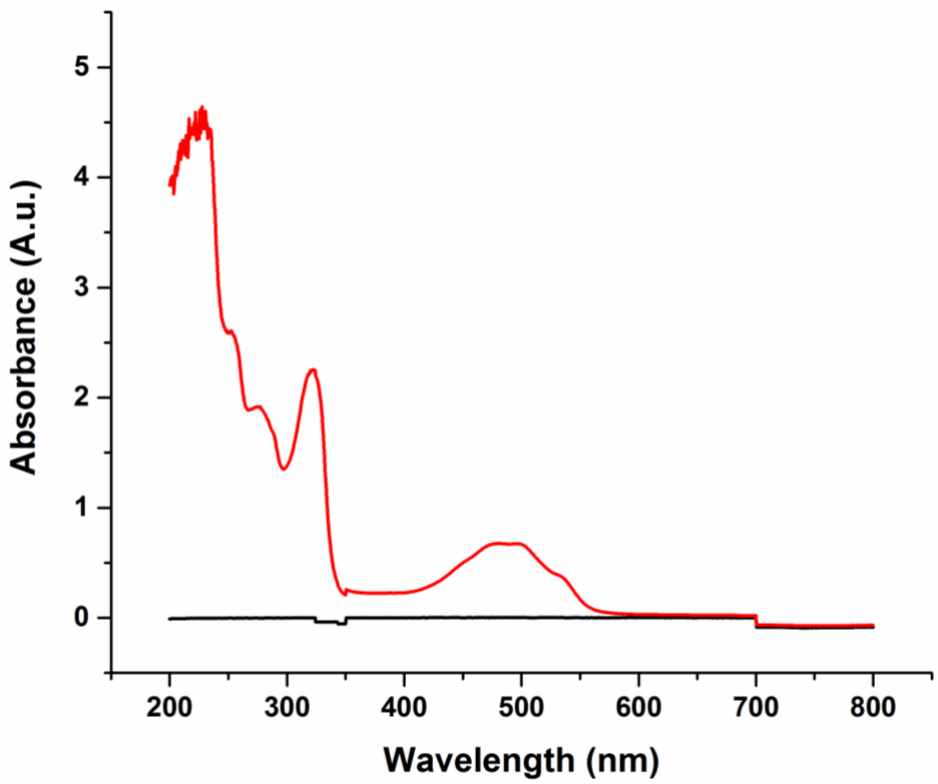


Figure 2.9. Absorbance of peptide-Dox conjugate using spectrophotometer.

2.4.4 Stability of Peptide-Dox Conjugate.

The stability of conjugate in aqueous conditions, aqueous acidic conditions and in the cell culture media was evaluated. While the conjugate was mostly stable in aqueous conditions (pH 7.0) when incubated for up to 48 hours (>80% intact), the stability in the aqueous acidic conditions (pH 5) and cell culture medium was much less (**Figure 2.10 A**). A solution of conjugate at pH 5 or in cell culture media (DMEM/F12 containing HEPES, 100 μM) was incubated at 37 °C, and aliquots were analyzed by RP-HPLC. The HPLC peak (area under the curve) for the conjugate slowly decreased over time with a half-life of 24 hours at pH 5 and

~48 hours in media. The loss of conjugate under acidic conditions is most likely due to the hydrolysis of the acetal group present in Dox (**Figure 2.10 C**). This is supported by the appearance of acetal hydrolysis fragment with a mass of 1764.4 (calculated mass 1764.7) in aqueous acidic conditions (**Figure 2.11**).

Next, the stability of conjugate in human serum was conducted by incubating conjugate (100 μM) with human serum at 37 °C. Aliquots from the incubation mixture were taken at regular intervals and subjected to precipitation by adding methanol to remove serum proteins. The supernatant was analyzed by HPLC and mass spectrometry. As shown in **Figures 2.10 A and 2.10 B**, the conjugate was fairly stable in human serum ($t_{1/2}$ ~ 18 hours), and 25% of the conjugate was still intact at 48 hours. The depletion of conjugate under different conditions is apparent as it is a succinimidyl thioether that is produced by Michael addition reaction of a thiolate with the double bond of the MCC-Dox maleimide.[45, 46] This reaction is reversible, and therefore can undergo the retro-Michael reaction to give back the free thiol and the MCC-Dox maleimide (**Figure 2.10 C**). In human serum, the presence of other proteins can enhance the formation of other protein-MCC-Dox adducts and over time, the conjugate is depleted. In addition, the formation of peptide dimer (disulfide formation) is also observed when the conjugate is incubated with the human serum (37 °C). Peptide dimer was observed ($[M+H]^+$ 2794.8) in the precipitate fraction when the aliquots at 6, 12, and 24 hours were precipitated with methanol. The succinimidyl thioether conjugates have become popular over the past few years with the approval of ADCs like brentuximab vedotin (Adcetris) and ado-trastuzumab emtansine (Kadcyla) that utilize the same chemistry.[3, 4] These conjugates function well over a short period of time (1-2 days), however, may not be the best when a prolonged drug circulation is required.[46] Over a long time these conjugates will undergo either thiol exchange reaction or a stabilizing succinimide ring opening reaction. Several strategies to

synthesize the ring opened conjugates with the hydrolysis of the succinimide ring have been proposed.[45-47]

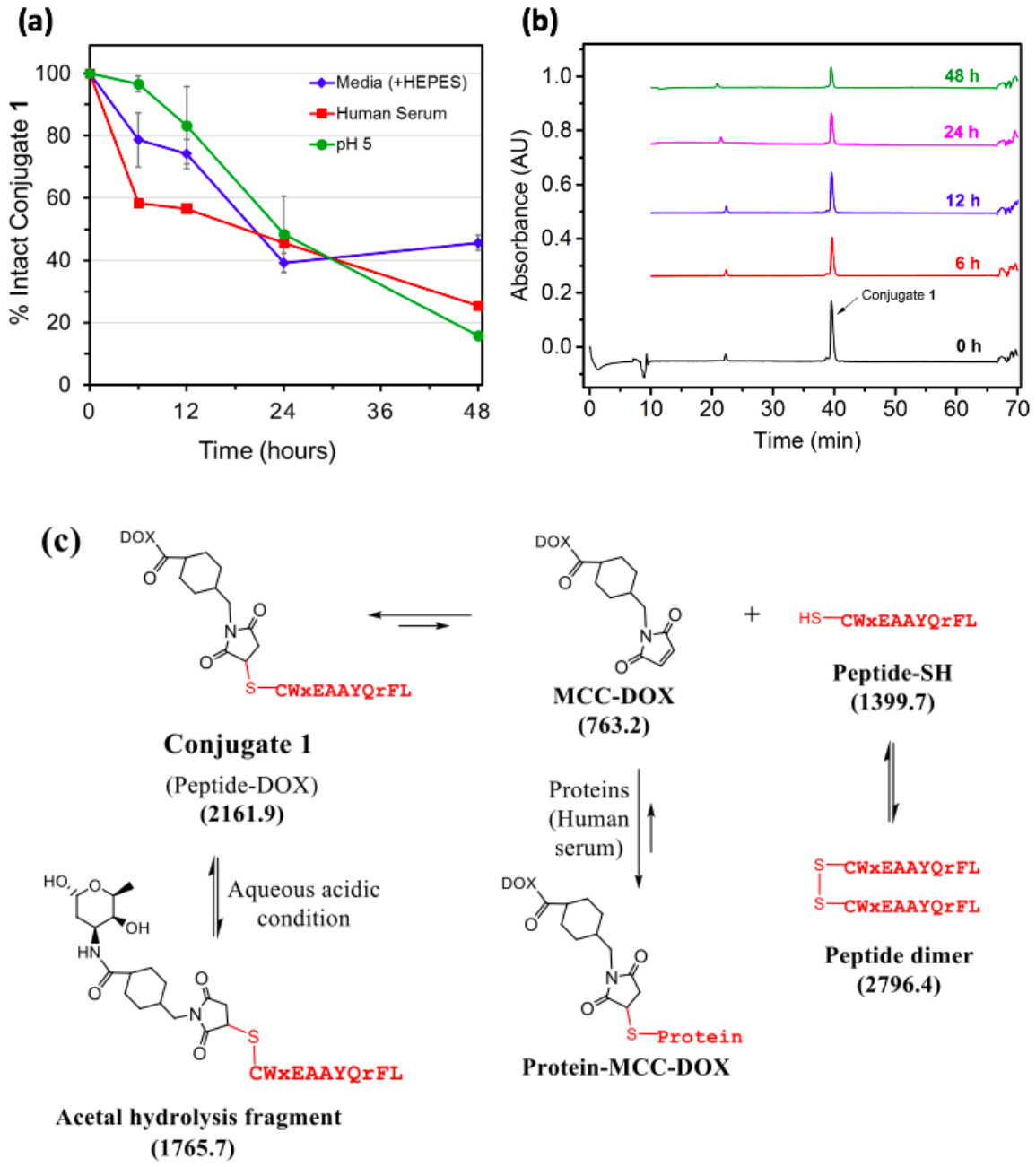


Figure 2.10. (A) The stability of conjugate in aqueous solution, cell culture media, and human serum at 37 °C. Each experiment was repeated twice and error bars show standard deviation. (B) Analysis of different moieties present in solution during incubation of conjugate with human serum using RP-HPLC. (C) Structures of the possible molecules/adducts when

conjugate is incubated in human serum (due to the reversible thiol-maleimide reaction) or in acidic conditions. The numbers in parenthesis are the calculated mass $[M+H]^+$.

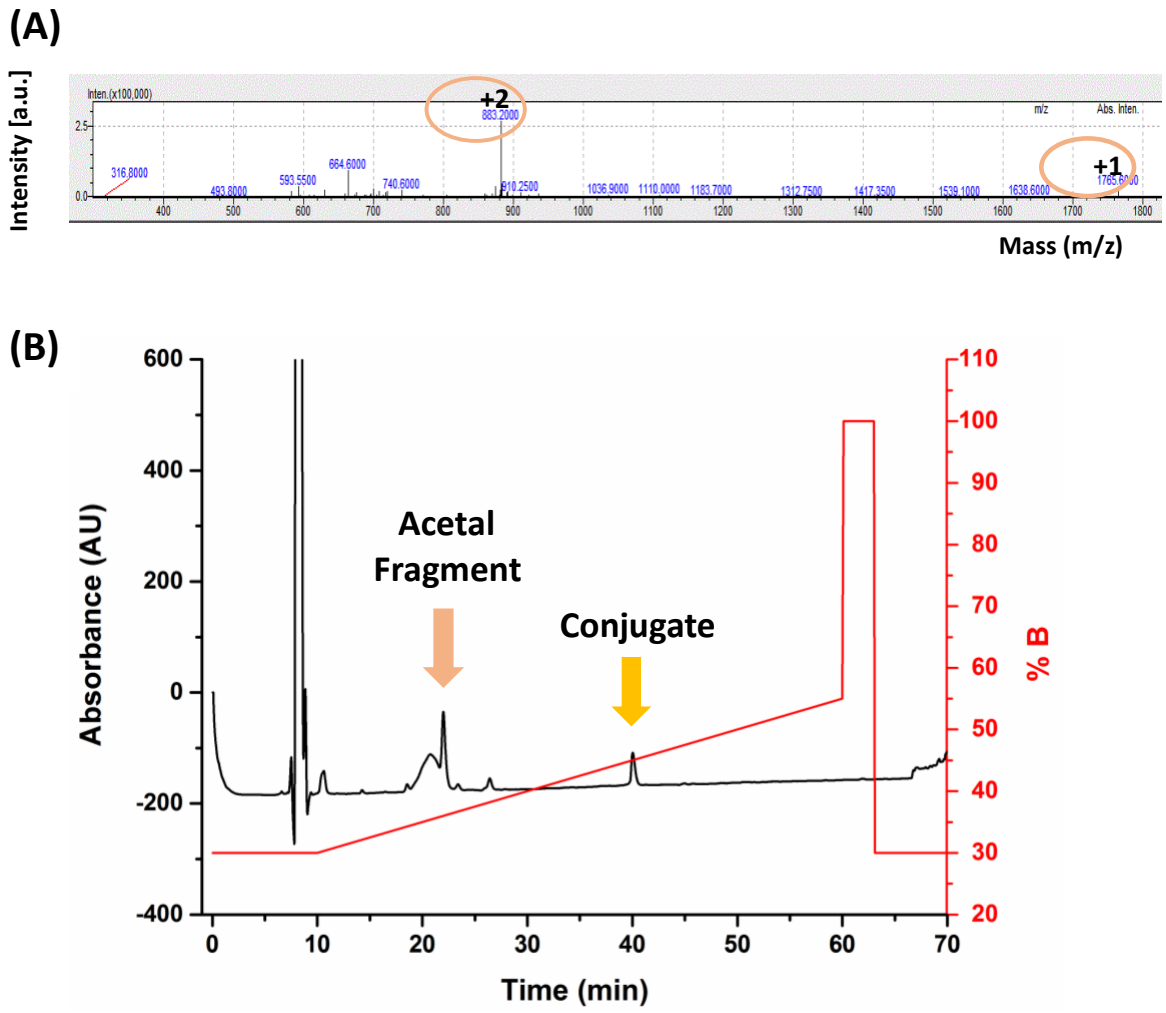
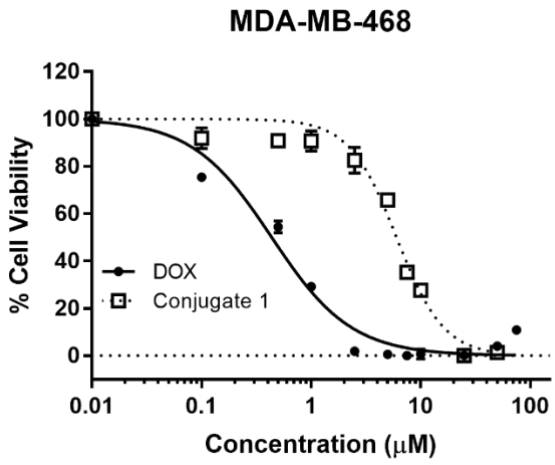


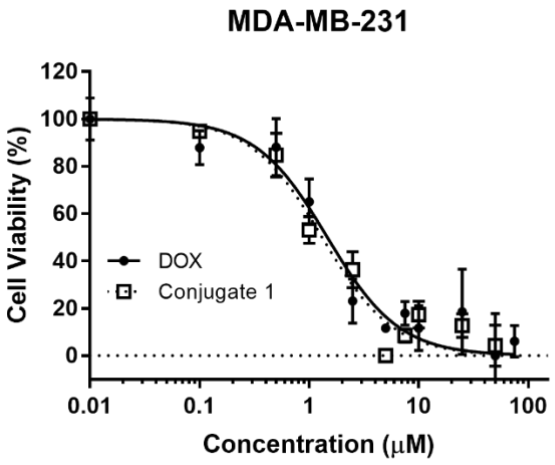
Figure 2.11. (A) Q-TOF mass spec for acetal fragment, calc. 1764.7 and found 1765.6. (B) RP-HPLC chromatogram of peptide-Dox conjugate and acetal fragment in in aqueous acidic condition.

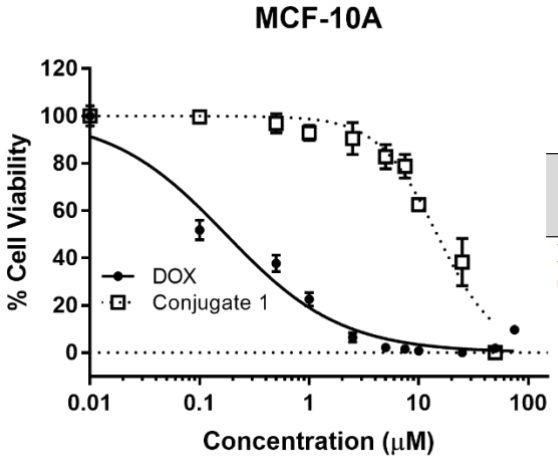
2.4.5 Cytotoxicity of Conjugate.

The main treatment for TNBC is chemotherapy.[32] Therefore, to enhance the therapeutic efficacy of Dox the conjugate was prepared and it's cell cytotoxicity was evaluated using two TNBC cell lines (MDA-MB-231 and MDA-MB-468) and one noncancerous mammary epithelial cell line (MCF-10A). To assess the cell viability via MTT assay and compare the IC_{50} values, cells in serum-free media were used during the assay to prevent early release of Dox from the conjugate (**Figure 2.12**).

The results showed that the cytotoxicity of conjugate (IC_{50} = 1.3 μM), as well as the free Dox (IC_{50} = 1.5 μM) on MDA-MB-231 breast cancer cell line, were in the low micromolar range (**Figure 2.12**). For the breast cancer cell line MDA-MB-468, free Dox (IC_{50} = 0.35 μM) was slightly more toxic compared to the conjugate (4.7 μM). For the non-cancerous cell line MCF 10A, the free Dox was highly toxic (IC_{50} = 0.24 μM) whereas conjugates displayed much-reduced toxicity (IC_{50} = 38.6). The conjugate was ~30 times less toxic toward non-cancerous breast cells compared to the breast cancer cells, while free Dox showed either the same or more toxicity towards noncancerous cells compared to the breast cancer cells. The variability in toxicity of conjugate to different cell lines could be attributed to the differential expression of keratin 1 among the cell lines, which is the target receptor for peptide **18-4**.[17]







Treatment	$IC_{50} \pm SD$ (μM)		
	MDA-MB-231	MDA-MB-468	MCF-10A
Free Dox	1.5 ± 0.2	0.35 ± 0.04	0.24±0.03
Conjugate **1**	1.3 ± 0.2	4.7 ± 0.31	38.6 ± 1.1

Figure 2.12. Cytotoxicity of PDC MTT assay. The percent cell viability is plotted against conjugate or Dox (control) concentration and the data are fitted using non-linear fit of normalized data to obtain IC50 values using GraphPad Prism 7.04. The plots show a representative experiment for each cell line used, namely, MDA-MB-231, MDA-MB-468, and MCF-10A. The table lists corresponding mean IC50 values and the standard deviation for Dox, and conjugate for the three cell lines. Each experimental point was done in triplicates and the experiment was repeated once. The horizontal dotted line at 0% cell viability serves as a baseline.

2.4.6 Cellular Uptake of Conjugate in Triple Negative Breast Cancer Cell Line MDA-MB-468.

It was previously shown that the uptake of the peptide takes place via receptor mediated endocytosis.[11] Cancer cells MDA-MB-435 were incubated with FITC-labeled 18-4 peptide (10^{-5} mol/L) in the presence of excess unlabeled 18-4 (50-fold), and the fluorescence of the cell (using flow cytometry) was found to decrease by 50% in the presence of excess unlabeled peptide. To confirm that the conjugate is internalized via the endocytic pathway, TNBC MDA-MB-468 cells were visualized by confocal fluorescence microscopy after incubation with conjugate **1** for 4 h at 37 ºC. As expected, the conjugate (red) colocalized with the lysosomal marker (green) (**Figure 2.13**).[29]

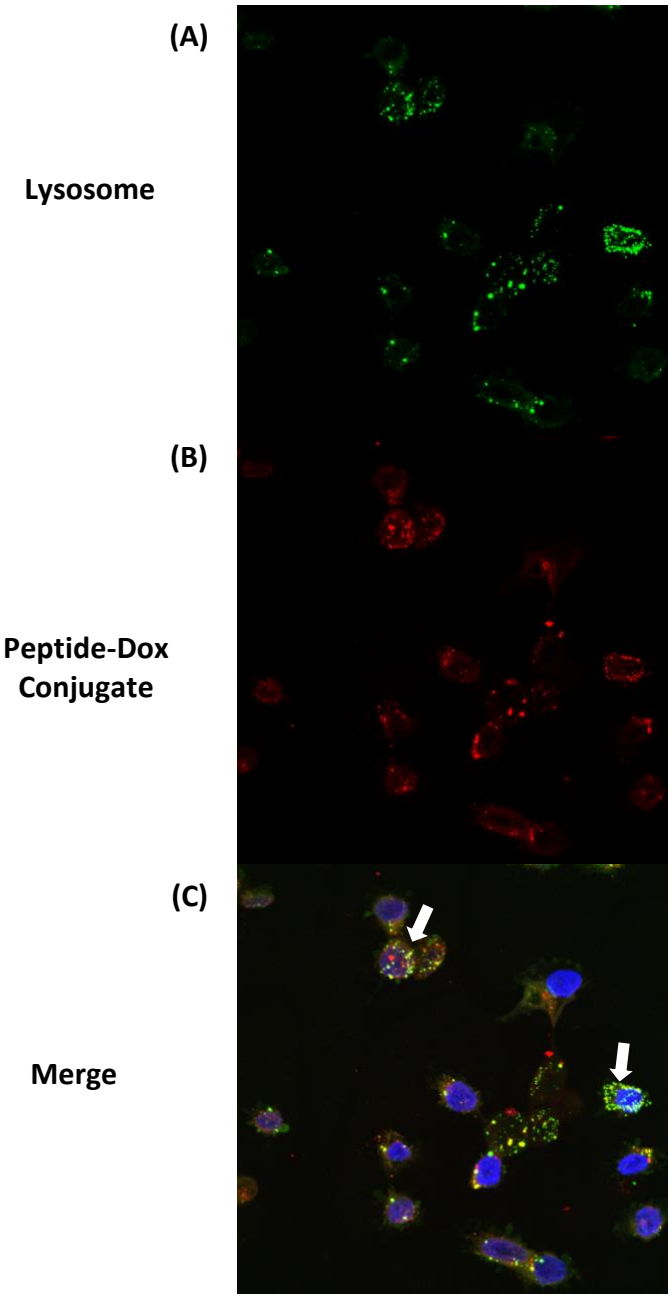


Figure 2.13. Cellular uptake of conjugate. MDA-MB-468 cells were incubated for 4 h with conjugate (red) at 37 °C, followed by incubation with LysoView (green) to stain lysosomes and DAPI (blue) to stain nuclei. Some of the regions of colocalized conjugate and LysoView (yellow) are indicated with arrowheads in the figure.

2.5 CONCLUSIONS

The use of succinimidyl thioether linker for the synthesis of PDC gave conjugate which has optimal characteristics such as decent stability and high selectivity for targeted delivery of Dox to TNBC cells. The cause of instability of conjugate was that it is a reversible maleimide-thiol adduct which can convert back to the free thiolated peptide and maleimide-Dox overtime. For instance, when conjugate was incubated with human serum, only 45% intact conjugate was left after 24 hours (**Figure 2.10**). The peptide **18-4** (WxEAAYQrFL) component of the conjugate is stable in human serum as the peptide has been engineered to be proteolytically stable by exchange of the labile amino acids with unnatural D-amino acids.[11] Furthermore, the targeting capability of the peptide **18-4**, and the uptake of the peptide and peptide-Dox amide/ester conjugates via specific receptor-mediated endocytosis in breast cancer cells is reported previously.[1, 11, 17] Peptide **18-4** binds keratin 1 on the breast cancer cell surface[17] and keratin 1 portrays an important role in breast [48] and other cancers or disease conditions.[21, 49-51] The purpose here was to develop linker chemistry for the conjugation of peptide to Dox to obtain a PDC with superior in vitro and in vivo efficacy. The maleimide-thiol reaction which forms succinimidyl thioether linker has been used previously for FDA approved ADCs,[3] and with the promising in vitro results presented here with the new PDC that use similar linker chemistry, we conjecture that this conjugate will lead to improved efficacy and lesser side effects for TNBC treatment. The in vitro studies of the novel PDC that use thioether linker chemistry suggest that it can serve as a potential anticancer agent, especially for TNBC treatment. The in vivo efficacy experiments with conjugate are currently in progress using MDA-MB-231 subcutaneous xenograft in NOD-SCID mice.

CHAPTER 3: Analysis of Expression of Keratin-1 in Different Breast Cell Lines

3.1 INTRODUCTION

Breast cancer cell targeting peptide 18-4 was engineered starting from the lead peptide p160 (**Figure 1.3**) identified by Zhang et al using ex vivo phage display. Zhang et al found that peptide p160 bound to several cancer cell lines including neuroblastoma WAC2 cells and MDA-MB-435 cells but showed no binding to primary HUVECs, erythrocytes, lymphocytes, and monocytes.[24] The second-generation peptide 18-4 (Figure 1.3) was screened to bind specifically the breast cancer cells with minimal or no binding to non-cancerous breast tissue derived epithelial cells MCF-10A. Further, it was found that the cancer cell targeting peptides p160 and 18-4 bind to keratin 1 on the surface of cancer cells. Using affinity purification, liquid chromatography-tandem mass spectrometry, and proteomics Kaur group identified type II keratin 1 (K1) as the target receptor on breast cancer cells for p160 peptide(s) [17]. Western blot and immunocytochemistry in MCF-7 breast cancer cells confirmed the identity of K1. We demonstrated that the p160 binding to breast cancer cells is dependent on the expression of K1, and confirmed peptide-K1 binding specificity using SPR experiments (Kd ~1.1 μM). Our results with p160 peptides establish K1, a 67 kDa protein, as a new marker for breast cancer targeting [17].

Keratins are markers of epithelial tissue that maintain cell structural integrity and regulate several cellular functions. There are 28 Type I (low molecular weight and acidic) and 26 Type II (high molecular weight and basic or neutral) keratin proteins [51, 52]. Type I and Type II keratin proteins form heterodimers that constitute intermediate filaments which shape the

cytoskeleton of epithelial cells. Numerous studies have reported the role of keratins in cancer cell invasion, metastasis, and treatment responsiveness [19, 51, 53]. Expression of several keratins, especially K1, K8, K17, K18, and K19, has been associated with cancer prognosis and is being used as a diagnostic marker [19, 20, 50, 51, 53-55].

In a proteomic study done by Blanckaert V. *et al*., it was concluded that expression of K1 as a functional protein in the triple negative breast cancer cell line MDA-MB-231 cell line was inhibited in the cells treated with 100 μM docosahexaenoic acid (DHA), an anti-invasive drug.[56] In an *in vitro* invasion assay, using siRNA against KRT1, they were able to suppress the anti-invasive activity of DHC in the triple negative breast cancer cells.[56] Collard C. *et al*. concluded that oxidative stress increases cytokeratin 1 (CK1) expression on human umbilical vein endothelial (HUVEC) cells.[57] The expression of cell-surface CK1 in HUVEC cells after oxidative stress was significantly upregulated compared to normoxic cells.[57] Using immunofluorescent confocal microscopy and Z-section scanning, Collard C. *et al*. confirmed the cell-membrane CK1 expression after endothelial oxidative stress induction.[57] Furthermore, immunoprecipitation and western blot of endothelial revealed a 67-kd band which is consistent with human CK1.[57]

Subiros-Funosas, et al. used cyclic analogue of peptide 18-4 in which they inserted a red-emitting Trp-based fluorogenic amino acid, and used the peptide to image K1 protein which is over-expressed in aggressive TNBC tumors.[58] In a flowcytometry analysis, they confirmed presence of K1 expression in a TNBC cell line, MDA-MB-231, and absence of K1 expression in HUVEC cells incubated with the red-emitting fluorogenic peptide.[58]

The goal of this chapter was to evaluate the cell-surface K1 expression in TNBC cells and compare it with the expression in non-cancerous cells. Two TNBC cell lines and one normal

mammary cell line MCF-12A was selected for the study. The level of K1 expression was determined using immunofluorescence confocal microscopy.

3.2 MATERIALS AND METHODS

Materials. Human TNBC cell lines, MDA-MB-231 and MDA-MB-468, and normal mammary tissue derived cells MCF-12A (ATCC, USA), Hank's balanced salt solution or HBSS without calcium chloride, magnesium chloride and magnesium sulfate (Gibco, USA), cell culture medium DMEM/F-12 (cat # 11330-032, Corning, Virginia, USA), cell culture medium DMEM (1×) + GlutaMAX-l media (10567-014, Gibco, USA), cell culture medium DMEM/ F-12 with HEPES (cat # MT-10-092-, Corning, Virginia, USA), human serum (Novus Biologicals, USA), hydrocortisone (Sigma, USA), insulin (Sigma, USA) and cholera toxin (Sigma, USA), 4-chamber Falcon cell culture slide (Corning™ Falcon™, USA), CellBrite Fix 555 Membrane Stain (Biotium, USA), primary antibody KRT 1 (cat # LS-C343835, LSBio, USA), secondary antibody Alexa 488 goat anti-mouse IgG (H+L) (Invitrogen, USA), Bovine serum albumin or BSA purified by a heat shock process, and protease-free powder (fisher bioreagents, USA), DAPI containing mounting medium (Abcam, USA), coverslip (Thermo Scientific, Germany), Nikon Eclipse confocal microscope.

3.2.1 Cell Culture

The study was done using human breast cancer cell lines MDA-MB-231 and MDA-MB-468 which were maintained at 37 °C in a 5% CO_2 -95% O_2 incubator and growth media were replaced every 48 h. Human breast cancer cell line MDA-MB-231 was cultured in DMEM/F-12 medium, and human breast cancer cell line MDA-MB-468 was cultured in DMEM medium. Both media were supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Human mammary cell line MCF-12A was cultured in DMEM medium. The

media was supplemented with 5% horse serum (HS), hEGF, hydrocortisone, cholera toxin, insulin, penicillin and streptomycin.

After removing cells from liquid nitrogen and thawing, under the hood, warm media (1 mL) was added to the cells and then transferred to a 15 ml centrifuge vial to centrifuge, spin down in 1500 rpm for 5 mins at 4 ℃ in order to remove previous media that contained DMSO. The supernatant was removed, and warm media (15 mL) was added to the cell pellet and re-suspend the cells by vigorously pipetting the media up and down. The cell suspension was transferred to a 100 mm tissue culture plate and placed in cell culture incubator (37°C, 5% CO2) undisturbed overnight. Next day the old media was removed and fresh media was added to the plate. The cells were fed with fresh media every two days till they reach 60% confluence.

3.2.1.1 Cell Subculture.

Human TNBC cell lines, MDA-MB-231 and MDA-MB-468, and normal mammary tissue derived cells MCF-12A were maintained at 37 °C in a 5% CO_2 -95% O_2 incubator and growth media were replaced every 48 h. All cell lines were obtained from ATCC. The MDA-MB-231 cells were cultured in DMEM/F-12 (ham) (1:1) (1X) with L-glutamine medium and 15 mM HEPES (Gibco, USA), and MDA-MB-468 cells were cultured in DMEM (1X) + GlutaMax dulbecco's modified of eagle's medium with 1 g/L D-glucose and 110 mg/L sodium pyrovate (Gibco, USA) medium. Both media were supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. The MCF-10A cells were cultured in DMEM/F-12 50/50, 1X (dulbecco's modified of eagle's medium / ham's F-12 50/50 Mix) with L-glutamine (Corning, USA) medium. The media for MCF-12A cells was supplemented with 5% horse serum (Gibco, USA), 1% penicillin and streptomycin. The medium for MDA-MB-231 and MDA-MB-468 cells were supplemented with 10% FBS (Corning, USA), 1% penicillin and streptomycin

(Gibco, USA). Hank's balanced salt solution (HBSS) (without calcium chloride, magnesium chloride and magnesium sulfate) (Gibco, USA) was used to wash the cells before trypsinization.

After removing cell culture plate from cell culture incubator, under the hood, the old media was discarded and cells were rinsed with fresh warm media (2 mL) three times. Next, 0.25% trypsin (3 mL) was added to the plate and the plate was placed into the cell culture incubator 37°C for 5 minutes. Then, the cells were checked under the microscope to ensure that most cells are detached from the plate and floating in the solution. Next, under the hood, neutralization media (6 mL) containing FBS or Horse serum was added and mixed with the cells by pipetting the solution. The solution containing cells was pipetted into a 50 mL centrifuge tube with cap, and centrifuged at 1500 RPM for 5 minutes at 4°C. Normal breast MCF-12A cells were centrifuged at 125 ×g for 8 minutes at R.T. After aspirating the supernatant, media (2 mL) was added to pelleted cells, and mixed cells by pipetting the solution 20 times.

Next, the cells were counted using hemocytometer by adding 10 μL of the cells to the hemocytometer while holding the coverslip in position. The chamber was placed in the microscope under a 10× objective. Only the cells located in the large, central gridded square were counted. Lastly, the cell count was multiplied by 10^4 to estimate the number of cells/mL. After transferring desired amount of cells in media to new plates, the plates were placed in cell culture incubator overnight. After 24 hr, the media was replaced with fresh warm media. This protocol was repeated once cells were ~80% confluent.

3.2.2 Immunofluorescent Confocal Microscopy of K1 Expression in Triple Negative Breast Cancer Cell Lines MDA-MB-231 and MDA-MB-468

Experiments were carried out with 1×10^5 cells/chamber on a 4-chamber Falcon cell culture slide (Corning™ Falcon™, USA) overnight. Next day, DMEM/ F12 (1:1) culture medium (Gibco, USA) was removed and cells were rinsed with sterile PBS (1X). Pre-staining solution (1 μl, Biotium, USA) was diluted in HBSS (1 mL), and MemBrite solution (Biotium, USA) was prepared by mixing MemBrite dye (1 μl) with HBSS (1 mL). Then, pre-staining solution (500 μl/well) was added and incubated for 5 min at 37 °C. Next, pre-staining solution was removed and MemBrite solution (500 μl/ chamber) was added and incubated for 5 min at 37 °C in dark. Chambers were rinsed with HBSS buffer and cells were fixed with 4% formaldehyde for 10 min at R.T. Cells were washed with PBS (three times, each 10 min). Next, 5% BSA in PBS (200 μl/ chamber) was added to cells and incubated in moist chamber (30 min, R.T.) in dark. Primary antibody keratin 1 (150 μl/ chamber, LSBio) in PBS (1:20) was added to each chamber to cover the cells completely and incubated in moist chamber (90 min, R.T.) in dark. Chambers were rinsed with PBS (×3) after primary antibody withdrawal. Secondary antibody Alexa 488 goat anti-mouse IgG (H+L) (Invitrogen by Thermo Fisher Scientific, USA) in PBS (1:250) was added to each chamber (150 μl/ chamber) and incubated in moist chamber (60 min, R.T.) in dark. After washing the slides with PBS (×3), chambers were removed, the slides were coated with DAPI (abcam, USA), covered with coverslip (Thermo Scientific, Germany), and analyzed with a Nikon confocal microscope. For each chamber, 10 randomized fields were photographed and analyzed with NIS-Elements AR software. Three independent experiments were performed in duplicate.

3.2.3 Immunofluorescent Confocal Microscopy of K1 Expression in Normal Breast Cell Line MCF-12A.

Experiments were carried out with 1×10^5 cells/chamber on a 4-chamber Falcon cell culture slide (Corning™ Falcon™, USA) over 48 hours. After 48 hours, media was removed and cells were rinsed with sterile HBSS. Keratin 1 expression (green) was evaluated with a FITC-conjugated goat anti-mouse IgG (H+L) antibody. Cell membrane expression (red) was evaluated using MemBrite fix cell surface staining kit, and nucleus (blue) was stained with DAPI. Pre-staining solution (1 μl, Biotium, USA) was diluted in HBSS (1 mL), and MemBrite solution (Biotium, USA) was prepared by mixing MemBrite dye (1 μl) with HBSS (1 mL). Then, pre-staining solution (500 μl/ chamber) was added and incubated for 5 min at 37 °C. Next, pre-staining solution was removed and MemBrite solution (500 μl/ chamber) was added and incubated for 7 min at 37 °C in dark. The slides were then washed in HBSS (three times, each 10 min) and fixed in 4% paraformaldehyde for 10 min at R.T., washed again (three times, each 10 min), and blocked with 5% BSA in PBS (1X) (200 μl/ chamber) for 30 min at R.T. After incubation with the primary antibody keratin 1 (150 μl/well, LSBio) in PBS (1:20) for 90 min at R.T., the slides were washed (3 times, 10 minutes each) and incubated with the secondary antibody Alexa 488 goat anti-mouse IgG (H+L) (Invitrogen by Thermo Fisher Scientific, USA) in PBS (1:250) for 60 min at R.T., and washed again (3 times, 10 minutes each). The slides were coated with DAPI (abcam, USA), covered with coverslip (Thermo Scientific, Germany), and analyzed with a Nikon confocal microscope. For each chamber, 10 randomized fields were photographed and analyzed with NIS-Elements AR software. Three independent experiments were performed in duplicate.

3.3 RESULT AND DISCUSSION

3.3.1 Differential Epithelial Cell-surface K1 Protein Expression in Different Cell Lines

Immunofluorescence confocal microscopy was used to determine the expression level of K1 in cancer and non-cancerous cells. The plasma membrane of the cells was labeled with a membrane staining dye and primary anti-K1 mouse antibody followed by secondary antibody was used to label and visualize the cell-surface K1 (**Figure 3.1**).

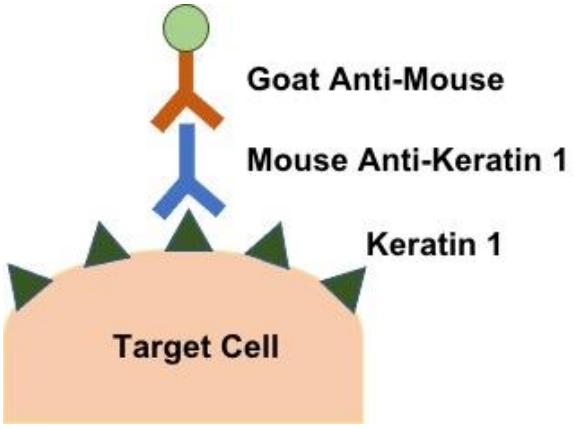


Figure 3.1. Schematic representation of K1 staining using mouse anti-keratin 1 primary antibody and Alexa 488 goat anti-mouse secondary antibody.

Human epithelial cell-surface K1 protein expression in TNBC cell lines MDA-MB-231, and MDA-MB-468, and normal mammary cell line MCF-10A was determined by confocal microscopy (**Figure 3.2**). K1 expression (green) was evaluated with a FITC-conjugated goat anti-mouse IgG (H+L) antibody. Cell membrane expression (red) was evaluated using MemBrite fix cell surface staining dye, and nucleus (blue) was stained with DAPI. Immunofluorescent confocal microscopy demonstrated a significantly higher K1 expression in triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 compared to normal

mammary cell line MCF-12A (**Figure 3.2**). Images with Z-section scanning further confirmed higher surface K1 expression in TNBC cell lines compared to normal breast cell line.

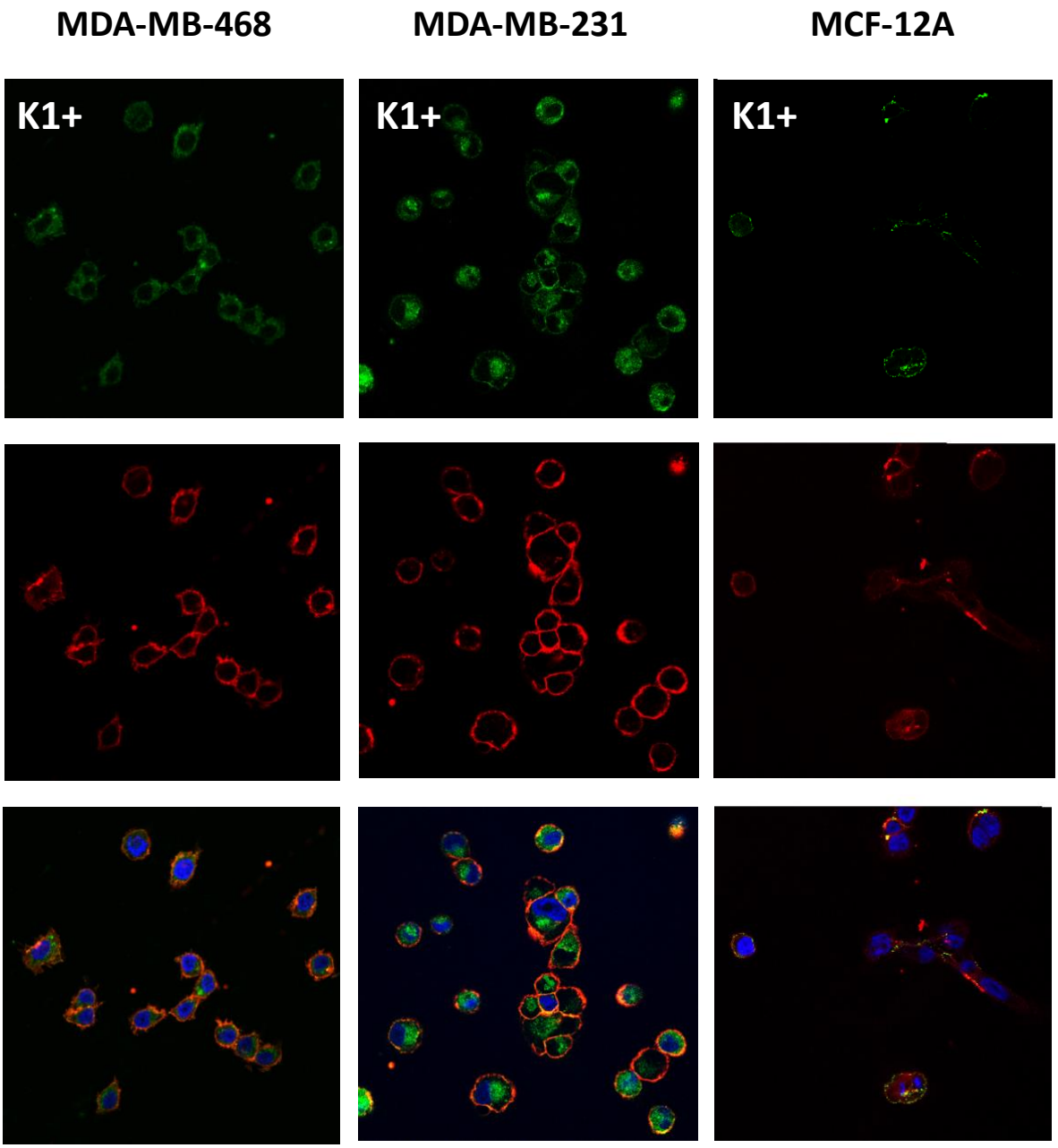


Figure 3.2. Epithelial cell-surface K1 protein expression in TNBC cell lines MDA-MB-231, and MDA_MB-468, and normal mammary cell line MCF-12A. K1 protein expression was determined by Immunofluorescent confocal microscopy. K1 expression (green) was

significantly higher K1 in triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 compared to normal mammary cell line MCF-12A.

3.4 CONCLUSIONS

Human epithelial cell-surface K1 protein expression in TNBC cell lines MDA-MB-231, and MDA_MB-468, and normal mammary cell line MCF-12A was evaluated using immunohistochemistry staining. Immunofluorescent confocal microscopy demonstrated a significantly higher K1 expression in triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 compared to normal mammary cell line MCF-12A. Z-section scanning further confirmed higher cell membrane K1 expression in TNBC cell lines compared to normal breast cell line. Further experiments evaluating cell-surface expression of K1 in HUVEC cell line and TNBC cell line MDA-MB-436 are currently in progress.

CHAPTER 4: Preparation of Peptide-Dox Conjugate for In Vivo Mice Efficacy Studies

4.1 INTRODUCTION

Breast cancer, a malignant disease, is one the most common types of cancer and second leading cause of death among women worldwide.[12] Breast cancer is divided into four subtypes based on the level of expression of three types of receptors including, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor (HER2).[12] The fourth subtype is triple negative breast cancer (TNBC) which lacks all three receptors.[12]

Using animal model systems in research has brought about significant advancement into developing and evaluating pre-clinical therapies for various diseases including breast cancer.[59] In breast cancer research, mouse models offer a critical approach in investigating the efficacy of a potential drug, in addition to understanding the genetic pathways and mechanisms of disease progression.[12] As primary tools for breast cancer research, mouse models are divided into 3 groups: Xenograft models, Ionization radiation (IR)-induced models, and genetically engineered mice (GEMs).[60] In xenograft models, tumor is transplanted in the body to grow and reach the desired size prior to investigation.[12] Xenograft models are used to evaluate the therapeutic efficacy and toxicity, and to make preclinical toxicology assessment on a candidate anticancer drug.[12] There are two types of xenograft models, patient-derived xenograft (PDX) model, and cell-line-derived xenograft (CDX) model.[12, 60] CDX model is often used to investigate breast cancer and metastatic progress, because it is easier to monitor the tumor growth.[12] While in CDX transplantation model, cell-line-derived cancer cells are transplanted in mice subcutaneously or intravenously, in PDX transplantation model, primary human breast carcinomas or fragments of tumor are subcutaneously or orthotopically implanted

into immune-compromised mice such as NOD-SCID, NOD-SCID IL_2-receptor null (NSG), or nude mice.[12] Philips G. *et al.* surgically transplanted FO5 or F2#1282 HER2 xenograft tumor tissues into mammary fat pad of female beige nude mice for their in vivo efficacy and pharmacokinetic studies.[10] Mice bearing tumor were given single i.v. injection of antibody drug conjugates (ADC) weekly, and tumor growth was monitored for 25 days.[10] Ahmed F. *et al*. orthotopically injected a basal MDA-MB-231 triple negative human breast tumor into mammary fat pads of NOD SCID mice to evaluate efficacy of their peptide drug conjugate (PDC).[38] After developing breast tumor of appropriate size, mice received i.v. dose of drug 3 times a week for 14 days.[38]

Triple negative breast cancer (TNBC), an aggressive subtype of breast cancer, is difficult to target, as this breast cancer subtype lacks expression of hormone receptors (estrogen or progesterone) as well as lacks overexpression of human epidermal growth factor receptor 2 (HER2).[61, 62] Chemotherapy is the mainstay treatment for TNBC. Several cancer targeting peptides have been proposed for targeting overexpressed receptors in breast cancer.[61] For instance, peptide 18−4 binds keratin 1 (K1) and peptide GE11 binds epidermal growth factor receptor (EGFR or ErbB1) overexpressed on breast cancer cells, and both keratin 1 and EGFR are suggested to play key roles in TNBC. The linker is an important component of the PDC and allows timely release of the drug after it is internalized. Linkers like esters, amides, disulfides, and acid-labile hydrazones have been explored that get cleaved in the intracellular tumor environment. In addition, thioether linker has been used where the drug is released from the peptide or antibody by proteolytic degradation (intracellular) of the peptide/antibody. The succinimidyl thioether linker is gaining attention as it is present in two clinically used ADCs, Kadcyla and Adcetris. The succinimidyl thioether linkage shows superior in vivo efficacy compared to disulfide-bonded ADCs. For the cytotoxic agent, it is recommended to use a

highly toxic agent in the PDC with IC_{50} values in the sub-nanomolar range such as drugs like maytansine derivatives, auristatin and doxorubicin (Dox). In this toxicology study, we aimed to explore the ability of peptide-Dox conjugate to deliver Dox to TNBC cells specifically. In conjugate , Dox is conjugated to the peptide via a succinimidyl thioether linkage. In previous studies the stability of the conjugate in different environments was studied, followed by in vitro cytotoxicity using TNBC and normal breast tissue-derived cells were explored. The results showed that conjugate is stable in human serum, and displays much higher cytotoxicity toward TNBC cells compared to normal breast cells. The in vitro study of the novel peptide-Dox conjugate suggested that it may serve as potential anticancer agent, especially for TNBC treatment.

The goal of this chapter was to synthesize and purify conjugate 1 for in vivo efficacy and biodistribution studies. In addition, the solubility of the conjugate at the concentrations required for in vivo experiments was assessed.

4.2 MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride (Dox.HCl) salt and aldoxorubicin were bought from LC Laboratories (MA, USA) and MedChem express (NJ, USA), respectively. N,N-Dimethylformamide (DMF), trifluoroacetic acid (TFA), were bought from Sigma- Aldrich. Sulfo SMCC (sulfosuccinimidyl 4-[N-maleimidometh- yl] cyclohexane-1-carboxylate) was purchased from Thermo- fisher Scientific (NY, USA). Absolute ethanol, sterilde water, sterile DMSO, sterile normal saline, filter, tween 20.

4.2.1 Optimization of Peptide-Dox Conjugate Synthesis to Increase Yield

Two different approaches were used to react the maleimide group on MCC-Dox with the sulfhydryl group on the peptide. In the first approach, peptide was reacted to MCC-Dox in presence of DIPEA. A solution of peptide (1.1 mg, 0.78 μmol) in DMF (0.26 mL) was added to the MCC-Dox (0.8 mg, 1 μmol) in DMF (0.5 mL), and additional DMF (0.74 mL) was added. Lastly, DIPEA (19 μl, 1% of weight) was added, and the reaction mixture was stirred at r.t. under nitrogen overnight. The crude mixture was injected into RP-HPLC for characterization.

In the second approach, peptide was reacted to MCC-Dox in presence of PBS (100 mM, pH 7.4). A solution of peptide (1.1 mg, 0.78 μmol) in DMF (0.26 mL) was added to the MCC-Dox (0.8 mg, 1 μmol) in DMF (0.5 mL), and additional DMF (0.24 mL) was added (**Figure 4.1**). Lastly, PBS (500 ul, 100 mM, pH 7.4) was added, and the reaction mixture was stirred at r.t. under nitrogen overnight. The reaction progress was monitored using RP-HPLC.

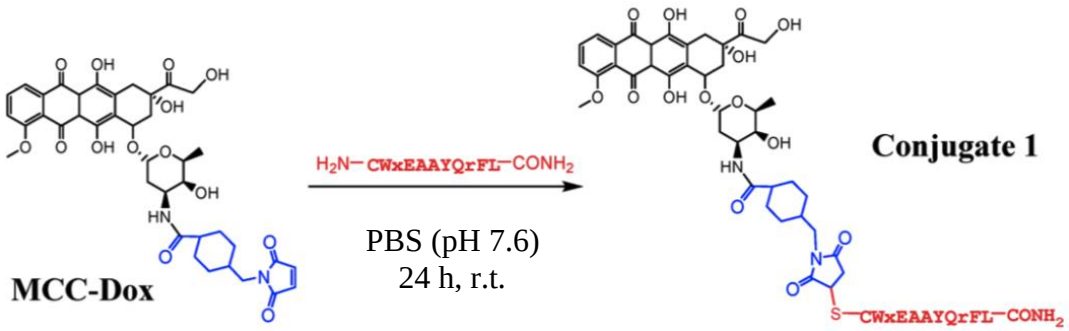


Figure 4.1. Chemical synthesis of peptide-Dox conjugate. The reagents used were MCC-Dox, DMF, and peptide (NH_2-CWxEAAYQrFL-$CONH_2$), DMF, PBS (pH 7.6, 100 mM).

4.2.2 Purification of Peptide-Dox Conjugate

The crude mixture of the conjugate obtained using the above procedure (**Figure 4.1**) was purified using RP-HPLC (30−45% acetonitrile/water containing 0.05% TFA, 2 mL/min flow rate, 60 min run time, t_R= 35 min) one day after the reaction. Q-TOF found 1081.43 charge +2; therefore, unprotonated mass found (1081.43 − 1) × 2 = 2160.86, calc. mass = 2160.9; MALDI-TOF $[M + H]^+$ found 2161.5, calc. 2161.9. Until used, the conjugate was stored as a dry powder at −20 °C. In order to obtain the amount of pure peptide-Dox conjugate needed for the in vivo studies, total of 6 experiments were done to synthesize crude conjugate (19.5 mL). The first two synthesis (synthesis 1 and 2) were done in presence of DIPEA with scales of 1 and 3 respectively. The last four synthesis were done in presence of PBS (100mM, pH 7.4) (synthesis 3, 4, 5 and 6) with scales of 1, 2, 2 and 4 respectively. In double scale synthesis, A solution of peptide (2.2 mg, 1.56 μmol) in DMF (0.52 mL) was added to the MCC-Dox (1.6 mg, 2 μmol) in DMF (1 mL), and additional DMF (0.48 mL) was added. Lastly, PBS (1 mL, 100 mM, pH 7.4) was added, and the reaction mixture was stirred at r.t. under nitrogen overnight.

4.2.3 Calculation of Amount of Conjugate Needed for Seven Mice During 6 Weekly Injections.

Average mice weight (24.3 g) was determined. Since each mice should have received one injection per week containing conjugate (0.1043 μmol/injection) dissolved in aqueous solution (200 μl /injection), the amount of total conjugate (10.5 mL, 522 μM) needed for seven mice during 6 weeks of injection was determined. To obtain pure conjugate, approximately 162 runs of purification were done using RP-HPLC system Prominence-i (Shimadzu Corp., Kyoto, Japan). In each HPLC run (60 min/run), crude conjugate (120 μl) was injected in C18 semi-preparative (10 mm × 250 mm, 5 μm). Pure peptide-Dox conjugate eluted in two separate peaks (t_R= 35 min, and t_R= 37 min) which were collected together in the same vial.

4.2.4 Concentration of Peptide-Dox Conjugate

To dissolve the conjugate, acetonitrile and water (1:1, 100 μl) were added to the round bottom flask containing pure conjugate powder. The conjugate in aqueous solution (3 μl) was transferred to an Eppendorf tube to measure its concentration using Quickdrop. First, the instrument was switched on, and from the main screen, [Read Type] was selected by followed by selecting [Single Wavelength] method. Next, [Mode] was set to [Absorbance], and [Wavelength] and [Integration Time] were set to 481 nm and 2 seconds respectively. After finishing method parameters, the reference solution containing water/acetonitrile (1:1) (2 μl) was loaded in the sample port followed by taking the reference measurement by touching the reference icon (blue) on the touch screen. After removing the reference by wiping the sample port, pure conjugate (2 μl) was loaded in the sample port followed by taking the sample measurement by touching the sample icon (red) on the touch screen. The sample was removed by wiping the sample port, and the sample measurement was repeated two more times to obtain

total of three absorbance measurements. To calculate the concentration of conjugate from obtained absorbances the following equation was used:

$$\text{Conc.} = \frac{\text{Avg absorbance}}{10410\left(\frac{L}{mol.cm}\right) \times 0.05\ cm}$$

4.2.5 Aliquots for Injections and Solubility

To prepare the injection solution for seven mice during 6 weeks of injection, conjugate (522 μM, 10.5 mL) was aliquoted in three round-bottom flasks (**Table 4.1**) so each flask can be used for 2 weeks (14 injections). First, the dried conjugate powder was dissolved in sterile water/acetonitrile (6:4), then equal amount of conjugate aqueous solution (522 μM, 3.5 mL) was transferred to each of the 3 round bottom flasks. Each round bottom flask should contain the amount of conjugate required for 14 injections. At last, the solvents were removed using the rotary vapor. Until used, the conjugate was stored as dry powder at −20 °C. To prepare the injection solution, every two weeks, 24 hours prior to injection, the conjugate in a round bottom flask was

Table 4.1. Aliquots of peptide-Dox conjugate in three round bottom flasks during 6 weeks of injection.

	Concentration (μM)	**Injections**
Round Bottom flask 1	522	Injection 1 & 2
Round Bottom flask 2	522	Injection 3 & 4
Round Bottom flask 3	522	Injection 5 & 6

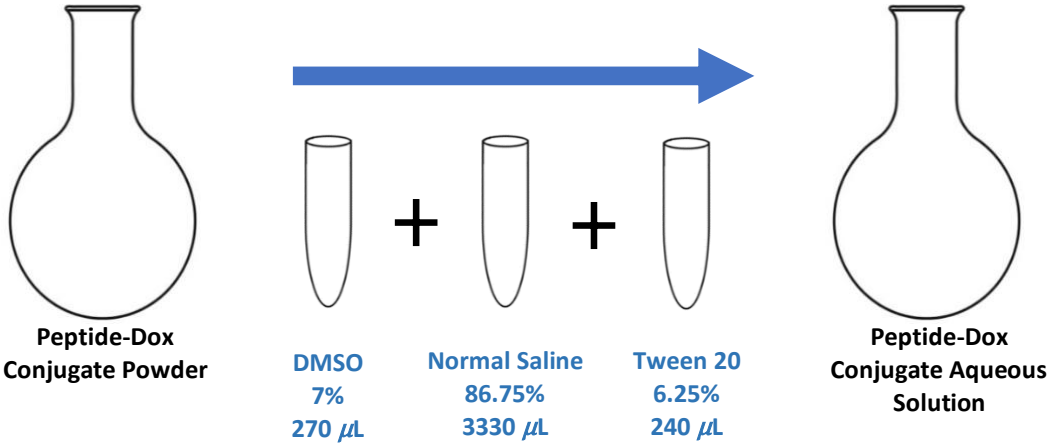


Figure 4.2. Schematic presentation of peptide-Dox conjugate aqueous solution preparation for in vivo studies. Dried conjugate powder was first dissolved in DMSO (7%, 270 μL), and then normal saline (3.33 mL) was added, followed by addition of tween 20 (6.25 %, 240 μL) to the solution.

solubilized. Under the biosafety cabinet hood, to a round bottom flask DMSO (0.7:9.3, v/v, 270 μL) was added. After dissolving the conjugate in DMSO completely, sterile normal saline (3330 μL), and Tween 20 (0.625: 9.375, v/v, 240 μL) were added gradually (**Figure 4.2**). The conjugate mixture was aliquoted and transferred into two sterile Eppendorf vials, and stored at 4 °C. half an hour prior to injection, the vial was removed from the fridge, and placed in sonicator for 10 min to improve the solubilization of conjugate.

4.2.6 In Vivo Study

Twenty-one NOD-SCID female mice were used. All animal experiments were carried by Azam Saghaeidehkordi in accordance with IACUC guidelines. MDA-MB-231 cells were first collected in a concentration of 2×10^7/mL and inoculated subcutaneously into the right flank

of each mouse. When the tumor size reached a volume of 100 mm^3-200 mm^3, the mice were randomly divided into three groups for the treatment with 2.5 mg Dox equivalent/kg of the conjugate, free Dox, or normal saline once every week for 6 times. Free Dox was used as positive control, and normal saline was used as negative control. The mice weight and tumor size were measured twice a week. The day of the i.v. injection, it was realized that the conjugate is not fully solubilizing. Unfortunately, due to pandemic starting at the same time (Mar 2020) and complications observed with the solubility of the conjugate, it was decided not to proceed with the injections at that time.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Optimization of Peptide-Dox Conjugate Synthesis to Increase Yield.

Sulfo-SMCC is heterobifunctional cross-linker that allows covalent conjugation of molecules containing amines and sulfhydryls through its *N*- hydroxysuccinimide (NHS) ester and maleimide groups, respectively.[31] Maleimide groups react with sulfhydryl at pH 6.5-7.5 to form stable thioether bond. In order to increase the yield of peptide-Dox conjugate, two different approaches were used to react the maleimide group on MCC-Dox with the sulfhydryl group on the peptide. In the first approach, peptide was reacted to MCC-Dox in presence of DIPEA. The crude mixture was injected into RP-HPLC for characterization. The conjugate eluted at 35 min (40% acetonitrile) using RP-HPLC (30−45% acetonitrile/water containing 0.05% TFA, 2 mL/min flow rate, 60 min run time, t_R= 35 min) to give pure peptide−Dox conjugate as TFA salt with 63.5 % yield and 98% purity one day after the reaction. In the second approach, peptide was reacted to MCC-Dox in presence of PBS (100 mM, pH 7.4). The crude mixture was injected into RP-HPLC for characterization. The conjugate eluted at 35 min (40% acetonitrile) using RP-HPLC (30−45% acetonitrile/water containing 0.05% TFA, 2 mL/min flow rate, 60 min run time, t_R= 35 min) to give pure peptide−Dox conjugate as TFA salt with 81.4 % yield one day after the reaction. As shown in **Figure 4.3**, the yield of the conjugate was higher with the second approach as the AUC for the conjugate peaks using the second approach (1.31×10^6) was ~5 times more than that for the first approach 2.4×10^5. For each conjugate, absorbance at 481 nm was measured using Quickdrop. The absorbance spectra of conjugate synthesized in presence of DIPEA (A_{avg} 0.526) and conjugate synthesized in presence of PBS (A_{avg} 0.795) were measured (**Figure 4.4**).

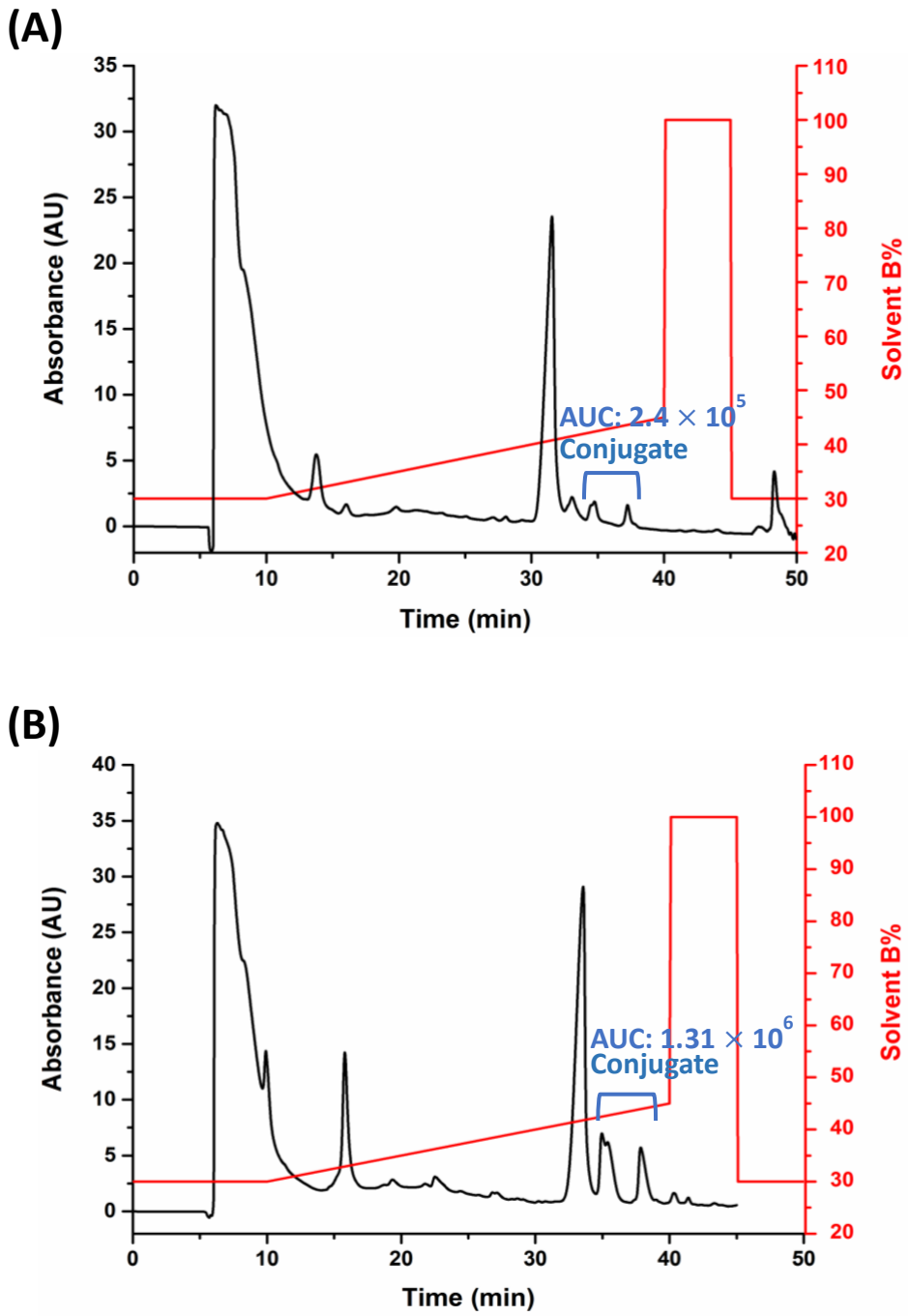


Figure 4.3. (A) RP-HPLC chromatogram of crude peptide-Dox conjugate synthesized in presence of DIPEA for 24 h. **(B)** RP-HPLC chromatogram of crude peptide-Dox conjugate synthesized in presence of PBS (pH 7.4) for 24 h. The area under the curve for conjugate peaks from the synthesis in presence of PBS (AUC 1.31×10^6) was higher than the area from the synthesis in presence of DIPEA (AUC 2.4×10^5) .

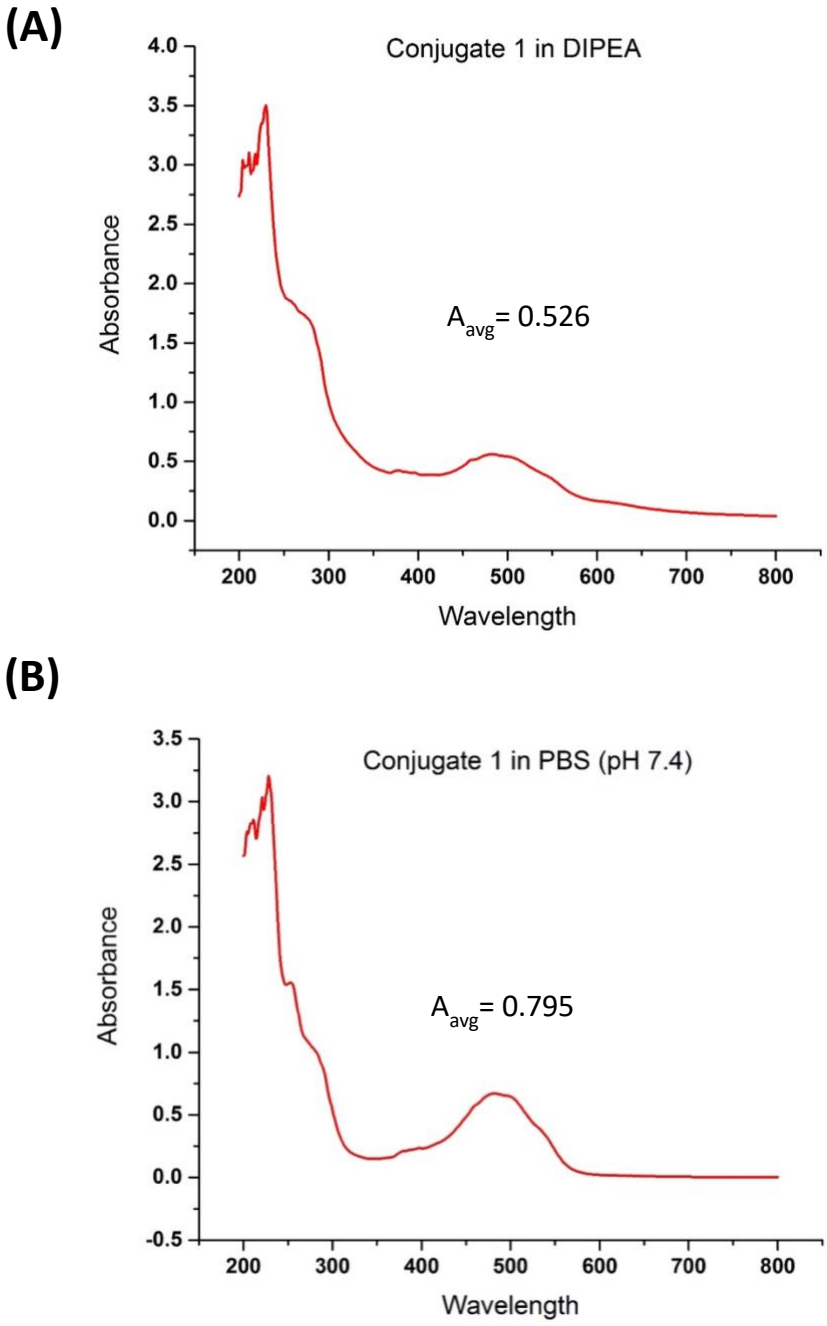


Figure 4.4. (A) Absorbance of peptide-Dox conjugate synthesized in presence of DIPEA. **(B)** Absorbance of peptide-Dox conjugate synthesized in presence of PBS.

4.3.2 Peptide-Dox Conjugate Formation Over a Long Time.

A solution of peptide in DMF was added to the MCC-Dox in DMF, and additional DMF was added. Lastly, PBS (100 mM, pH 7.4) was added, and the reaction mixture was stirred at r.t.

under nitrogen overnight. The reaction progress was monitored using RP-HPLC over time (1, 10, 20 and 30 days) stored at room temperature. Each time, an aliquot of crude mixture was injected into RP-HPLC for characterization. The conjugate eluted at 35 min (40% acetonitrile) using RP-HPLC (30−45% acetonitrile/water containing 0.05% TFA, 2 mL/min flow rate, 60 min run time, t_R= 35 min) to give pure peptide−Dox conjugate as TFA salt. The area under the curve for conjugate's peaks (2 and 3) were significantly increased over time (**Figure 4.5**). Hence, it was concluded that the yield of the reaction is increased with time when the conjugate is stored at r.t. up to 30 days (**Figure 4.5**). Q-TOF found 1081.43 charge +2; therefore, unprotonated mass found (1081.43 − 1) × 2 = 2160.86, calc. mass = 2160.9; MALDI-TOF $[M + H]^+$ found 2161.5, calc. 2161.9.

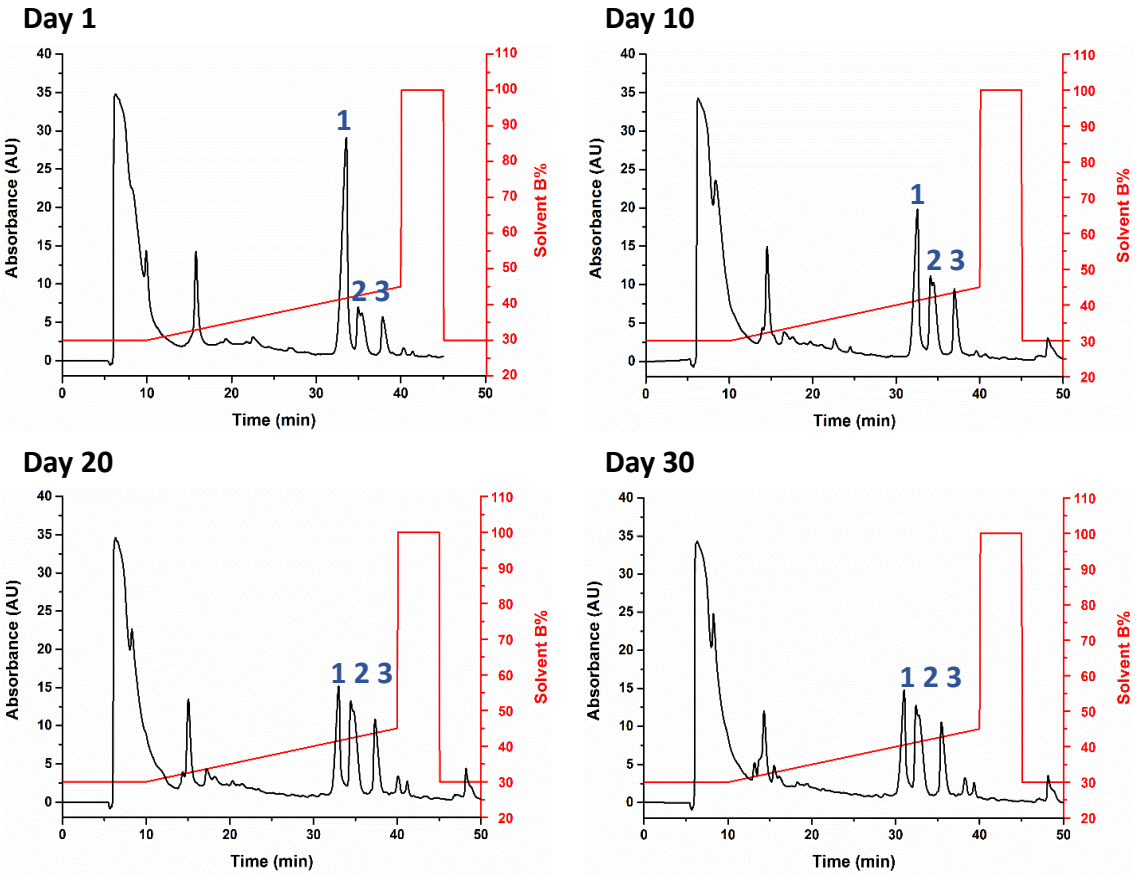


Figure 4.5. HPLC chromatogram showing an increase in AUC which indicates an increase in the yield of the conjugate after synthesis when stored at r.t. in 30 days after the synthesis. Each time an aliquot of conjugate (100 ul) was injected into RP-HPLC. Peptide-Dox conjugate elutes in two separate peaks (2 and 3) at t_R= 35 min, and t_R= 37 min. Both peaks were collected together in the same vial. MCC-Dox elutes in one peak (1) at t_R= 34 min.

4.3.3 Conjugate Preparation for the In Vivo Mice Experiments.

Design of PDCs involves selection of a proper targeting peptide, linker that allows sufficient circulation time and is mainly cleaved at the tumor site, and a highly toxic chemotherapeutic agent. The linker is an important component of the PDC and allows timely release of the drug after it is internalized. In this chapter my goal is to evaluate the efficacy of peptide-Dox conjugate in vivo using mice as cancer cell xenograft model.

The crude peptide-Dox conjugate was purified using RP-HPLC and was obtained in 81.4 % yield (98% purity). The liquid chromatography showed two separate peaks for the peptide-Dox conjugate with the first peak eluting one min after the peak for MCC-Dox. To preserve the purity of peptide-Dox conjugate eluent and avoiding coelution with MCC-Dox, every time only a small amount of crude conjugate (100 μL) was injected into HPLC. Therefore, the purification of peptide-Dox conjugate for the in vivo studies took a few months. To purify the required amount of peptide-Dox conjugate (522 μM, 10.5 mL) for animal studies, 189 HPLC runs were performed. The pure peptide-Dox conjugate was collected and dried using rotary vapor after each run and stored at -80 °C.

4.3.4 Solubility of the Conjugate at the Concentration Required for IV Injections

To solubilize the conjugate four different conditions were tried (**Figure 4.6**). In the first trial, conjugate powder was first completely dissolved in DMSO (1:9, v/v, 410 μL), and sterile normal saline (9:1, v/v, 3950 μL) was added to the solution later. However, the conjugate mixture precipitated immediately upon addition of normal saline. In the second trial, conjugate powder was first completely dissolved in DMSO (0.67:9.33, v/v, 117.5 μL), and sterile normal saline (1457.5μL) was added to the solution. Since, the conjugate mixture precipitated immediately upon addition of normal saline, absolute ethanol (1:9, v/v, 175 μL) was added dropwise to the conjugate mixture to improve solubility. However, addition of ethanol did not help the solubility of conjugate in aqueous solution. In the third trial, conjugate powder was first dissolved in DMSO (0.75:9.25, v/v, 270 μL) completely, followed by sterile PBS (pH 7.4) (1:7.2, v/v, 500 μL) was added to conjugate mixture, and at last sterile water (2830 μL) was added gradually. The third trial also failed, because the conjugate mixture precipitated after addition of sterile water to the mixture.

Ahmed F. *et al*. used 10% tween 20 to dilute their drug for in vivo studies, therefore, in the final trial, Tween 20 (0.625: 9.375, v/v, 240 μL) was gradually added to conjugate mixture in DMSO (0.7:9.3, v/v, 270 μL), and sterile normal saline (3330 μL) (**Figure 4.6 D**). Addition of tween 20 improved the conjugate's solubility in aqueous solution, and it was used for the injection of conjugate in in-vivo studies.[38] Although addition of tween 20 helped conjugate's solubility in aqueous solution, its presence in the solution for injection caused a few complications. Tween 20 increased the viscosity of the conjugate solution which in turn made it more difficult to inject the solution into the tail vein. Also, when the solution was stored in 4 °C for 7 days prior to injection, minor aggregation of conjugate was observed in the solution. To remove the aggregated particles, the mixture was passed through filter which in turn decreased the concentration of the solution due to adsorption of conjugate into the filter material.

Later, it was suggested to change the order of solvents and dissolve conjugate in DMSO followed by addition of tween 20 in second step and normal saline in third step could improve the solubility of the conjugate. The suggested formulation will be tried in our future in vivo studies.

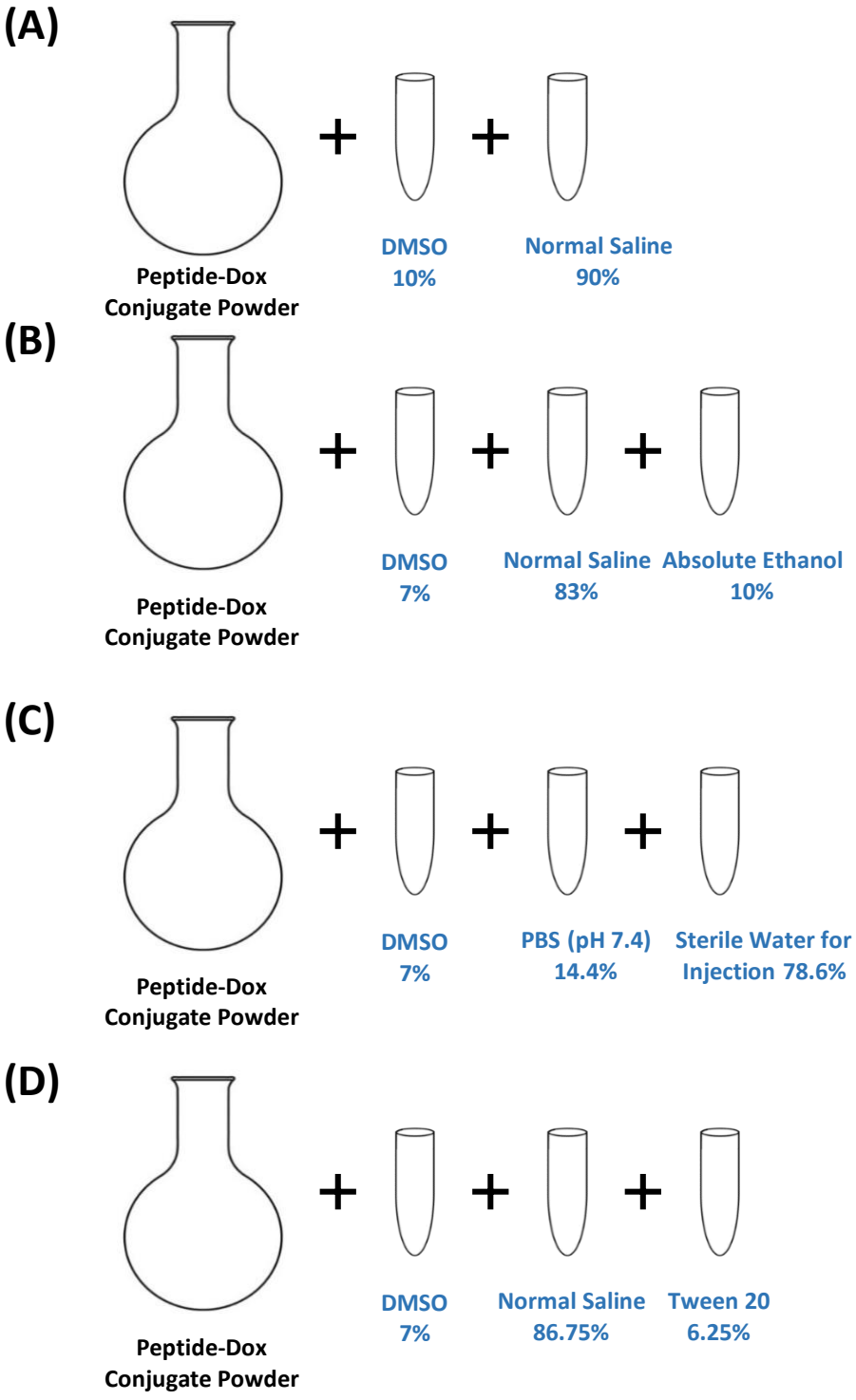


Figure 4.6. Schematic presentation of 4 different formulations to solubilize peptide-Dox conjugate in sterile normal saline. **(A)** In the first formulation, DMSO (10%) was added first to dried conjugate powder, then normal saline (93%) was added the conjugate solution in

DMSO. **(B)** In the second formulation, dried conjugate powder was first dissolved in DMSO (7%), and then normal saline (83%) was added, followed by addition of absolute ethanol (10 %) to the solution. **(C)** In the third formulation, after dissolving the conjugate powder in DMSO (7%), PBS (pH 7.4, 14.4%) was added, followed by addition of sterile water for injection (78.6%) to the solution. **(D)** In the last formulation, dried conjugate powder was first dissolved in DMSO (7%, 270 μL), and then normal saline (3.33 mL) was added, followed by addition of tween 20 (6.25 %, 240 μL) to the solution.

In the process of drug design and development, several issues of drug delivery, such as drug solubility, must be resolved.[63] In order to achieve a desired pharmacological effect in the body, it is important to obtain the required concentration of a drug in systemic circulation. [64] Hence, solubility of drug, dissolution of drug in solvent to obtain a homogenous solution, is an important parameter in drug delivery. [64] In formulation development of new drugs, low water solubility is the major challenge encountered.[64] Low aqueous solubility of a compound leads to many disadvantages including low absorption and bioavailability, poor solubility for IV dosing, poor patient compliance due to frequent high-dose administration, extension of development time and cost , and so forth. [64] To enhance the solubility of poorly soluble drugs many different techniques such as particle size reduction, salt formulation, use of surfactant, solid dispersion, crystal engineering, and complexation are used. [64] For instance, hydrophobic drugs such as the taxanes must be formulated in hydrophilic matrix such as castor oil-derived solvent Cremophor EL (CrEL) or polysorbate-80 (P-80) to enhance their solubility and bioavailability.[63, 64] Ahmed F. *et al.* used 10% tween 80 to formulate their Paclitaxel (PTX) conjugate for in vivo studies.[38] Pi-Ping L. *et al.* developed a tumor targeting delivery system for PTX by PEGylated *O*-carboxymethyl-chitosan (CMC) nanoparticles.[65] And, Mostafa S. *et al.* physically encapsulated PTX into PEO-*b*-PCL or PEO-*b*-PBCL micelles. [66]

However, use of surfactants such as P-80 and CrEL could cause solvent-mediated complications such as hypersensitivity reactions which may cause adverse reactions when administered to patients, or forming micelles in aqueous solution which reduces availability of the drug to target cells.[63]

4.4 CONCLUSIONS

The solubility of peptide-Dox conjugate in aqueous solution was challenging due to high hydrophobicity of the conjugate. The cause of insolubility of the conjugate could be due to its molecular structure and reduced hydrogen bonding ability since the amino group of the sugar moiety of Dox was covalently bonded to the linker and not available to water molecules. Four different formulations were tried to solubilize the conjugate in aqueous solution (**Table 4.2**).

Table 4.2. Different formulations (1-4) to solubilize the conjugate in aqueous solution

Formulation #1	Conjugate in DMSO (10%) and normal saline (90%)
Formulation #2	Conjugate in DMSO (7%) and normal saline (83%) and absolute ethanol (10%)
Formulation #3	Conjugate in DMSO (7%) and PBS (14.4%) and sterile water for injection (78.6%)
Formulation #4	Conjugate in DMSO (7%) and normal saline (86.75%) and tween 20 (6.25%)

While conjugate dissolved in DMSO (7% or higher), it immediately precipitated upon addition of an aqueous solvent such as normal saline or PBS solution (pH 7.4) to the solution. And addition of an organic solvent such as absolute ethanol did not increase the solubility. Finally, when tween 20 (in fourth formulation) was added to the conjugate solution in DMSO (7%) and normal saline, the conjugate dissolved giving a viscous clear solution which will be used for future in vivo efficacy studies. The purpose here was to develop a formulation of conjugate solution for in vivo studies. The in vivo efficacy experiments with conjugate using MDA-MB-231 subcutaneous xenograft in NOD-SCID mice are planned for the future studies.

CHAPTER 5: Future Directions

Following the successful in vitro studies of thioether peptide-Dox conjugate, in vivo studies are currently in progress. Calculating the tumor volume and mice survival rate after conjugate treatment will enable us to evaluate the therapeutic efficacy of the conjugate in mice. In addition, the in vivo biodistribution experiments will help to identify the efficiency of conjugate in delivering the drug to tumor while sparing other normal tissues. These experiments could be carried out in MDA-MB-231 subcutaneous xenograft in NOD-SCID mice model, where peptide-Dox conjugate is injected in mice followed by determining the amount of fluorescence that is emitted from Dox in different organs. Subsequently, the ultimate goal of in vivo studies is to identify if peptide-Dox conjugate could be potentially tested in the clinical trials.

Furthermore, for further optimization of PDCs, other forms of peptide and drug can be used in the synthesis of a PDC and evaluated in vitro and in vivo. For instance, a cyclic analogue of peptide 18-4 that showed better uptake at tumor site in in vivo studies can be attached to Dox through MCC linker similarly.[26] This conjugate containing the cyclic peptide is believed to have better selectivity in targeting TNBC cells and more stability in vivo.[26]

Another approach that needs to be explored is using other chemotherapeutics with more potency than Dox in design of PDC.[10] Due to high toxicity, some cytotoxic drugs cannot be used alone as a treatment for cancer.[30] For instance, drug maytansine (DM) is so potent that induces mitotic arrest and apoptosis at sub-nanomolar concentrations.[67] However, incorporating a very potent cytotoxic drug in PDC or ADC not only improves the efficacy at lower concentrations, but also reduces toxicity and side effects of the drug. As previously

mentioned, Phillips L. *et al* synthesized an ADC using cytotoxic drug DM1 (maytansinoid) and a monoclonal antibody, trastuzumab (Herceptin), in order to determine efficacy and toxicity of the designed antibody-drug conjugates.[10] As a result of its superior pharmacokinetics and safety profile trastuzumab-MCC-DM1 was selected for clinical development and later approved by FDA in 2013.[10, 30]

In addition to the previously postulated future experiments, design and synthesis of ADC using K1 antibody as targeting ligand is highly encouraged. This could be approached by, initially, comparing K1 levels in all TNBC cell lines with that in normal tissue cells. This study is already in progress and a significant difference between K1 levels between two TNBC cell lines (MDA-MB-231 and MDA-MB468) and one normal breast cell line (MCF-12A) has been confirmed. However, further studies such as determining K1 levels in more cell lines (TNBC and normal) are required.

Such above mentioned studies will certainly improve the targeting ability of conjugates (PDC or ADC) in delivering the drug to tumor-site as well as lower the detrimental side effects of potent chemotherapeutics in order to develop safe and efficacious targeted anti-cancer therapies.

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