The Development of a Cancer-Targeting Peptide-Drug Conjugate for the Treatment of Melanoma

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The Development of a Cancer-Targeting
Peptide-Drug Conjugate for the Treatment of Melanoma

A Thesis by
Cassandra Dill

Chapman University
Irvine, CA
School of Pharmacy
Submitted in partial fulfillment of the requirements for the degree of
Master of Science in Pharmaceutical Sciences
January 2021

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Aftab Ahmed, Ph.D.
The thesis of Cassandra Dill is approved.

Kamaljit

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Aftab Ahmed, Ph.D.

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The Development of a Cancer-Targeting Peptide-Drug Conjugate for the Treatment of Melanoma

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The fruition of this journey would not have been possible without the support of many people.

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ABSTRACT

The Development of a Cancer-Targeting Peptide-Drug Conjugate
for the Treatment of Melanoma

by Cassandra Dill

Cancer is an ongoing global pandemic which has caused a dramatic shift in research priorities. One of the most aggressive and difficult to treat has invariably remained metastatic melanoma. Although encompassing only 4% of overall skin cancer diagnoses, chances for recovery were slim until recent revolutionary development of immunotherapy adding to its regimen spectrum. This is due to its resistance to many standard-of-care treatment methods, along with its relatively high-metastatic potential. Within the past decade, eight new targeted and immune checkpoint inhibitors have gained FDA approval. The median life survival has increased significantly from 9 months to over 2 years as a result of this concerted drug development effort; however, this rapid development of treatment technologies come with new severe adverse effects, as well as increased opportunity for toxicity and drug resistance. It is not uncommon for a treatment to switch before originally projected due to lack of patient therapeutic response to a typical cytotoxic drug. One novel method to avoid this effect is use of a peptide delivery system, essentially increasing its targeted delivery. In this thesis, a ligand, dubbed KK-11b (sequence: CVPWxEPAYQrFL), synthetically made to form a cell-specific, noncytotoxic 13-mer peptide residue, is conjugated to a linker, sulfo-SMCC, and chemotherapeutic drug doxorubicin. Together, these form a new family of targeted drug therapies: peptide-drug conjugates (PDCs). The development and characterization are discussed, its stability analyzed, and, finally, preliminary in vitro melanoma cell studies were conducted. Overall, KK-11b stability was constant, remaining present in serum-free cell media, water, and preliminary in vitro A375 melanoma cell studies. More analysis will be conducted to test the
cytotoxicity of KK-11b conjugate in alternate types of melanoma cells, as well as in the presence of human serum, before ultimately progressing to *in vivo* murine studies if results remain promising.
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<td>Acetonitrile</td>
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<tr>
<td>ADC</td>
<td>Antibody-drug conjugate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus-Calmette Guerin vaccine</td>
</tr>
<tr>
<td>BRAF</td>
<td>V-raf murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>CHCA</td>
<td>Cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell-penetrating peptide</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen-4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle’s medium</td>
</tr>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<tr>
<td>Dox</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HCTU</td>
<td>2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate</td>
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<td>ICI</td>
<td>Immune checkpoint inhibitors</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight spectrometry</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl blue tetrazolium bromide</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
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<td>Programmed cell death protein-1</td>
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<td>PDC</td>
<td>Peptide-drug conjugate</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole time-of-flight spectrometry</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high-pressure liquid chromatography</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SMCC</td>
<td>Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid-phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Trisopropylsilane</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>TVEC</td>
<td>Talimogene laherparepvec</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet radiation</td>
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CHAPTER I: INTRODUCTION

1.1 Development of Melanoma

**Etymology of Melanoma.** Melanoma is one of the top killers of both men and women aged 25-40, and numbers continue to rise annually. Of the various types, melanoma is by far the most severe. Although it only accounts for 4% of the total skin cancer diagnoses, it is the cause of about 80% of skin cancer-related deaths \[1\]. It is estimated at least one patient dies from melanoma-related causes every hour. Whether environmentally or genetically spawned, the prognoses have generally remained the same until this past decade with low median survival rates [five-year period after treatment] and poor response to treatments among the late-stage melanoma patients. However, the quality of care and prognosis have significantly increased with the development of innovative drug delivery strategies.

Skin, the body’s largest organ, acts as a natural yet crucial barrier between the outside world and its internal organs. Consisting of epidermis and dermis, most cells within these layers are involved in the regulation, sensation, or protection of the body. One such cell is the melanocyte. A dendritic cell, melanocytes make up only 8% of the lower epidermal layer. Unlike the plentiful squamous and basal cells in the epidermis, melanocytes are directly involved in the protection of deeper layers from harmful effects caused by UV radiation exposure. Because of this, they are arguably one of the most important cells in the epidermis.

Ultraviolet radiation (UVR) is the key etiologic agent to the development of melanoma. On the electromagnetic spectrum, UVR frequency ranges from 10 to 400 nm. Its wavelength is shorter than visible light, but longer than x-rays. Humanity’s greatest exposure to UVR is
inarguably from the sun. The subtypes of UVR are dependent on the wavelength’s energy level; the higher the energy, the greater the ionizing radiation $^{[2]}$. This radiation, meant to describe an agent’s ability to ionize an electron from a molecule, can turn deadly when the energy causes penetrating, lasting damage to cellular DNA.

This newly-reactive molecule, referred to as a reactive oxygen species (ROS), has the potential to build up thanks to UVR suppression of catalase, as well as up-regulation of nitric oxide synthase, both key factors in ROS production $^{[2]}$. When ROS buildup surpasses amount of antioxidant defenses, it can envelop skin cells in an oxidatively-stressed environment. The environment contributes to DNA susceptibility, exposing genetic material to a possible mutation. Along the skin’s surface, damage may appear as a suntan or sunburn; over a period of time, if the damage is severe, the affected area may provide the prime environment for a tumor to develop. Any heightened exposure to ultraviolet radiation- either via the sun or artificially- can greatly increase one’s chance of developing a long-lasting mutation with devastating effects.

Melanocytes are regularly exposed to potential stressors which may cause significant DNA damage internally; but unlike internal cells, melanocytes are specially formulated to resist absorption and intake of potentially harmful chemicals using the skin-darkening pigment melanin $^{[3]}$. Their highly hydrophobic cellular membranes, as well as compound-resistant mitochondria contribute to their abnormal resistance. Along with their resistance, melanocytes also boast a rapid turnover cycle of 30 days, which helps decrease the risk of prolonged exposure and damage caused by harmful stressors. Under normal conditions, skin regeneration can be perfectly orchestrated. However, there is a catch. The risk for genetic mutations and rapid tumor necrosis also rises due
to rapid cellular regeneration \[3\]. What’s meant to prevent tumor formation then leads to rapid tumor growth, along with a resistance to standard treatments.

Phenotypically, skin cancer begins as a mole. According to the American Cancer Society \[4\], a mole is a cluster of melanocytes believed to show as hyperpigmentation due to a hormonal or environmental shift. Most moles remain unproblematic; in fact, the moles one may have in adolescence are known to change shape and pigment as they grow older \[4\]. The characteristics to be aware of are a rapid change in shape or color, a sudden cluster of abnormally shaped moles, or persistent swelling, redness, and tenderness in an area around mole \[4\].

Melanoma can develop in two ways: noninvasive, which is confined to epidermis; or invasive, which penetrates from epidermis to the dermis \[2\]. Of these, invasive is characteristically harder to treat because of location. Left untreated, the tumor metastasizes through the bloodstream, requiring a viable systemic solution. When identified early enough, most melanoma can be eliminated with a wide excision surgery. This is the recommended first line of treatment for early stages of skin cancer \[4\]. Prognosis after surgery may also include a prescription for an anti-tumor topical cream imiquimod [brand name Zyclara] \[4\]. Biopsies are performed to confirm no metastasis, and patient relative survival rate is high.

However, this is not normally the case. Due to melanoma’s growth and metastasis potential, it leaves a narrow treatment window—about four to six weeks—of possible surgical remedy before the cancer progresses to an advanced stage \[5\]. By the time most lesions are detected, the cancer is multifocal and rapidly progressing, narrowing possibility for an effective treatment
window. A further nuisance to tackle with metastatic melanoma is the hereditary resistance of melanocytes against classic chemotherapies \[^5\]. The natural protection of skin cells against outside stressors also serves as an added complexity in malignant melanocytes. For this reason, treatment strategies utilizing small and large molecule complexes specifically targeting melanoma have expanded within the past decade \[^6\].

**General treatments for melanoma.** Advanced melanoma has evolved to include extensive and varied treatment regimens dependent on patient’s response and level of care required. Along with standard antineoplastic regimen, eight regulatory-approved treatment methods have been added as first- or second-line standard treatments just in the past decade \[^7\], including immune checkpoint inhibitors (ICIs), targeted therapies, and even a therapeutic vaccine. Thanks to these concerted efforts, treatment and prognosis of melanoma have significantly improved, with patient median survival rates increasing from less than nine months to its current level of greater than two years \[^6\].

Chemotherapeutics remained the reigning standard of care for many advanced malignancies for over the last thirty years. However, in melanoma, because of the drug resistance and multilocality of lesions, only two classic chemotherapies, dacarbazine and temozolomide, have been approved for melanoma therapy in conjunction with an immunotherapy to bolster a recipients' immune response during treatment. Dacarbazine is a purine analog which competitively inhibits DNA replication, serving to slow and eventually stop cancer growth \[^8\]. Temozolomide, an alkylating agent, effectively breaks down DNA and prevent replication in highly proliferative cells \[^8\]. For both treatments, response rates remain reported as 11% during clinical study \[^8\].
Immunotherapeutic options have greatly expanded over the last decade. The area was previously dominated by treatment with interleukin-2 (IL-2) therapeutic vaccine [9]. The vaccine bolsters a patient’s immune response by stimulating leukocyte production. The leukocytes would travel systemically through the blood to attack any affected melanocytes. A high and consistent dose of vaccine was required to produce a response. It produced modestly positive response rates in clinical studies but is still an important alternative standard-of-care. The scope has since expanded to include Bacille Calmette-Guerin (BCG) vaccine, the prevalent treatment for tuberculosis; talimogene laherparepvec (TVEC) vaccine, the first oncolytic virus approved for metastatic melanoma treatment; and gp100:209-217(210M) peptide vaccine, which has recently shown to be equal or better to IL-2 vaccine response [9].

Treatment has since transitioned from only cytokine-dependent treatments to now include antibody-mediated immune checkpoint inhibitors (ICIs). These have been found crucial for moderating immune responses. Instead of relying on simple overstimulation, these regulate immune cells. ICIs bind to checkpoints which otherwise compete with immune cells for cancer detection, allowing those cells to present a highly-concentrated attack against the cancer. Currently, two are approved for first- or second-line of care. Combination regimen has been found to produce a greater response and higher median survival rate.

Ipilimumab was approved as second-line treatment for advanced melanomas in 2011 [7]. It is a fully human monoclonal IgG1 ant-CTLA-4 (cytotoxic T-lymphocyte antigen-4) antibody [7]. As of 2020, the FDA has only approved this treatment for second line use along with a standard chemotherapeutic. Ipilimumab binds to two ligands key to activating the CTLA-4 pathway. Its
Activation inhibits IL-2 secretion; when bound, the antibody indirectly stimulates leukocyte production \[^6\]. Effective treatment regimen can lead to long-lasting anti-tumor effects in approximately 20% of patients \[^6\].

Nivolumab and pembrolizumab are new additions to ICIs. These target another pathway, programmed death-1/ligand-1 (PD-1/L-1) \[^6\]. This pathway prevents T cell proliferation and propagation of immune response. The combination of anti-PD-L1/PD-1 and anti-CTLA-4 antibodies has produced positive overall median survival rates in recent clinical studies \[^7\]. These drugs have evolved to first line of treatment if no BRAF genetic mutation is identified through biopsy. Furthermore, combination of CTLA-4 and PD-1 inhibitors has showed significantly improved outcome compared to monotherapies, especially in PD-1 negative tumors \[^6\].

Targeted therapies offer an alternative solution to melanoma resistance by activating or inhibiting tumor-promoting pathways. It has been estimated that genetic mutation of certain pathways - including BRAF, MEK, and CDK2NA - account for 40-50% of melanoma diagnoses \[^1\]. Mutation leads to a constitutively activated pathway, which can directly affect the tumor growth rate, as well as indirectly affect the patient’s immune response.

Targeted therapy is unique in that it targets specifically to melanoma cells carrying the certain mutation, while avoiding a constituent of normal cells. Instead of all cells receiving treatment, as in chemotherapy, this targeted effect aims for cells which continuously operate growth pathways. This limits uptake in normal cells while ensuring a large enough dose is administered to affected cells. No standard of care is yet established, but immunotherapies are
generally used first to limit immune response along with promoting cellular uptake before administering targeted treatment \[^7\].

Two current options are BRAF inhibitors dabrafenib and vemurafenib. BRAF, v-raf murine sarcoma viral oncogene homolog B1, is a serine/threonine protein kinase which plays a crucial role in the MAPK, or mitogen-activated protein kinase, cell signaling pathway \[^{10}\]. MAPK is involved in mitigation of gene expression, cell growth and overall survival \[^{10}\]. An overactive pathway can lead to increased tumor division and growth, as well as altered gene expression and cell secretion. A similar protein to target is the MEK protein, a downstream signal of BRAF in the MAPK pathway \[^{10}\]. Current approved MEK inhibitors, trametinib and cobimetinib, demonstrated a superior monotherapy response than BRAF inhibitors alone, along with less severe adverse events with long term use. Therefore, these are now used in combination therapies for BRAF-positive mutations in melanoma \[^7\].

**Limitations of current treatment methods.** As with all drugs, every therapy has its limitations. There is no perfect treatment pathway, but one needs to weight the relative risk-benefit when considering which regimen(s) to undergo. Melanoma is an aggressive cancer to tackle due to its resistance and rapid regeneration; therefore, the available treatment therapies have historically been equally aggressive. This can wreak varied levels of havoc on one’s body. Chemotherapies are by far the most severe, as a higher dose is usually required to produce any positive response. Side effects include nausea, vomiting, constipation, headache, fatigue, and hair loss \[^8\]. The higher drug dosage can also lead to more severe long-term complications such as cardiotoxicity or neurological degeneration. Along with these, melanoma is also immanently resistant to classic
chemotherapy, so less drug may interact with tumor DNA versus what may be required to kill the cell. It would only slow, instead of stop, tumor growth.

Immunotherapies also come with their own limitations. Immune-related toxicity is associated with higher doses \cite{7}. Patients are also privy to develop immune resistance, limiting treatment length and drug efficacy. 20-27\% patients report initial increase in tumor burden or new tumor lesions before immune response can be tracked \cite{7}.

And finally, targeted therapies are revolutionary, but difficult to formulate \cite{10}. One must understand the structure and function of the target on a comprehensive level in order to build an effectively targeted drug. An additional hurdle for targeted therapies is melanoma resistance. Over time, the targeted pathway will lose its affinity to interact well with the drug; or, more common, the tumor will switch to another pathway that does not interact with the drug. Therefore, combination regimens are recommended; but too much exposure can cause thrombocytopenia, skin rash or depigmentation, or gastrointestinal problems \cite{10}.

1.2 Doxorubicin

Originally developed from the bacteria \textit{Streptomyces peucetius}, doxorubicin has been listed as an essential medicine since 1974 for its antitumor activity \cite{11}. Doxorubicin (Dox) is a current chemotherapeutic option for a plethora of cancers; however, it is not currently recommended for use in melanoma. A brilliant red powder, doxorubicin absorbance peaks at 495 nm \([\varepsilon = 10410 \text{ cm} \cdot \text{L}]\). Dox is cell cycle nonspecific, so can work its way into a cancer cell at any point. There are two binding sites for linking Dox, as indicated in Figure 1-1.
Its two-fold method of action is well-documented and studied. When exposed to cancer cells it’s highly effective. First, doxorubicin intercalates into DNA, causing irreversible DNA damage to the cell \[^{11}\]. This effectively breaks DNA linkage in cell nuclei. Second, doxorubicin prevents a pathway necessary for cell division and growth \[^{11}\]. When Dox blocks topoisomerase II enzyme, this leads to elevated oxidative stress and DNA damage, finally causing irreversible mitochondrial damage.

Doxorubicin is not currently approved for melanoma treatment due to its high resistance and lack of efficacy to treatment. The increasing non-targeted dose regimen necessary for cytotoxic efficacy has shown dose-dependent cardiotoxicity which can cause severe heart failure later in life. Lifetime limit dosage is less than 550 mg/m\(^2\) to reduce this risk \[^{12}\]. To combat this dose restriction along with receiving an effective dosage, combination treatments have already been developed for Dox with biomolecules. However, due to the limitations listed above (section 1.1) we must weigh the treatment benefit and potential risk and long-term toxicities very carefully when using Dox for chemotherapy.

**Figure 1-1.** Structure of Doxorubicin. The hydroxyl and amine groups (indicated by arrows) allow conjugation of ligands for its targeting to cancer site.
1.3 Conjugates for Drug Targeting

In order to combat limitations, a new line of targeted drug therapies has emerged. This area focuses not on creation of new cytotoxic agents, but rather re-purposing them to act as a targeted anticancer tool. These can be useful as science shifts towards a personalized therapeutic approach; instead of a new drug to treat each affliction one can repurpose a drug with new standards after altering its design. One such design is a conjugate. Scientists found if they take the strengths of a therapy and combine it with another therapy, they can build a targeted, less-limiting molecule. This three-component molecule is generally made of a ligand, drug, and a biocompatible linker to conjugate the two main parts \[13\]. The method of action is straightforward and moderately simple to replicate once the conjugate system is designed. The delivery ligand binds to a specified receptor believed to be overexpressed in the tumor cell. The entire complex is believed to be ingested by the cell via endocytosis \[14\]. Once inside the cell, the drug, attached to the ligand via a chemical linker, is released via breakdown proteins in the cytoplasm. It is now free to follow its original function and attack the tumor cell.

The conjugates to be discussed combine a biomolecule with a chemotherapeutic drug \[13\]. This biomolecule may exhibit cytotoxicity, as in antibodies; or, is fashioned to be no more than a delivery ligand for the conjugated drug, such as a peptide. Both methods look to limit the risk associated with treatment, as well as increase overall efficacy of the drug.

**Antibody-drug Conjugates.** Antibody-drug conjugates (ADCs) are carriers designed to deliver drugs directly to a tumor. These highly specific ligands boast a few advantages to current delivery methods, including cytotoxicity and delivery of a higher load \[13\]. Because it is an antibody, its
biocompatibility is high; so, the ADC is believed to internalize into cancer cells easier than other drug delivery systems. However, there are also disadvantages. Because of their large size, large-scale manufacturing is difficult and highly regulated. Antibody design is complex and requires specified chemistry during synthesis, and there is less control over the biomolecule-to-drug ratio. Lastly, an ADC is large and prone to higher number of inaccuracies during synthesis and purification steps [14]. The scope is growing, as a couple have recently been clinically-approved—although none have yet been approved for melanoma.

An example of an ADC is monoclonal antibody (mAb) -425, conjugated to doxorubicin directed against epidermal growth factor receptor (EGFR) [13]. The immunoreactivity was conserved, with an average of six to eight Dox molecules per mAb molecule. mAb-425 targeted metastatic melanoma cells in in vitro studies. In addition, preclinical therapeutic efficacy studies found immunosuppression and prolonged lifespan in mice with its use [13]. An equivalent dose of free Dox was found to be ineffective and had no effect in vivo.

**Peptide-drug Conjugates.** Peptide-drug conjugates (PDCs) combine a noncytotoxic peptide chain with a cytotoxic agent using some chemical linker. This agent can be a classic chemotherapeutic, pro-apoptotic peptide, or oligonucleotides [14]. A well-designed PDC is one which the peptide displays high affinity for the overexpressed target, the drug has a high potency and known mechanism of action, and the linker easily cleaved to release the free unmodified drug at the tumor site. Via this method, a concentrated amount of drug can be delivered while bypassing anti-target effects usually observed from an equal concentration of free drug alone, thus increasing the therapeutic window.
Advantages of using a PDC as a delivery system include their availability and versatility, with the cost-per-conjugate maintaining a trivial amount in comparison to similar, but more complex molecular designs. With the proper amino acid sequence, potential targets are limitless, as well as affinity to each can be specified during design. Due to their miniscule size, there is greater window for the PDC to enter through a channel in cell membrane, instead of reliance on a specific receptor to bind. However, there are also disadvantages. Metabolically, these conjugates have been known to remain relatively instable with a short circulating half-life, causing more doses to be administered. Their charged functional groups and low lipophilicity limit their bioavailability if passing through the GI tract; therefore, parenteral route is the most common method of administration. And lastly, they do maintain a rapid renal clearance, causing sensitivity to treatment over time.

One such PDC has already been designed, and its derivatives explored. P160, a 12-mer peptide, was discovered in 2007 using \textit{in vitro} phage display peptide library that was testing for activity against WAC2 neuroblastoma cells \cite{15}. Following this study, it was found p160 had high affinity and specificity to certain types of breast cancer cells. To determine the best peptide sequences for use against cancers, a synthetic peptide array was built containing 70 linear peptides. Quite a few peptides were revealed to have high affinity for various types of cancers, including melanoma; however, their stability \textit{in vitro} was low at first.
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<td>VPWXEPAYQRFL</td>
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<td>Soudy [17, 20], Ziaei [18]</td>
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<tr>
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<td>cy-WXEAAYQkFL</td>
<td>Raghuwanshi, Y</td>
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<td>X= norleucine; lower case = D-amino acids</td>
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</table>

*Figure 1-2.* Sequence of peptide p160 and its analogues.

When p160 peptide stability was analyzed, it was found to have low relative stability in media of only one hour. To combat this, the stability of p160 and each subsequent generation of peptide was optimized. As seen in *Figure 1-2*, methionine residue in original P160 phage display was replaced with L-norleucine. The generations have since evolved, including replacing two residues with their unnatural D-amino acid counterpart, and even testing the relative stability of the equivalent cyclic peptide.

Once each peptide’s affinity and stability in various environments were characterized, a peptide-drug conjugate was created. For the drug component, researchers decided to use doxorubicin. Dox was already used to treat a plethora of cancers and had a well-known bilateral mechanism of action (section 1.2). To conjugate the drug with peptide, the molecule is joined via a succinimidyl thioether linkage. It contains a maleimide group which readily reacts with sulfhydryl groups.
The first of these conjugates successfully tested for drug delivery was peptide 18 (p18), a first generation p160 peptide derivative \[^{17}\]. Following the conjugate design described above, cytotoxicity studies comparing p18 to free Dox uptake were conducted. Researchers found p18 exhibited high selectivity, as uptake was 6 to 10 times higher in cancerous versus noncancerous cell lines \[^{17}\]. It was also found to be 40 times less toxic than free Dox when in a noncancerous cellular environment. Further optimization was conducted, changing two amino acid analogues with their unnatural counterparts to increase proteolytic linear stability \textit{in vitro}. This optimized peptide, called 18-4, was also recently studied.

Following a similar design model, cytotoxicity studies in triple negative breast cancer (TNBC) cells found 18-4 peptide-drug conjugate was equal or greater than toxicity of free Dox \[^{18}\]. Uptake in noncancerous cells was low, however remained high in various TNBC cell lines. This suggested 18-4 PDC had translational potential to treat TNBC. The conjugate is currently awaiting further \textit{in vivo} exploration to confirm its efficacy and toxicology in mice.

\subsection*{1.4 Rationale, Hypothesis and Objectives}

Distant metastases cause low median survival rates due to systemic circulation of the drug in order to reach its target. This forces the therapeutic dosage to increase, inadvertently causing adverse systemic effects. Currently, doxorubicin is not the drug of choice to treat melanoma due to the high dosage required to have any significant cytotoxic effect on the cancer. Doxorubicin, a cytotoxic anthracycline antibiotic, has been found to cause significant cardiovascular issues due to inherent resistance of melanoma DNA. On the other hand, its well-documented mechanism of
action makes it a viable choice for tumor treatment could it be delivered specifically to the cells in question.

Conjugation of doxorubicin to a ligand such as a peptide accounts for both factors. This can be accomplished with a synthetic peptide specifically engineered as a delivery ligand. Because of the peptide specificity, the dosage of doxorubicin necessary for achieving sufficient cytotoxic activity can be significantly decreased. This could limit, as a result, adverse effects- including cardio-related complications. Furthermore, peptides are not cytotoxic, meaning the amount of peptide-drug conjugate (PDC) administered has a wider therapeutic window for treatment. Peptide KK-11b will be linked to doxorubicin via sulfo-SMCC, a heterobifunctional cross-linker which has been used in clinically approved antibody-drug conjugates. It forms a linker via a maleimide group which reacts with sulfhydryls. The one-pot reaction requires no additional heat and occurs at pH 7.4, so is viable for in vivo study.

My **hypothesis** is that the conjugation of KK-11b peptide to doxorubicin will improve the therapeutic efficacy of the drug and reduce its cardiotoxic side effects. In order to decisively conclude if KK-11 peptide-drug conjugate was effective, the **objectives** of my thesis were to: synthesize and characterize the conjugate using mass spectrometry and reversed-phase liquid chromatography; evaluate the stability of conjugate in water as a potential short-term storage vehicle; evaluate the stability of conjugate in serum-free media; and, evaluate preliminary cytotoxicity studies of PDC in a melanoma cell line.
CHAPTER II: METHODOLOGY

2.1 Materials and Methods

Fmoc-Leucine-Wang resin (loading 0.69 mmol/g) and Fmoc-(D)-amino acids were purchased from Merck (Germany). All Fmoc-(L)-amino acids and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were obtained from NovaBiochem (California, USA). The protecting side chains on amino acids were as follows: pentafluorophenyl (Pbf) for Arginine; trityl (Trt) for Cysteine and Glutamine, respectively; tert-butoxy (tBu) for Glutamic Acid; tert-butyl (tBu) for Tyrosine; and t-butyloxycarbonyl (Boc) for Tryptophan. Doxorubicin-hydrochloride salt was obtained from LC Laboratories (Massachusetts, USA). Trifluoroacetic acid (TFA) was purchased from Honeywell (Germany). Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (Sulfo-SMCC) was obtained from Thermofisher Scientific (New York, USA). Acetonitrile was purchased from Avantor Performance Materials (Pennsylvania, USA). Triisopropylsilane (TIS) was purchased from Chem-Impex International Inc. (Illinois, USA). N,N-dimethylformamide (DMF) and diethyl ether were purchased from Sigma-Aldrich (Germany). N-methylmorpholine (NMM) and piperidine were purchased from Merck. Alpha-Cyano-4-hydroxycinnamic acid (CHCA) matrix was purchased from Fluka.

Equipment. Solid-phase peptide synthesis (SPPS) on Wang resin was performed using automated peptide Tribute synthesizer from Protein Technologies (Protein Technology Inc, Arizona, USA) with HCTU and NMM as coupling reagents. HPLC characterization and purification of peptide and conjugates was conducted using VydaC C18 analytical (4.6 cm x 25 cm, 5 µm) and semi-
preparative (1cm x 25 cm, 5 µm) columns on a reversed-phase (RP) HPLC system Prominence-can (Shimadzu, Kyoto, Japan). Mass spectra was recorded using an autoflex speed matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker, USA). Bruker Impact II Ultra-High-Resolution Q-TOF LC/MS (Bruker, USA) was used to characterize peptide and its conjugate at various absorption wavelengths. Q-TOF characterization was conducted using ACQUITY UPLC Peptide CSH C18 Column (2.1 mm x 150 mm, 1.7 µm, Waters, Ireland). NanoDrop (ThermoFisher Scientific, USA) was used to measure absorption spectrum of stock samples. Microplate absorbance spectrophotometer (BioRad, USA) was used to analyze the absorbance of treated cells after MTT assay. Pure water was maintained through the MilliPore Sigma Milli-Q Ultrapure water system (FisherScientific, USA).

2.2 Cell Culture

Human melanoma cell line A375 (CRL-1619) was obtained from American Type Culture Collection [23] (ATCC, Virginia, United States). Under normal conditions, A375 was cultured in Dulbecco modified Eagle’s medium (DMEM, high glucose, catalog no. 10-013-CV, Sigma Aldrich) supplemented with 10% fetal bovine serum. For experiments with serum-free condition, serum was not added to the culture media. All cells were maintained at 37 °C and 5% CO₂. Fetal bovine serum (FBS) and Hanks’ balanced salt solution (HBSS) were obtained from ThermoFisher Scientific (USA).
2.3 Solid Phase Synthesis of KK-11b Peptide

KK-11b peptide (NH₂-CVPWxEPAYQrFL-CONH₂) was synthesized using standard Fmoc SPPS via TRIBUTE automatic peptide synthesizer (Protein Technology Inc., Arizona, USA).

To synthesize the peptide using TRIBUTE the following procedure was used. Briefly: vessel containing Wang resin pre-loaded with Fmoc-Leu (Fmoc-Leu-Wang resin, loading 0.69 mmol/g, 0.1 mmol scale) was loaded into RV2 slot. From the Load Synthesis screen, position 1 was selected to load the first amino acid (Phe). Dissolve time- amount of time DMF mixes in the vial- was set to seven minutes. The first method selected was a swelling method, meant to swell the resin before synthesis. After each amino acid tube was loaded, the screen moved the position forward, and a method was selected. The method was dependent on if the amino acid was the first or second tube of double-coupled pairs. For the final method, Fmoc was removed from the last amino acid. Once all amino acids were loaded, Run Synthesis was selected from the main screen.

Following SPPS techniques, to start the synthesis first the resin was swelled in a solution of DMF. Second, the Fmoc-protecting group was cleaved during deprotection in a solution of 20% piperidine in DMF. Next, the peptide chain was then built upon the free leucine via the resin. To do so, coupling of each amino acid was done using solutions of Fmoc amino acid, NMM, HCTU and DMF. Once coupled, the amino acid was double coupled to ensure all free residues were bound. The resin was washed with DMF three times to eliminate any leftover residue before continuing to the deprotection step. These three steps- deprotection, coupling, double coupling- were repeated until the final amino acid was bound to the peptide chain.
The methods were used alternately to designate if the amino acid which followed was a new amino acid to the chain, or a double couple. All methods began with five-minute treatment with 20% piperidine in DMF for Fmoc removal, 30 seconds to wash piperidine from resin, two hours for amino acid conjugation, and another 30 seconds to wash with DMF three times. The final method ends with Fmoc removal and three sets of washes. All amino acids of the 13-mer peptide were double coupled, increasing the total cycles to 24 as the first amino acid Leu was already present on the resin. Synthesis took about three days once started.

Once complete, the peptide was cleaved from the resin using a 10-ml cocktail of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (90:5:5). The solution was rotated for two hours, then filtrated into cold diethyl ether (20 ml), followed by centrifugation for 5 minutes (4500 rpm). The supernatant was discarded, and precipitated peptide dissolved in acetonitrile and water for purification and characterization.

2.4 Purification and Characterization of KK-11b Peptide

The identity of KK11b peptide was confirmed using MALDI-TOF mass spectrometry. For MALDI-TOF mass analysis, a stock matrix of CHCA was prepared following this procedure. In an Eppendorf tube, 10 mg of MALDI TOF matrix CHCA was weighed and a mix of water/acetonitrile (1 ml, 1:1) was added. The sol was vortexed for a few minutes until most of the solid particles dissolved. To this, 1 µl of TFA was added to obtain a 1% TFA sol. The mix was stored in an amber bottle at 4°C and replaced every 8-10 weeks to avoid contaminants.
From the stock matrix solution, 2 µl of sol was collected in a clean Eppendorf tube. To this, 2 µl of peptide sol (dissolved in acetonitrile/water, 1:1) was added, then vortexed for at least thirty seconds. Finally, 2 µl from the mixed sample solution was placed on a specific sample slot on the MALDI TOF plate and was allowed to dry completely. The MALDI plate was inserted in the machine, and a low-intensity (LC) method was loaded for low-molecular mass peptides (500-3500 Da). This sample preparation method was followed for all MALDI-TOF analyses conducted.

To purify the peptide, RP-HPLC running with a gradient of 30-65%ACN containing 0.05% TFA with 1 ml/min flow rate and 110 min run time (t_R = 51 min) was used. Peptide was injected several times to purify all the crude peptide solution. Appropriate fractions with the peptide peak were collected, dried and stored at -20 °C. The purity of peptide was determined using area under the curve from RP-HPLC chromatogram.

2.5. Synthesis of the precursor MCC-Dox

Sulfo-SMCC in excess with doxorubicin was used to produce the necessary precursor MCC-Dox needed for the synthesis of the conjugate. Previous method was improved by reducing the molar ratio of sulfo-SMCC crosslinker to drug in sample from 3:1 to 1.5:1. This method was evolved to not only make the reaction more cost-effective but was found to give better yield than the prior method. Using the improved method, MCC-Dox no longer needed purification before reacting with purified KK-11b (see section 2.6).

Sulfo-SMCC (5 mg, 11.5 µmol) was dissolved in a solution of DMF/water (1 ml, 1:1) and PBS (200 µl, 100 mM, pH 7.6). Doxorubicin-HCl (4.3 mg, 7.7 µmol) dissolved in DMF/water
(1.2 ml, 1:1) was added. The reaction mixture was stirred at 28 °C for 3 h (Figure 2-1). MALDI analysis was conducted at 0 h, 1, 2, and 3 hours to confirm all doxorubicin was used to make conjugate. An aliquot of the newly formed MCC-DOX was characterized using RP-HPLC at 495 nm with a gradient ACN-H₂O (30-65% containing 0.05% TFA, 2 ml/min flow rate, 55 min run time, tᵣ = 20.01 min). It was further characterized using MALDI-TOF mass analysis. Calculated [M+Na]+₁ was 785.1 Da; found [M+Na]+₁ was 785.3 Da. The crude product was stored at -20°C until further use.

![Diagram](MCC-DOX)

**Figure 2-1.** MCC-Dox synthesis reaction.

### 2.6 Synthesis and Characterization of Peptide-Doxorubicin Conjugate

1 eq. of 5.55 mmol peptide dissolved in 1 ml DMF was reacted with excess 10 equivalents of 630 mmol crude MCC-Dox (1.5:1, MCC:Dox) also dissolved in 1 ml DMF (Figure 2-2). Reaction conditions were kept constant at 32°C and stirred for 4 hours.
MALDI-TOF was used to confirm the presence of the conjugate. Expected [M+H]$^+$ for the conjugate was 2384.1 Da; found mass values were three major peaks corresponding to MCC-DOX ([M+Na]$^+$= 785.2 Da), peptide-MCC ([M+H]$^+$= 1987.8 Da), and peptide-drug conjugate [M+H]$^+$ 2384.2 Da.

PDC was also confirmed with Bruker Q-TOF LC/MS. 10 µl of PDC dissolved in water/acetonitrile with 1% formic acid was injected into Q-TOF using ACQUITY UPLC peptide column. Elution with a gradient of ACN-H$_2$O (10-70% containing 0.1% formic acid, 0.150 ml/min flow rate) showed conjugate at $t_R = 13.01$ min and 56% ACN, $\lambda = 495$ nm. [M+H]$^+$ was 2384.1 Da (1192.5 Da [M] • 2 = 2384.1 Da [calc. M]).

2.7 Stability Studies

Stability under aqueous conditions at 4°C. To determine the stability for long-term storage of conjugate solution, 7.5 µl of 993 µM pure PDC was suspended in 142.5 µl water (Final conjugate concentration = 49.95 µM). Approximate pH of MQ water is reported as 6.998. Sample vial was
stored at 4°C and analyzed at time 0, 24, 48, 72, and 120 hours. Aliquots (10 µl) of sample were injected into Bruker Q-TOF LC/MS via ACQUITY UPLC peptide column and the peak for the conjugate was monitored using the LC chromatogram at wavelengths 220, 280, and 495 nm and mass analysis ([M]= 1192.5 Da). The gradient used for the elution was ACN-H₂O (10-70% containing 0.1% formic acid, 0.150 ml/min flow rate, t<sub>R</sub> = 13.01 min and 56% ACN). AUC was obtained to determine percent intact conjugate at each time point. This experiment was repeated three times and average with SD were reported. Additionally, a paired t-test statistical analysis was performed to compare the relative validity of the raw data.

**Stability in serum-free cell culture media at 37°C.** To study stability in media, 50 µl of 561 µM pure conjugate was suspended in 550 µl serum-free DMEM (Final concentration = 46.8 µM). Serum-free media (high-glucose) was used to determine the best outcome. Approximate pH of serum-free DMEM is reported as 7.4. Sample vial was incubated at 37°C for 24 h. Aliquots (20 µl) of sample at time 0, 6, 10, and 24 hours were injected into the Shimadzu RP-HPLC via Vydac analytical column using a gradient elution of ACN-H₂O (30-65% containing 0.05% TFA, 45 min run time, 0.75 ml/min flow rate, t<sub>R</sub> = 21 min, λ = 495 nm). AUC was obtained to determine percent intact conjugate at each time point. This experiment was repeated three times and average with SD were reported. Additionally, a paired t-test and ANOVA statistical analyses were performed to compare the relative validity of the raw data.

### 2.8 Preliminary MTT Cytotoxic Assay

First, A375 cells were grown in incubator over a period of three to five days to 90% confluence and confirmed with microscope. Under the biosafety hood, the cells were washed with
1.5 ml HBSS to remove serum, then 2 ml Trypsin. These were incubated for 5 min, and cell detachment was confirmed with microscope. 4 ml media was added to stop Trypsin, then decanted to a clean centrifuge tube. The cells were centrifuged at 1200 rpm, 25°C for five min. Supernatant was discarded in proper waste. 2 ml of serum-containing media (DMEM + 10% FBS) was added to cell pellet in the tube, then pipetted up and down 20 times. To count the cells, 10 µl Trypan Blue Dye was mixed with 10 µl of cell-media solution. 10 µl blue cell solution was placed in chamber of counting slide. Cells were counted using Bio Rad cell counter. Cells were suspended in DMEM and seeded at 5,000 cells per well.

From this point, the cells were incubated in the 96-well plate with DMEM + 10% FBS for 24 hours. After confirming cells had adhered to the walls of their respective cells, the serum-containing media was removed from each well. The media was then replaced with the same DMEM plain solution without FBS. Amounts of this serum-free media was placed with calculated amount of drug to make each respective concentration (0-25 µM). Total volume in each well was 200 µl and each concentration was done in triplicates. The plate was gently shaken for a few minutes to ensure drug was evenly dispersed within the media, then incubated at 37°C for 48 hours. The plate was completely wrapped in aluminum foil to avoid light exposure.

After 48 hours of treatment, 20 µl MTT solution (5 mg • ml⁻¹) was added to each well. The well plate was gently shaken for a few seconds, wrapped in foil and again incubated. After two hours, the culture medium was removed and 100 µl of MTT solubilization sol was added to each well. After gently shaking the plate for 5 min, absorbance was measured at 570 nm using the BioRad microplate reader.
CHAPTER III: RESULTS & DISCUSSION

3.1 Design of KK-11b Peptide-drug Conjugate

**Design of KK-11b Peptide.** KK11b (sequence CVPWxEPAYQrFL) is an engineered analogue of peptide P160 (sequence VPWMEPAYQrFL, Figure 3-1). As mentioned in Section 1.3, this parent peptide was discovered in 2007 using phage display [15]. After high throughput screening, P160 was found to have affinity to various types of cancer, but most definite to breast cancer. It was found that the new analogues where the sequence was altered, the affinity greatly improved. However, stability remained low when incubated with media and human serum. P160 peptide was undetectable within one hour; whereas the engineered KK-11 peptide (VPWxEPAYQrFL) was relatively more stable and lasted for five hours in human serum. In order to increase stability, two L-amino acids in the sequence were traded for their unnatural D configuration. Peptide KK-11 was discovered previously in Kaur lab (unpublished results) by screening a library of peptides for high uptake by malignant and primary melanoma cell lines at low concentration (1 µM).

![Chemical structure of peptide P160, sequence VPWMEPAYQrFL.](image)

**Figure 3-1.** Chemical structure of peptide P160, sequence VPWMEPAYQrFL.

Next, we plan to use this peptide for targeted delivery of doxorubicin to the cancer cells, specifically malignant melanocytes. A peptide KK-11 - drug conjugate was designed to increase
the therapeutic window of a standard chemotherapeutic drug while also increasing the efficacy of systemic therapy. By combining a drug such as doxorubicin with a highly specific peptide, its toxicity to normal cells is significantly reduced. Doxorubicin’s method of action is established as highly potent to cancer cells; but when treating melanoma, the dosage needed becomes significantly cardiotoxic.

In the designed peptide-drug conjugate, a maleimide linker, sulfo-SMCC was used to conjugate the peptide to doxorubicin. The amine group of doxorubicin is attached to the linker via an amide bond and the thiol (sulfhydryl) from N-terminal cysteine of the peptide is attached to the maleimide of linker sulfo-SMCC as shown in Figure 3-2 below. An additional cysteine group was added to the N-terminus of peptide KK11, and the resulting peptide is called peptide KK11b (CVPWxEPAYQrFL) which was used for peptide-doxorubicin conjugate formation.

Figure 3-2. Schematic of Conjugate formation. Doxorubicin reacts with sulfo-SMCC to form MCC-Dox conjugate (1). MCC-Dox reacts with KK-11b peptide in excess to make peptide-drug conjugate (2).
**Synthesis and characterization of peptide.** For the synthesis of KK-11b peptide, solid-phase peptide synthesis was used via Tribute automatic synthesizer. The completed peptide was then cleaved from resin and purified using reversed phase-HPLC, as described in Methods section 2.3. Purification was first done to collect two significant peaks at 1623 Da and 1634 Da. It was found the method used to purify crude peptide was too fast, preventing good separation of two different peptides. The improved method increased the run time and allowed for better separation of the peaks. This allowed the peptide to purify at 56% acetonitrile and the peptide eluted at 56 mins using a gradient of 10-80% acetonitrile/water for 110 mins (**Figure 3-3 A**). The pure peptide was optimized, and later characterized by HPLC in which it eluted at 20.01 mins as a single peptide in the chromatogram (**Figure 3-3 B**). The peptide was also characterized with MALDI-TOF mass spectrometry. As seen in **Figure 3-4**, the calculated [M+H]$^+$ was 1621.9 Da; found [M+H]$^+$ was 1621.69 Da.

**Figure 3-3.** (a) HPLC chromatogram of crude KK-11b peptide. Pure peptide eluted at 56% ACN ($t_R = 51$ min). (b) HPLC chromatogram of pure KK-11b peptide.
Figure 3-4. (a) MALDI-TOF mass spectrum of crude KK-11b peptide. Expected [M+H]$^+$ is 1621.9 Da, found [M+H]$^+$ is 1621.85 Da. (b) Pure KK-11b peptide. Used a low-molecular mass (LC) method for analysis via MALDI-TOF mass spectrometry. Expected [M+H]$^+$ is 1621.9 Da, found [M+H]$^+$ is 1621.7 Da

Synthesis and characterization of peptide-drug conjugate

Synthesis of MCC-Dox precursor. According to the suggested conditions of sulfo-SMCC, it works best under slight basic conditions, so pH was maintained at 7.4 using PBS. When reacted with doxorubicin, the solution turned a bright red color. The first attempt to make MCC-Dox involved using a molar ratio of 3:1, as well as heating the solution at 40°C for 3 h. This produced a small yield and very little product was returned.

When the amount of excess MCC:Dox was reduced to 1.5:1, the yield increased significantly to 91%. Furthermore, it was found the reaction occurred at room temperature (27°C) using this new ratio, and excessive heat was not necessary. The solution was believed to be more
stable, and therefore did not need to undergo purification- and significant sample loss- before reaction with the purified peptide. The RP-HPLC chromatogram for crude and HPLC purified MCC-Dox are shown in Figure 3-5. The compound was also characterized with MALDI-TOF mass spectrometry. As seen in Figure 3-6, the calculated [M+H]^+1 was 786.1 Da; found [M+H]^+1 was 785.3 Da. The [M+Na]^+1 peak at 806.4 was also observed.

**Figure 3-5.** (a) Crude MCC-Dox. Elution occurred at t_R = 39 min (61% ACN) (b) HPLC of pure MCC-Dox. Elution occurred at t_R = 17.45 min (54% ACN)
Figure 3-6. Pure MCC-Dox. Used a low-molecular mass (LC) method for analysis via MALDI-TOF mass spectrometry. Expected [M+H]$^{+1}$ is 786.1 Da, found [M+H]$^{+}$ is 785.3 Da.

The [M+Na]$^{+1}$ peak at 806.4 was also observed.

**Synthesis of Peptide-drug Conjugate.** The thiolated peptide 11b was reacted with the MCC-Dox to obtain the peptide-doxorubicin conjugate. At first, peptide KK11b was reacted at a significantly lower scale of peptide to MCC-Dox (1:100). However, this method found not much product formation. In order to increase the yield, the ratio was increased to 1:10, which significantly improved the amount of PDC produced with a yield of 72%. The RP-HPLC of the crude PDC is shown in Figure 3-7 A. The gradient used here was 30-65% ACN + 0.05% TFA, 55 min run time, 2 ml/min flow rate, $\lambda = 495$ nm for purification of the crude conjugate. The major peak eluting at time 20.01 minutes (55% ACN) was identified as the conjugate peak. The minor peaks eluting at
Figure 3-7. (a) RP-HPLC chromatogram of crude PDC. Used 30-65% ACN + 0.05% TFA to purify. $\lambda = 495$ nm. Elution occurred at 20 minutes (51% acetonitrile). (b) RP-HPLC chromatogram of pure PDC. Used 30-65% ACN + 0.05% TFA to purify. $\lambda = 495$ nm. Elution occurred at 20.01 minutes.
8.1 min and 13 min were identified as MCC-Dox and peptide-MCC, respectively. There were also other minor peaks at 19.0 and 19.5 min, which were most likely impure peptide fragments. The main peak of conjugate at 20.01 minutes was collected and characterized by RP-HPLC (Figure 3-7 B) and mass spectrometry (Figure 3-8). Interestingly, no dimer (disulfide) due to the oxidation of the thiolated peptide was observed.

The mass of PDC was confirmed not only using MALDI-TOF, but also with Q-TOF LC/MS. The MALDI-TOF showed several peaks for the pure conjugate most likely due to fragmentation of the conjugate by the laser used. Along with the conjugate, peaks for MCC-Dox, peptide, and MCC-peptide were also observed (Figure 3-8 A). Next, mass spectrometric analysis was attempted using Q-TOF to characterize the conjugate. Fortunately, the mass obtained using Q-TOF showed only one peak suggesting that the pure conjugate was present (Figure 3-8 B).

The conjugate was further characterized using UV spectroscopy. A UV spectrum of a solution of pure conjugate was obtained using Nanodrop. Several characteristic peaks were observed including a broad peak at ~ 490 nm (Figure 3-9). Peak at 490 nm was also used to calculate the concentration of stock solutions of the conjugate.
Figure 3-8. (a) MALDI-TOF MS of pure KK-11b peptide-drug conjugate. Expected [M+H]$^+$ is 2384.1 Da. Laser split the conjugate into 3 components plus the whole compound: MCC-Dox ([M+Na]$^+$ = 785.1 Da); KK-11 peptide ([M+H]$^+$ = 1623.1 Da); peptide-MCC ([M+H]$^+$ = 1988.2 Da); and whole conjugate, [M+H]$^+$ = 2384.1 Da. (b) Q-TOF MS of KK-11 peptide-drug conjugate. Expected [M+2] is 1192.1 Da.
3.2 Stability of the Conjugate

The stability of conjugate was evaluated in water (aqueous, pH 6.998) and media (pH 7.4) for long-term storage and *in vitro* cytotoxicity experimental conditions, respectively.

**Stability of the Conjugate in Aqueous Conditions.** The stability of conjugate was observed in aqueous conditions to understand the effects of long-term storage. A solution of PDC at a final concentration of 49.95 µM in water was kept at 4 °C for several days. A small aliquot was removed at regular intervals (0, 1, 3, and 5 days) and injected into Q-TOF LC/MS to monitor stability. The conjugate was found to be fairly stable in water as shown in **Figure 3-10**. At day 5, there was still 73.5 ± 14.1% intact conjugate present.

![Figure 3-9. UV spectrum of pure conjugate using Nanodrop.](image)
Figure 3-10. Aqueous stability of the PDC in water at 4 °C. Aliquots were removed at regular intervals and intact conjugate was determined using Q-TOF LC/MS. Peak heights of PDC elution ($t_R = 13.0$ min) at 220 nm and 495 nm are compared over a period of 5 days. Experiment was repeated three times and the values (% intact conjugate) are average ± standard deviation.

Additionally, statistical analysis was performed to test the null hypothesis that there was no significant difference between the intact conjugate over the five days. The t-test: paired two samples for means showed that the hypothesis was valid and there is no significant difference between the % intact conjugate over different days as the p values were found to be > 0.05.

**Stability of the Conjugate in Serum-free Media.** Before in vitro evaluation of the conjugate could begin, it was important to understand the behavior of PDC under conditions similar to what it would be exposed to during testing. Therefore, trials were conducted to expose levels of conjugate to serum-free media (DMEM, high glucose) for 24 h.
Figure 3-11. RP-HPLC chromatograms for the conjugate stability. Conjugate was incubated in serum-free media at 37 °C and aliquots at regular intervals were monitored using RP-HPLC. Peak heights (AUC) of PDC eluting at $t_R = 20.01$ min at 220 nm are compared over a period of 24 h. This figure shows one representative experiment out of three repeats.
A solution of PDC at a final concentration of 46.8 µM in media was kept at 37 °C for a day. A small aliquot was removed at regular intervals (0, 6, 10, and 24 hours) and injected into reversed-phase HPLC to monitor stability as shown in Figure 3-11. Under these conditions, the half-life of conjugate was estimated to be around 12 h. It was also observed that conjugate degradation began between 0 to 6 h, with a peak observed at 17 minutes after the initial conjugate run (time 0 h). At 6, 10 and 24 hours, there was 89.3 ± 7.4, 60.0 ± 6.3% and 7.2 ± 3.6% intact conjugate present, respectively (Figure 3-12). Although, the conjugate was degrading with time as observed by the decrease in the AUC peak for the conjugate, we did not observe any additional peaks in the RP-HPLC over time to identify any degradation products/fragments. Further investigation is needed to find the mechanism for the degradation of the conjugate in the presence of DMEM media.

Figure 3-12. Stability of the conjugate in serum-free media at 37 °C. Aliquots were removed at regular intervals and intact conjugate was determined using RP-HPLC (AUC for the peak at 20.01 minutes). Experiment was repeated three times and the values (% intact conjugate) are average ± SD.
Further, statistical analysis using t-test: paired two samples for means showed that there is no significant difference between the % intact conjugate at 0 and 6 hours. However, the % intact contact at 10 and 24 hours was significantly different from % intact conjugate at 0 hours (p < 0.05). This suggests that the conjugate concentration dropped significantly after 10 hours of incubation with media.

Additionally, ANOVA statistical analysis was performed to test the null hypothesis that there was no significant difference between the data generated from the three trials performed. The means of each trial were 59, 64.875, and 66.25, respectively. Degrees of freedom between groups was 2, and within the groups was 9, for a total of 11. The total sum of squares was 16002.556. The p-value was 0.967, and F-statistic value was 0.03361. Due to this p-value, it was determined the null hypothesis was valid, and the data was not significantly different. Therefore, the stability method used was found to be an accurate gauge of stability of conjugate in media.

3.3 Preliminary In Vitro Cytotoxicity Studies

In vitro study results in melanoma A375 cells were preliminary at best, and not yet repeated for confirmation. However, it was observed cell viability in free Doxorubicin was significantly decreased, compared to KK11b conjugate (Figure 3-13). The higher Dox dosage left fewer cells to metabolize the NADPH-dependent MTT solution, leaving the solution red. However, the higher the concentration of cells alive the greater the reduction of MTT, a yellow dye, to formazan, a blue product.
This may be due to the incubation time allotted for the cytotoxicity study. The initial stability study in media (section 3.2) only analyzed conjugate degradation over a period of 24 h; during this time, the conjugate’s half-life was concluded to peak somewhere between 12-14 h of incubation before declining at a fast rate. Indeed, at the 24 h mark only trace amount of PDC was detected. However, the protocol for this cytotoxicity study was designed for a 48-h incubation period. The incubation period may have been far too long for any detected oxidative stress that may have occurred earlier in the experiment. Furthermore, the concentrations of each sample were not verified once sample was suspended among the cells. Revisions to the protocol are necessary so the conjugate performance can be observed and characterized.

Figure 3-13. MTT cell toxicity assay results in A375 cells when incubated with different concentration of the conjugate (0-25 µM).
CHAPTER IV: CONCLUSION

This study resulted in the design and synthesis of a peptide-drug conjugate which is expected to increase the efficacy of doxorubicin for melanoma treatment. A synthetic protocol for the conjugate synthesis to produce large amount for future in vitro and in vivo studies was established here. In addition, the conjugate was found to be stable under aqueous conditions for storage over time. The stability in media was moderate, with a half-life of about 12 hours. The in vitro cytotoxicity studies are warranted to establish if the conjugate will be a promising candidate for the delivery of doxorubicin to melanoma.
CHAPTER V: FUTURE DIRECTIONS

The progression of this project was hindered due to timeline of campus restrictions put in place during the COVID-19 pandemic. This pause in my research required an adjustment in priorities that otherwise would have been addressed. Therefore, the first line of direction would be to complete stability studies so that the chemical behavior of KK-11b drug-conjugate in various environments can be understood. Stability studies in aqueous conditions and cell media were completed; however, human serum was not. This step is necessary to understand the character of the conjugate if pursuing in vivo studies. The half-life of the compound also needs to be determined so incubation time can be adjusted, if necessary.

Once characterized, the compound can move to comprehensive in vitro cytotoxicity studies. This would follow the format set in section 2.8 of Methods for MTT cytotoxicity assay, but with a few minor enhancements which would promote better continuity and understanding, such as significantly decreasing drug incubation time. Studies would continue using different melanoma cell lines (A375, SK-MEL-28, WM3211) to confirm the anti-tumor activity and stability of the conjugate in vitro. Conjugate efficacy will be analyzed using flow cytometry and compared to free doxorubicin as well as untreated cells.

Furthermore, it is still unknown which melanoma membrane receptor promotes binding to KK11b. It has been theorized keratin-1, a surface receptor protein expressed by both skin and breast cells, is a possible target of proliferation when in active distress. In prior studies using p160 peptide(s), keratin-1 was shown to bind to p160 and is dependent on expression levels in breast
cancer cells \cite{25}. In order to confirm specific binding of the protein, keratin-1 expression will be quantified on the surfaces of various melanoma cancer cell lines.

Lastly, provided that all previous experiments show sufficient results, the final study will analyze behavior of the conjugate \textit{in vivo}. Studies would be done with mice in order to observe efficacy and safety of the conjugate. \textit{In vivo} studies are imperative to provide a realistic narrative of how the peptide-drug conjugate may respond when in a human body. Utilizing mice allows for creation of a blueprint of the concentrations needed, as well as determines the therapeutic window in which the conjugate can safely and effectively operate.
REFERENCES


