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Tumor imaging using the pacifastin family of peptides

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2018

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Program Authorized to Offer Degree:

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Abstract

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Cancer treatment involves multiple treatment modalities from the more traditional surgery, chemotherapy, and radiation treatment approaches to relatively modern forays into immunotherapy and the use of molecularly targeted agents. Survival rates continue to increase for many cancers as new therapies are introduced. The use of monoclonal antibodies targeting cancer, for instance, has played a major role in increased survival rates for subtypes of breast cancer as well as certain leukemias and lymphomas. Monoclonal antibodies, however, have limitations and several groups have been exploring peptides as both cancer imaging agents and cancer therapeutics. These peptide agents offer theoretical and practical advantages over antibody-based therapeutics and are showing promise in numerous clinical trials across a range of indications.

In this work, I created a novel peptide capable of delivering an imaging agent, attempted to broaden its utility to deliver a chemotherapeutic, and sought to determine its mechanism of binding. I have shown that variants of LCMI-II, a naturally occurring serine protease inhibitor of the pacifastin family, targets multiple types of flank xenograft tumors in mice. We discovered that a modified version of the peptide, THP1, delivered the Alexa Fluor 647 fluorescent dye to tumors *in vivo*, which subsequently internalized into the cancer cells. The examination of closely related peptides revealed residues critical for tumor accumulation and enabled the engineering of a variant with improved tumor targeting. A THP1 conjugate carrying the microtubule inhibitor MMAE showed limited activity *in vitro* and no difference *in vivo* when compared to the vehicle control. While these studies reveal some of the obstacles to developing LCMI-II derived peptides for therapeutic delivery, they highlight the promise they show for cancer imaging. Additionally, this work sets the stage for future testing of next generation therapeutic peptides based on the pacifastin family.

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ACKNOWLEDGEMENTS

This thesis would not have been possible without support from many people:

I have been extremely fortunate to have an excellent advisor in Jim Olson. My previous research mentor suggested I meet with him during my first interview at the University of Washington and I knew from Day 1 that I wanted to work with someone with such passion for cancer research and patient care. He has been a caring mentor who encouraged me when I struggled during my Ph.D. (and my first 100-mile bike ride) and shared with me his zest for life and sheer enthusiasm for live music. I consider Jim not only a mentor but also a friend and look forward to a long-lasting friendship.

My lab mates in the Olson Lab have also been a tremendous source of support. In particular, I am indebted to Andrew Mhyre for sharing his extensive knowledge and experience with me. His guidance was critical in helping me develop as a scientist. I thank Eric Nealy for our many thought provoking discussions and our continuing friendship. I thank many other members of the lab including Zachary Crook, Emily Girard, Carrie Myers, Fiona Pakiam, Matt Biery, Andy Strand, Shelli Morris, Albe Chan, Midori Clarke, Mesfin Gewe, Madison Nakamoto, Colin Correnti, William Johnson, Gene Hopping, Willem de van der Schueren, Ryan Steele, and Greg Sevilla for your friendship, support, and the things we were able to accomplish together.

My sincerest thanks to my thesis committee members – Nephi Stella, Shao-En Ong, Marshall Horwitz, Doug Fowler, and Roland Strong – for their enduring advice, support, and encouragement.

Thank you also to my friends for the laughs we shared and your companionship over the years. I especially want to thank Siva Kasinathan, Michael Duyzend, Kurt Terrani, and Mark Scott for

being like brothers to me. I am grateful to my family as well for believing in me and inspiring me. I have appreciated every phone call and visit to Seattle and have felt very loved and supported throughout this process.

Finally, thank you to my incredible wife Kimberly who has been my editor, my champion, my confidant, and my best friend. She has been with me all these years in Seattle and has made them the best years of my life. I love you more than you will ever know.

DEDICATION

To my son.
(I can’t wait to meet you.)

Chapter 1. INTRODUCTION

Development of peptide-based cancer agents

There has been an expanding repertoire of peptides used in the development of novel cancer agents for imaging and therapeutic purposes. These peptide-based agents are being integrated with current standard of care (surgery, radiation chemotherapy, targeted agents, and immunotherapy) towards generating greater response rates and cures in currently incurable cancers (Monk et al., 2016). These peptides can act as signaling modifiers (both agonists and antagonists) or be utilized to deliver imaging or therapeutic agents to target cells (Barbieri et al., 2013; Kenny et al., 2008). These peptides can potentially address some of the limitations of current therapies and a large number of trials have been undertaken worldwide to gain approval for agents deemed safe and effective. This review article briefly summarizes what's been done, compares antibodies and peptides, and highlights the promising body of clinical trial data that is emerging.

Although our understanding of cancer has improved, so too has cancer's ability to evolve and continue to evade surgical, radiological, and therapeutic interventions. As the population of the United States ages, the incidence of cancer continues to rise and millions of people are diagnosed and treated each year: Approximately 2 in 5 people in the United States will be diagnosed with cancer during their lifetime, with 1.7 million people newly diagnosed in 2016. Cancer is the second most common cause of death in the United States and in 2016, 600,000 people died from cancer (NCI 2017). Cancer affects patients of all ages and is a heterogeneous disease consisting of over 100 types and numerous subtypes. The goals of cancer therapies are to specifically disrupt these hallmarks of cancer in order to prevent the growth and spread of cancer cells while

minimizing harm to the normal cells and tissues of patients. Currently, the basic pillars of cancer therapy involve surgery, radiation, chemotherapy, and molecularly targeted agents.

Immunotherapy: strengths and weaknesses

A modern approach to cancer therapy is seeking to harness the immune system to treat cancer. This approach has seen a lot of successes in recent years. Monoclonal antibody-drug conjugates (ADCs), for instance, offer a number of advantages over traditional chemotherapy. The first monoclonal antibody for the treatment of cancer was rituximab, approved in 1997 for the treatment of drug resistant B cell non-Hodgkin's lymphoma by targeting CD20 antibodies (McLaughlin et al., 1998). In addition to triggering immune-mediated cell death, antibodies have been used to deliver radiation therapy or cytotoxic drugs such as trastuzumab-DM1 (Verma et al., 2012). Additional immunological approaches have been the development of immune checkpoint inhibitors such as antibodies against PD-1 and PD-L1 that take the brakes off immune-mediated killing of cancer cells (Hodi et al., 2010; Robert et al., 2015). There have also been cellular approaches undertaken where chimeric antigen receptors (CAR) on T-cells targeting cancer specific receptors lead to the killing of cancer cells and tumor regression (Turtle et al., 2017).

There are significant drawbacks and challenges in the use of antibody therapies for cancer imaging and treatment despite the number of successes that have been seen in their use. One of these challenges has been heterogeneous distribution of antibodies throughout the tumor due to differences in vascular density and permeability (Jain and Baxter, 1988; Dreher et al., 2006). This perfusion-limited delivery is greater for antibodies as compared to peptides due to the relatively high molecular weight of antibodies. It has been shown with mathematical modeling

that while the size of IgG antibodies has the highest potential tumor uptake for molecules with diameters from 5nm to 50nm. However, molecules with a diameter smaller than 5nm have the potential for significantly higher tumor uptake than larger molecules (Wittrup et al., 2012). Another limitation in the ability of antibodies to access tumors has been termed the “binding site barrier” whereby high affinity binding of antibodies to proximal sites prevents diffusion further into the tumor (Fujimori et al., 1990; Juweid et al., 1992). Peptides have the potential for overcoming this barrier due to more rapid extravasation to binding sites without the need for high affinity binding that might impede compound penetration into the tumor (Adams et al., 2001).

One of the critical factors regulating the efficacy of treatments is the ratio of delivery to cancerous cells versus delivery to normal tissue. Delivery to normal tissue can cause unintended toxicity. Antibodies have a circulating half-life of about 20 days and this leads to greater exposure for normal tissue that can lead to increased toxicity (Brekke et al., 2003; Vriesendorp et al., 1996). This is most pronounced in the case of antibody radiotherapeutics due to the mechanism of action: The therapeutic radionucleides are able to damage the DNA of cells by merely being in proximity to them and not requiring uptake (Willins and Sgouros, 1995). This can also be seen in the case of antibody-drug conjugates where free drug can be released into circulation leading to uptake in normal tissues over the long half-life of the molecule (Patterson et al., 2014). In the case of smaller peptide therapeutics, that generally have a significantly shorter half-life than antibodies, cancerous tissues can rapidly take up the peptides during a single pass through the tumor vasculature and are then rapidly excreted from systemic circulation preventing toxic exposure for normal tissues (Wittrup et al., 2012).

A key challenge for antibody therapies is targeting highly expressed cell surface targets present on all the cells of a tumor but absent or minimally expressed on normal cells. One significant success in this regard for monoclonal antibodies is trastizumab targeting HER2/Neu in breast cancer (Slamon et al., 2001). However, in the vast majority of cancers, specific and effective cellular receptors remain to be identified. Even in the case of FDA-approved rituximab targeting CD20 in non-Hodgkin's lymphoma and chronic lymphocytic leukemia, the antibody eliminates the vast majority of a patient’s B cell lineage (Leandro et al., 2006). The expression of receptors in normal tissues can lead to on-target toxicities and limit the dose of antibody therapies. In the case of certain tumor targeting peptides, the identification of a specific cellular protein receptor has been found to be unnecessary for accumulation – specifically in the case of a malaria-derived HSPG binding protein and chlorotoxin (Salanti et al., 2015; Veiseh et al., 2007). In the case of chlorotoxin:Cy5.5, it is found to accumulate in a variety of cancer types via a mechanism that remains to be fully elucidated but may lead to effective delivery to all the cells of a tumor.

In addition to previously stated issues with tumor penetration and target identification, the fact that antibody therapeutics are made as biologics leads to heterogeneity in production that can lead to suboptimal drug generation and activity (Dennler et al., 2015). In the case of antibody conjugates, the most common route to synthesis is through biological generation followed by chemical conjugation. Conjugation is a random, stochastic process leading to a range in the number of drugs bound to each antibody as well as in the sites of that conjugation (Hamblett et al., 2004). Since it is known that at least for certain antibodies, there is an optimal number of drug conjugates, this means that there is a pool of sub-optimally conjugated antibodies. In the case of peptide therapeutics, many peptides are small enough to be chemically synthesized

(Sugahara et al., 2010). This allows more specific and uniform production such that each molecule is able to be made best suited for its intended activity.

Limitations of peptide-based approaches for cancer

While there are many theoretical advantages of peptide-based cancer therapies, it is important to note certain challenges in their creation and optimization. Perhaps the biggest challenge facing peptide therapeutics is their short half-life compared with antibodies due to renal elimination (Ohlson et al., 2000; Mathur et al., 2016). However, small molecules drugs are also below the glomerular filtration cutoff. Following extensive testing and medicinal chemistry, there are beginning to be physiochemical properties that can predict renal clearance of small molecules (Varma et al., 2009). In some situations, extended serum half-life is desirable to increase tissue exposure to drug action. Antibodies have extended half-life due to both their large size and Fc receptor-mediated recycling mechanisms while small-molecules tend to have extended half-lives due to tissue distribution or serum protein binding (Datta-Mannan et al., 2007). There are a number of ways to extend the half-life of peptides including albumin-binding tags, carrier proteins, pegylation, X-ten tags, or other methods (Harris and Chess, 2003; Podust et al., 2016; Werle and Bernkop-Schnurch, 2006). These work by either allowing peptide binding to proteins that are large enough not to be filtered by the kidney or by increasing the hydrodynamic radius of the peptide so that the peptides act as if they are large enough to be excluded from glomerular filtration. In other cases, this extended half-life is not desired and no modifications need to be made to the peptide. This might be the case with imaging agents where the majority of uptake into the tumor would occur with the first time through circulation and additional circulating time may lead to background signal in blood and other normal tissues.

The smaller size of peptides as compared with antibody therapeutics limits the number of conjugates that can be attached without disrupting the function of the peptide. In ADC's, the optimal number of drug conjugates was shown to be 4 per antibody in some cases while for peptide drugs it is most common to only see a single therapeutic molecule per peptide (Hamblett et al., 2004). This may lead to insufficient drug delivery to tumor cells leading to a lack of response. It is possible, through novel chemistries, to attach multiple drug moieties to a single site on a peptide; this could potentially aid in the delivery of multiple payloads and higher potency peptide therapeutics for cancer (Thomas et al., 2008).

One concern is antigenicity of peptide therapeutics and recognition by the immune system. This concern is greatest for peptide agents derived from random libraries, non-human organisms, or mutagenesis. Immunogenicity has previously been an issue with antibody therapeutics and has been partly addressed through the use of humanized and fully human antibodies that work better than earlier mouse or chimeric antibodies (Bruggemann et al., 1989). In the case of peptides for imaging rather than therapeutic purposes, this is less of a concern. Following a single-dosing of an agent for imaging purposes, the chance of developing problematic anti-drug antibodies is quite low (Groot et al., 2007). However, in the case of cancer therapeutics that may require multiple doses this can be an issue. In this case, targeting peptides derived from native human peptides can be utilized such as somatostatin and EGF derived agents described below. In structured peptides such as cyclic peptides, knottins or other disulfide-rich peptides serum-stability and protease resistance is seen to a greater extent as compared with linear peptides and may lead to less efficient presentation to the immune system (Howell et al., 2014).

It is a challenge for both antibodies and peptides to effectively penetrate tumor tissue and the intracellular environment. Theoretically, tumor accumulation can be greater for small peptides

than for antibodies due to the smaller size of the former leading to more rapid extravasation into the tumor parenchyma (Schmidt and Wittrup, 2009). In addition, certain peptides have a known ability to penetrate tumor tissues and can even facilitate the delivery of co-administered drugs (Sugahara et al., 2010). Others have been identified that are capable of crossing the blood-brain-barrier (Ang-2) or even penetrating into the intracellular environment (modified CTX, maurocalcein, imperatoxin) (Demeule et al., 2008; Boisseau et al., 2006; Ojeda et al., 2017).

Additionally, when compared with small molecules, there is a reduced potential for oral bioavailability of peptide therapies. However, there is data suggesting that certain peptides are able to cross into the bloodstream intact following oral intake especially when formulated appropriately such as parathyroid hormones (Gardner, 1988; Karsdal et al., 2014). Alternatively, in the case of cancers of the GI tract where there is little need for the peptide to cross into the bloodstream following oral intake, binding can occur to cancer specific receptors in the lumen of the GI tract allowing tumor imaging or therapeutic delivery to microscopic cancer lesions.

Another challenge with peptide therapeutics is the potential for renal accumulation. Peptides smaller than the glomerular filtration cutoff pass freely into the kidney nephron and can be taken up in the proximal tubule (Carone, 1979). This can potentially lead to toxicity if drugs are delivered to those cells. However, some peptides such as EETI 2.5F that are freely filtered have been found not to be taken up strongly into those cells (Moore et al., 2013). Additionally, drugs or conjugates that are active in tumor tissue may not be active or release in their active form in renal cells thereby preventing damage (Albright et al., 2005).

Choosing a receptor

A critical choice in the design and development of a peptide-based cancer agent is the choice of receptor or binding partner. If possible, starting with a known ligand-receptor interaction allows you proof-of-concept as to whether a receptor is a suitable target for delivery. This is seen with the somatostatin receptor and EGF receptor binding reagents – taking a native ligand or a part of a native ligand to use as a starting point (Gao et al., 2016; Barbieri et al., 2013). If you are beginning with the receptor rather than a receptor-ligand interaction, there are a few qualities to assess: 1) the relative expression of the receptor on cancer cells versus normal tissues. In the case of receptors like EGF receptor, there is significant expression in normal tissues but in certain cancers a very high level of expression can be utilized to design a therapeutic; 2) assess the normal sites of expression you can know better where to look for on-target toxicities; 3) the localization of the receptor (intracellular vs. extracellular, etc.) to confirm that it will be accessible to the peptide ligand (Maiti et al., 2013; Yano et al., 2003).

Additional factors need to be considered when designing a peptide-drug conjugate without utilizing a known ligand-receptor interaction. It is essential that all or a vast majority of the cancer cells express the receptor of interest. This is one of the reasons why antibody-drug conjugates have been especially successful for blood cancers because all of the target cells express the receptor of interest, and at a high level (Prevodnik et al., 2011). Additionally, it is important that receptor binding triggers internalization and proper intracellular trafficking if delivering a drug that needs to act within a specific cellular compartment (Bareford and Swaan, 2007). If receptor binding is retained on the cell surface or is internalized and sequestered in endosomes, it is still possible to design a peptide therapeutic but may require the use of a radionuclide rather than an intracellular chemotherapeutic (Gudkov et al., 2015). The

identification of a proper ligand and target may make it possible to administer a single agent or a pair of agents that act as theranostics able to both diagnose and treat the cancer. The true test of a therapy is whether responses are seen in human patients.

Overview of current clinical development

Peptide conjugates for imaging

A critical component of cancer surgery is visualization of the tumor before, during, and after surgery to assess feasibility, facilitate surgical removal, and confirm the extent of tissue resection. Imaging—such as MRI and PET scans—have been invaluable in visualizing the extent of tumors prior to surgery and evaluating the potential for a successful and complete resection. Antibodies have proven to be critical in identifying tumor cells in *ex vivo* tumor sections, but to date, solid tumor imaging has generally relied on small molecular probes such as gadolinium or ^{18}F-FDG (Duo et al., 2013). These probes allow better imaging of soft tissues such as tumors and tumor metabolic activity, respectively.

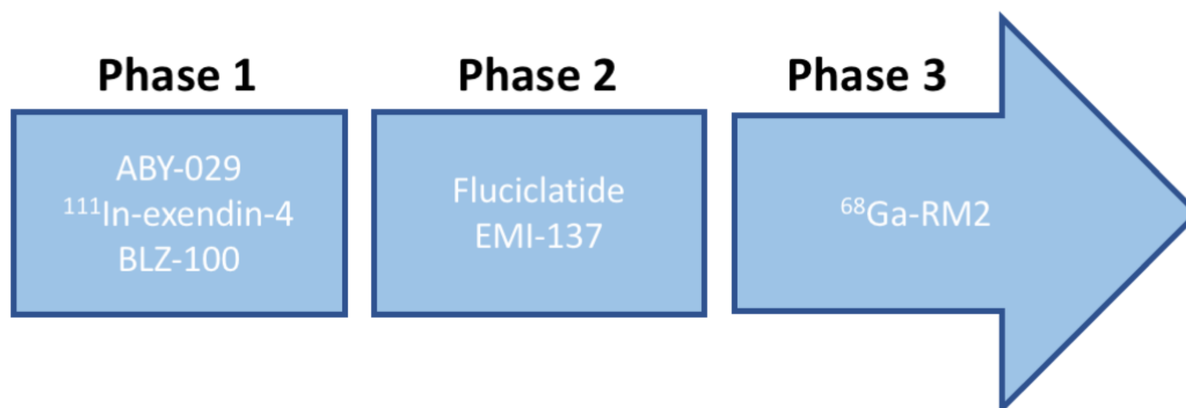


Figure 1.1: Peptides in clinical trials for tumor imaging

The limited use of antibody-based tumor imaging results from the challenge clinicians and researchers encountered when attempting to identify cancer-specific cell surface targets. Other challenges include the heterogeneity of target expression, increased interstitial pressure limiting solid tumor penetration, and other aspects of solid tumor physiology (Adams, 2012). Improvements in intra-operative tumor visualization have the potential for limiting the extent of healthy tissue resection leading to improved outcomes.

Dysregulated transcription of gene products is one of the identifying characteristics of cancers. Overexpressed cell-surface proteins such as receptor tyrosine kinases can be utilized as binding sites in order to deliver a payload to cancer cells. There is a lower bar for delivery of imaging agents to cancer cells as delivery to normal tissues can be tolerated with less concern about side effects such as with ^{18}F-FDG PET/CT and bladder accumulation (Bouchelouche et al., 2012). Bombesin receptors are GPCRs that exist in 3 forms, BB1/BB2/BB3, and are found to be aberrantly expressed in lung, colon, and prostate cancers (Moody et al., 1985; Reubi et al., 2002). A peptide derived from the native ligand bombesin, ^{68}Ga-RM2, has been tested for PET/MRI

imaging of recurrent prostate cancer (Minamimoto et al., 2016). ^{68}Ga-RM2 is currently recruiting for a Phase II/III trial for imaging biochemically recurrent prostate cancers (NCT02624518).

Receptors that have been exploited therapeutically by antibodies in some cases may be imaged better with a molecule with a shorter half-life due to a more rapid reduction in background signal. EGFR is a receptor tyrosine kinase that has been broadly studied and targeted in cancer with antibodies such as cetuximab (Cunningham et al., 2004). ABY-029 is a low-molecular weight affibody (a small peptide based on packed alpha helices with antibody-like affinities) that binds to the extracellular domain of EGFR and shown utility in imaging of EGFR(+) gliomas (Elliott et al., 2017). There are early Phase I trials testing the utility of targeting EGFR as a surgical aid using ABY-029 in sarcoma (NCT03154411), GBM (NCT02901925), and cancers of the head and neck (NCT03282461).

Integrins are a diverse family of cell adhesion molecules that support the proliferation and metastasis of tumor cells. Integrins have been an attractive cancer target due to peptide binding occurring through a simple RGD tripeptide motif (Ruoslahti, 1996). Through screening, low nanomolar ligands for cancer associated integrins such as $\alpha v\beta 3$ and $\alpha v\beta 5$ have been identified and used for imaging purposes (Indrevoll et al., 2006; Kimura et al., 2009). Fluciclatide is a phage-display derived peptide found to bind with high affinity to a number of cancer associated integrins (Kenny et al., 2008). Five Phase II trials evaluated the utility of fluciclatide for imaging a number of different cancer types but results have yet to be published.

GLP-1 is expressed in the pancreas, intestine and certain brain regions and is involved in blood glucose homeostasis (Molin 2016). While the vast majority of pancreatic cancers do not overexpress GLP-1R, there is a high-level of expression in benign insulinomas (Pattison and Hicks, 2017). Exenatide is the synthetic form of exendin-4 peptide in the saliva of the Gila

monster and acts as a GLP-1R agonist that is FDA approved for the treatment of type 2 diabetes (Drucker and Nauck, 2006). This has led to a Phase I trial investigating the use of ^{111}In-exendin-4 for the imaging of insulinomas (NCT00937079).

For certain well-established targets, peptides have the potential for greater stability and methods of administration that are not suitable for antibody therapies. One example is HER2/neu, a target for monoclonal antibody and ADC based cancer therapy. It is overexpressed in a significant fraction of breast and gastric cancers among others (Gravalos and Jimeno, 2008). In both breast and gastric cancers, HER2 expression is associated with poor patient outcomes. A completed Phase I trial evaluated the safety of an orally administered heptapeptide-dye conjugate targeting HER2/neu, KSP-910638G, to aid in the detection of GI malignancies (NCT03161418). Another peptide receptor target utilizing the increased stability of peptides in the GI tract as compared with antibodies is c-Met. c-Met is a receptor tyrosine kinase that is implicated in cancer angiogenesis. It has been found to be amplified in colon cancer and mediate resistance to EGFR inhibitors such as cetuximab (Bardelli et al., 2013). A cyclic peptide targeting c-Met conjugated to a fluorescent-dye, EMI-137, is being tested in a Phase II trial to aid in resection of colon adenocarcinoma (NCT03360461).

Chlorotoxin has been found to accumulate in a number of tumor types in preclinical models including brain tumors behind the blood-brain barrier. A number have targets have been proposed but the specific receptor remains to be conclusively identified (Dardevet et al., 2015). Additionally, a modified version of chlorotoxin conjugated to the dye ICG, tozuleristide, is being tested to aid in intraoperative cancer imaging in cases of pediatric and adult brain tumors (Fidel et al., 2015) (NCT02462629) (NCT02234297).

Unlabeled peptide therapeutics as signaling antagonists

A potential mechanism by which peptide therapeutics could be utilized in cancer is in the antagonism of signaling pathways that lead to cell growth, angiogenesis, and stabilization of a pro-tumor microenvironment. Antagonism is commonly used by other drug classes due to the greater ease by which signal transduction pathways can be turned off rather than enhanced. For peptides, this antagonism tends to result from binding to soluble factors such that they are unable to perform their physiologic function or via binding to receptors and blocking the ability of stimulatory molecules to bind to the receptor. In cancer, activation of Tie2 following binding to soluble angiopoietin results in tumor angiogenesis and can lead to cancer metastasis (Mazzieri et al., 2011). It has been proposed that antagonism of Tie2 can result in tumor suppression. A peptide-Fc fusion (peptibody) inhibitor of angiopoietin-1/-2 was generated through phage-display with sub-nM affinity that led to tumor regression (Oliner et al., 2004). This molecule, trebananib/AMG-386, has shown success in a Phase III trial (TRINOVA-1) for the treatment of recurrent epithelial ovarian cancer leading to increased progression-free survival and in a subgroup with ascites, increased overall survival (Monk et al., 2016). It has been tested in a number of cancer types including pediatric brain tumors where it saw some stable disease but no partial or complete response (Leary et al., 2017).

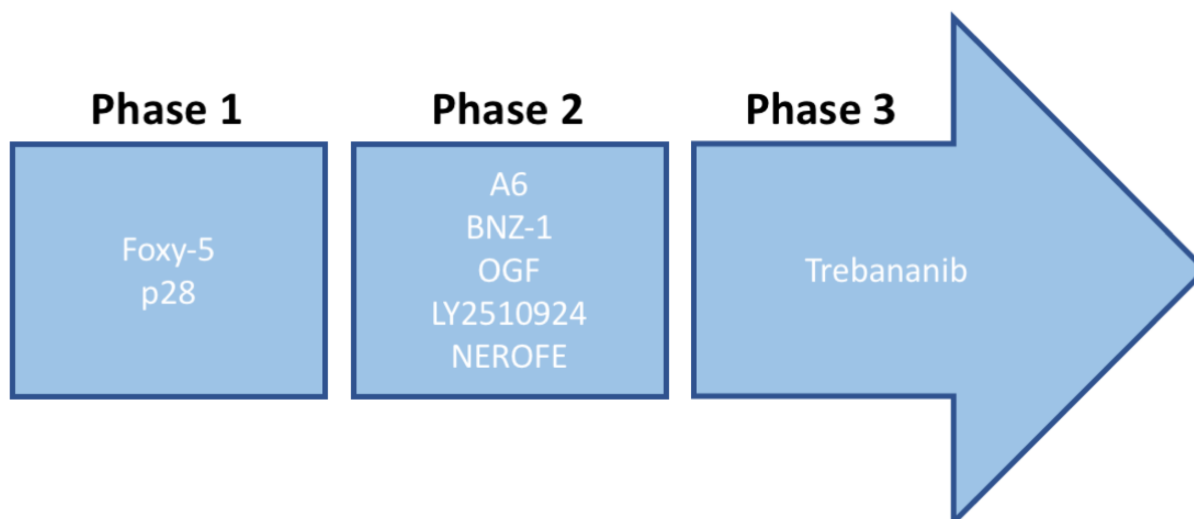


Figure 1.2: Unconjugated peptides in clinical trials as therapeutics

Another target for antagonists has been the cell surface protein CD44, which found in a range of human tissues and is known to interact with hyaluronic acid, MMPs, and other molecules. It is known to promote cell proliferation, invasion and survival in cancers such as lymphoma, where blocking antibodies have appeared to inhibit cell proliferation (Zahalka et al., 1995). It has been found that the peptide A6, an 8-amino acid peptide derived from urokinase plasminogen activator is capable of binding to CD44 and modifying signaling (Finlayson, 2015). A6 was tested in a Phase II trial for patients with chronic lymphocytic lymphoma but was terminated due to slow enrollment (NCT02046928).

The inflammatory microenvironment of a tumor has a significant effect on tumor cell growth and immune response to the cancer cells. T regulatory cells (T_{regs}) are important in establishing self-tolerance and preventing autoimmune disease. However, in cancer, these same T_{regs} lead to suppression of an immune response against cancer cells and prevent tumor eradication (Rochman et al., 2009). A rationally designed peptide-based cancer therapy, BNZ-1, capable of specifically

inhibiting IL-2, IL-9, and IL-15 was well tolerated in a Phase I trial in healthy adults and decreased the population of T_{regs} (Frohna et al., 2017). A Phase I-II trial is being run in leukemia and lymphoma (NCT03239392).

Unlabeled peptide therapeutics as signaling agonists

Peptide-based cancer therapies can mimic native ligands and exert their anti-cancer effects through known signaling pathways. A number of the peptides in clinical trials for cancer are derived from or mimic a physiologic ligand for a cancer cell receptor. Since many receptors natively bind peptide ligands in either physiologic conditions or disease states, the creation of a peptide-based therapy that signals through the target receptor can often be generated from sequences in the native ligand. Wnt signaling is aberrantly regulated in a wide range of cancers such as breast, colon, melanoma, and others (Klaus and Birchmeier, 2008). Wnt5a has polar opposite effects depending on the cancer type with excess signaling leading to migration of gastric cancer and melanoma, while in other cancers such as breast and colon Wnt5a signaling is inhibitory for metastasis. The Foxy-5 peptide, derived from Wnt5a, has been shown to inhibit breast cancer migration and metastasis *in vitro* and *in vivo* (Safholm et al., 2008). A Phase I trial with the Wnt5a-mimetic Foxy-5 for metastatic breast, colon, and prostate cancers has been completed (NCT02020291) with an additional Phase I trial currently recruiting (NCT02655952).

p53 is a known tumor suppressor and the most mutated gene in cancer (Hollstein et al., 1991). p53 triggers cell death via apoptosis when levels rise in response to stressors. Cancers with mutations or loss of p53 are often resistant to therapies as insults that would cause apoptosis are no longer able to do so. A 28-amino acid fragment of the bacterial and plant protein azurin, termed p28, was found to penetrate into cancer cells and inhibit ubiquitin-mediated degradation

of p53 leading to apoptosis (Yamada et al., 2009). P28 has undergone Phase I trials in refractory solid tumors and pediatric brain tumors and was well-tolerated (Lulla et al., 2016). In adult solid tumors, there were a number of patients with stable disease, partial response, and one with a complete response (Warso et al., 2013).

While Wnt and p53 signaling have been long recognized as cancer targets, other cancer relevant receptors have been identified more recently. Imidazoquiolines such as imiquimod exert some of their effects through the induction of opioid growth factor receptor (OGFr) and its signaling (Zagon et al., 2008). Activation OGFr signaling leads to G1 arrest via blockade of Rb phosphorylation. Interestingly, it has been seen that morphine, a ligand for OGFr, may suppress lung cancer proliferation in an OGFr-dependent manner (Kim et al., 2016). The use of OGF has been tested in Phase II trials for the treatment of pancreatic cancer (NCT00109941) and head and neck cancer (NCT00982696).

Similar to OGFr, other physiologic receptors can be co-opted in cancer. CXCR4 is a chemokine receptor best known as a receptor the HIV virus on T cells and the chemokine CXCL12/SDF-1. However, it has also been found to be expressed in a wide range of cancer types and to mediate cancer metastasis in some of those cancers (Sun et al., 2010). A novel cyclic peptide, LY2510924, that acts as a CXCR4 agonist was identified via screening and rational design (Peng et al., 2015). Two Phase II trials of LY2510924 have been completed – one in combination with sunitinib for renal cancer was terminated for lack of efficacy (NCT01391130). Another, for small cell lung cancer in combination with carboplatin and etoposide (NCT01439568) has been completed as well and results are pending.

The role of the immune system in cancer is continuing to become more appreciated. IL-33 is a cytokine expressed by a wide variety of cell types and acts via the interleukin 1 receptor-like 1,

also known as ST2. A ligand for ST2 was identified and termed Tumor-Cells Apoptosis Factor (TCApF), a peptide able to trigger caspase activation through ST2 signaling in proliferating AML cancer cells (Sandler et al., 2010). A 14-amino acid minipeptide derived from TCApF, called NEROFE, is being tested in a Phase II trial looking at the activity in acute myelogenous leukemia and myelodysplastic syndrome (NCT03059615).

Peptide-drug conjugates for therapeutic purposes

Cell-surface proteins that are uniquely overexpressed in cancer can be used to deliver therapeutic agents such as immunostimulatory molecules, radiotherapeutics, and chemotherapeutics. In these cases, it is critical that the receptor be highly expressed in the tumor cells as compared with normal tissue. Normal tissue that expresses the receptor need not be overly sensitive to the therapeutic agent and off-target delivery must be limited to tissues and levels that do not induce critical tissue damage. Annexin A2 has been studied as a marker of cancer metastasis and treatment resistance in a number of different cancer types (Lokman et al., 2011). The therapeutic targeting of Annexin A2 was evaluated for safety and efficacy using localized delivery for the treatment of recurrent glioma using a synthetic version of chlorotoxin TM-601 containing a ^{131}I (Mamelak et al., 2006). The single-dose was found to be safe in the 18 patients treated and at 18 months there were 4 patients with stable disease and 1 with a partial response.

The cholecystokinin receptors consist of 2 isoforms CCK1 and CCK2 which are expressed primarily in the GI tract and CNS, respectively. CCK2 is found to be overexpressed in a vast majority of medullary thyroid carcinoma (MTC) (Froberg et al., 2009). The pentagastrin-stimulated calcitonin test is used to aid in the diagnosis of MTC through CCK2 stimulated

release of calcitonin. A minigastrin analogue, ^{111}In-CP04, was developed for both diagnostic and treatment purposes utilizing the native ligand of the receptor as a starting point (Pawlak et al., 2016). This molecule is being tested in a Phase I trial as a theranostic for MTC (NCT03246659).

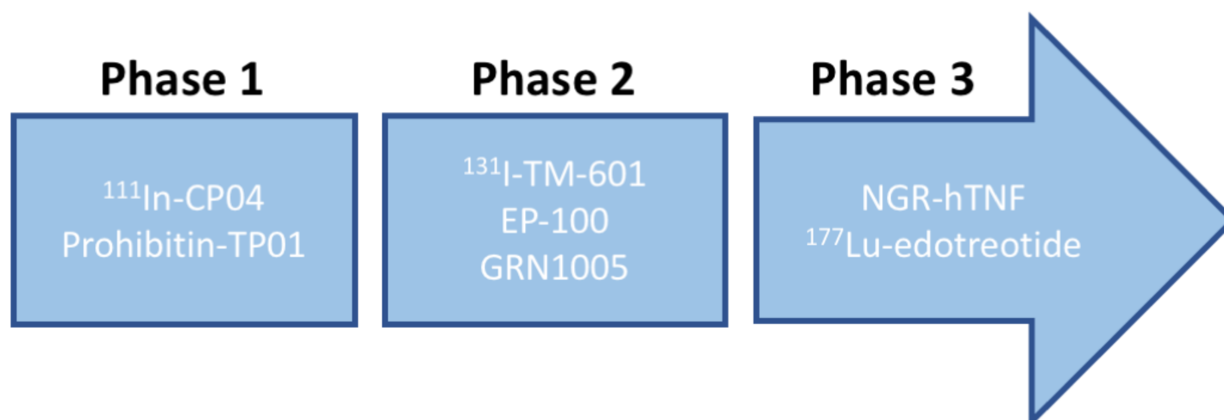


Figure 1.3: Peptide conjugates in clinical trials as therapeutics

CD13 is an aminopeptidase that is natively found expressed at highest levels in the liver, prostate, and kidney. CD13 is upregulated in endothelial cells during angiogenesis in many cancers. Bacteriophage that contained the NGR sequence motif bound the tumor vasculature through CD13 and CD13 antagonists were found to inhibit angiogenesis (Pasqualini et al., 2000). A fusion protein consisting of an NGR-tagged TNF, termed NGR015, was tested in a Phase III trial for the treatment of malignant pleural mesothelioma (NCT01098266) failed to meet its primary endpoint but in patients with a short treatment-free interval were found to have extended overall and progression free survival (Gregorc et al., 2015).

The expression of high levels of luteinizing hormone-releasing hormone (LHRH) is seen in a variety of cancers such as prostate, breast, ovarian and endometrial cancer. Interestingly, both agonists and antagonists of LHRH can have anti-cancer activity through activating a feedback loop blocking testosterone production or through shutting off testosterone production. LHRH

agonists, such as leuprolide, have long been used in androgen-deprivation therapy for the treatment of prostate cancer. In the past decade LHRH antagonists, such as degarelix, have been approved for similar patient populations (Lepor and Shore, 2012). A novel peptide, EP-100, fusing the natural LHRH ligand to a cationic lytic peptide has shown the ability to specifically lyse cancer cells due to their negatively charged cell surface due to high levels of phosphatidylserine (Curtis et al., 2014). EP-100 completed a Phase II trial for the treatment of recurrent ovarian cancer in combination with paclitaxel and led to a sustained treatment response (Nick et al., 2015) (NCT01485848).

Low density lipoprotein receptor-related protein 1 (LRP-1) is a receptor that is implicated in Alzheimer's disease and transport across the blood-brain barrier (BBB). LRP-1 expressed on brain endothelial cells is able to mediate transcytosis of ligands across the BBB (Pflanzner et al., 2010). A novel ligand, angiopep-2 was developed, that binds to LRP-1 and is able to transport molecules across the blood-brain barrier (Demeule et al., 2008). GRN1005 is novel peptide-drug conjugate tethering angiopep-2 to paclitaxel has been developed. It has been tested in patients with recurrent glioma and was able to reach therapeutic concentrations in tumor tissue (Drappatz et al., 2013). GRN1005 has completed three Phase II trials for breast cancer patients with brain metastases (NCT01480583) (NCT02048059) and GBM (NCT01967810). The results of NCT01480583 were presented in 2012 and led to the initiation of the two subsequent trials which have yet to report their findings.

Obesity has long been associated with poor outcomes among prostate cancer patients (Freedland and Aronson, 2004). The prohibitin receptor was identified as being expressed on the vasculature of white adipose tissue (Kolonin et al., 2004). Additionally, the prohibitin receptor is overexpressed in a number of cancer types that is able to activate ERK/MAPK signaling (Cao et

al., 2016). Prohibitin-TP01 is a novel fusion protein consisting of a prohibitin-binding peptide with a protein domain capable of triggering apoptosis. This peptide has a very unique mechanism of action where it triggers apoptosis of white adipose tissue and potentially better outcomes among prostate cancer patients. Prohibitin-TP01 is being tested in Phase I trials for obese patients with prostate cancer (NCT01262664).

The somatostatin receptor is found to be expressed at a high level in a number of tumor types as well as in tumor vasculature. Specifically, SSTR2 is most often expressed – its native ligand being the cyclic peptide somatostatin with a number of FDA-approved drugs acting as agonists, such as octreotide (Sun and Coy, 2011). There has been a lot of work done looking at using octreotide or similar somatostatin ligands for imaging purposes or peptide receptor radiotherapy (PRRT) (Bison et al., 2014). A Phase III trial of a somatostatin receptor ligand ^{177}Lu-edotreotide is currently recruiting for neuroendocrine tumors (NCT03049189).

An ideal tumor-homing peptide will be proteolytically stable, will achieve sufficient pharmacokinetic properties in serum, and will hit tumor cells at a higher ratio than normal tissue (Kuai et al., 2011). Cystine-knotted peptides address these challenges and are of interest for tumor delivery (Kolmar, 2009). An expanded array of tumor-homing peptides could be used as tools for basic science research in cancer biology or developed as clinical tools that will benefit patients.

With the goal of identifying peptides with novel activity targeting tumor cells, we investigated cysteine-knotted peptides for use as imaging agents. The pacifastin class of peptides is known to have protease-targeting activity (Breugelmans et al., 2009), and we theorized that increased protease expression driving metastasis in cancerous tissues could provide a target for

these peptides. Within this class, we focused on variants of LCMI-II, a high-affinity inhibitor of the serine proteases chymotrypsin and elastase, and derived from a component of the innate immune system of the migratory locust (Boigegrain et al., 1992; Simonet et al., 2002). We investigated the potential of LCMI-II variants as molecular scaffolds to target cancer cells and to deliver not only imaging agents but also a cytotoxic payload with therapeutic potential

Peptide-based cancer therapies offer certain advantages over traditional small molecule and antibody-based agents. The biggest strengths lie in utilizing known receptor-ligand interactions, to deliver molecules that will rapidly wash out of normal tissue, and are capable of greater penetration into solid tumors and tumors behind the blood-brain barrier. Additionally, their pharmacokinetic properties allow ease of generating theranostics capable of being utilized for both imaging and therapeutic purposes. While the field of peptide-based cancer agents has not yet fully blossomed, there are a number of agents that are showing promise in clinical trials with few results currently published and a significant number of novel agents still being developed in the preclinical pipeline.

Chapter 2. RESULTS

Identification of a tumor homing peptide

To identify a novel peptide for the purposes of cancer imaging, we tested a panel of 10 cystine-rich peptides for their ability to express at a high-level and as a single product with fully oxidized disulfide bonds indicating proper folding. Protein expression was done via viral transduction of mammalian cells (Bandaranayake et al., 2011). The expression construct produced the cystine-rich peptide fused to a siderocalin-carrier protein via a cleavable linker. The peptides were modified to enhance cleavage through modification of the N-terminus. They were optimized for further N-linked conjugation through mutation of the amino acid lysine to arginine. Of the 10 initial peptides for which transduction and expression was tested, 5 derivatives were produced in sufficient quantities of fully-oxidized peptide for further studies including chlorotoxin, hefutoxin, epiregulin, bubble, and PMP-C/LCMI-II. The decision was made to study the derivative of PMP-C/LCMI-II, termed THP1, in the context of tumor imaging due to its native function as a serine protease inhibitor. THP1 was further characterized by reduced and non-reduced HPLC to determine a single chemical species was produced and confirm the oxidized nature of the disulfide-bonds (Figure 1).

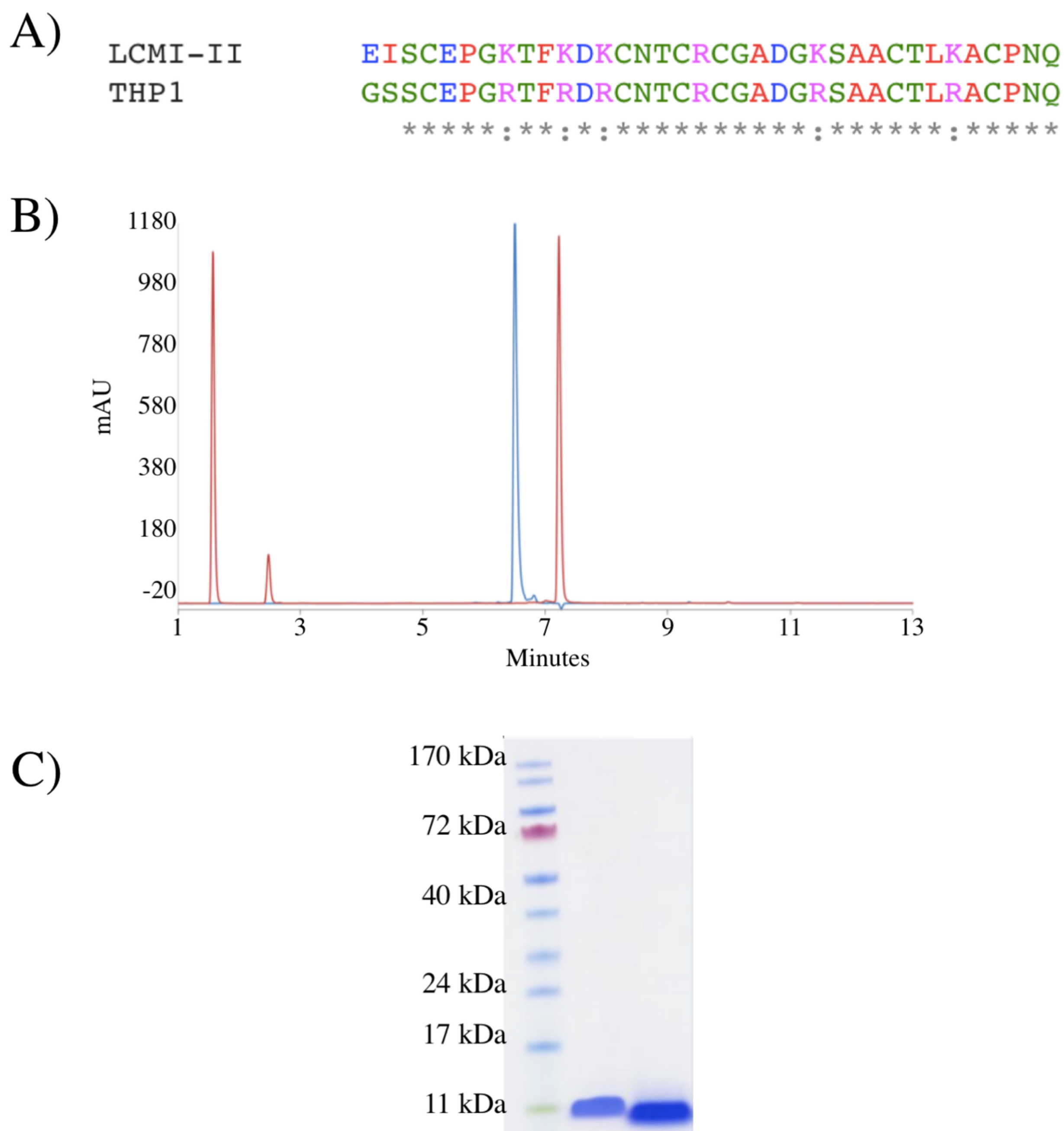


Figure 2.1: Sequence and quality control data on recombinant THP1 A) Sequence alignment of LCMI-II with THP1 B) Reverse phase-HPLC of non-reduced (blue) and reduced (red) THP1 showing a shift in retention time upon reduction. C) SDS-PAGE of non-reduced (left) and reduced (right) THP1 demonstrating a single-product with a shift in migration upon reduction.

The ability of THP1 to deliver an imaging agent to tumors in mouse xenograft models was tested. The purified THP1 peptide was conjugated via its N-terminal amine to Alexa Fluor 647 (AF647). Mice were dosed *via* tail vein injection with 10 nmol of AF647-labeled THP1 (THP1-AF647) and tissues were imaged *ex vivo* after 4 hours (Figure 2). The dosage and time point were selected based on previous experience demonstrating optimal signal-to-noise in tumor as compared with normal tissue for dye-conjugated chlorotoxin in mouse models. THP1 showed 3.2-fold higher (p<0.01) tumor uptake than AF647 alone in the RH28 sarcoma cell line. Additionally, tumor specific uptake had an 8.3-fold higher delivery to tumor than to liver. While THP1-AF647 has 60-fold higher fluorescent signal observed in the kidneys when compared with AF647 treated mice this accumulation is often seen following glomerular filtration of small peptides (Carone, 1979).

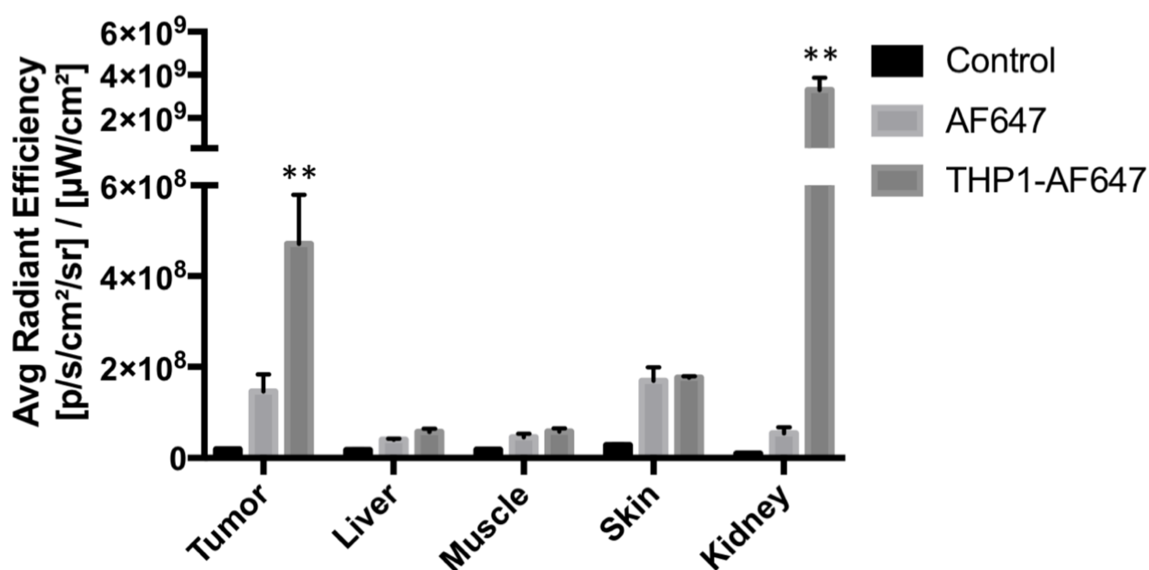


Figure 2.2: THP1-AF647 accumulation in RH28 bearing mice Mice bearing RH28 flank xenografts were dosed with 10 nmol of AF647 or 10 nmol of THP1-AF647 and euthanized 4 h following injection. Quantification of imaging signals, reported in average radiant efficiency comparing the tissue signals in mice bearing the RH28 xenograft. (**: p<0.01 as compared with AF647), n=3.

We next assessed whether THP1-AF647 was able to accumulate in additional tumor types. In RH28 sarcoma, Ramos lymphoma, and A375 melanoma, THP1-AF647 showed a 3.2-, 3.5-, and 2.2-fold higher signal relative to AF647 alone, respectively (Figure 3). Further, the THP1-AF647 signal was 5- to 8-fold higher in tumors compared to liver. Upon observing that the bulk tumor accumulated THP1-AF647, we determined the increase in tumor cell fluorescence following dosing.

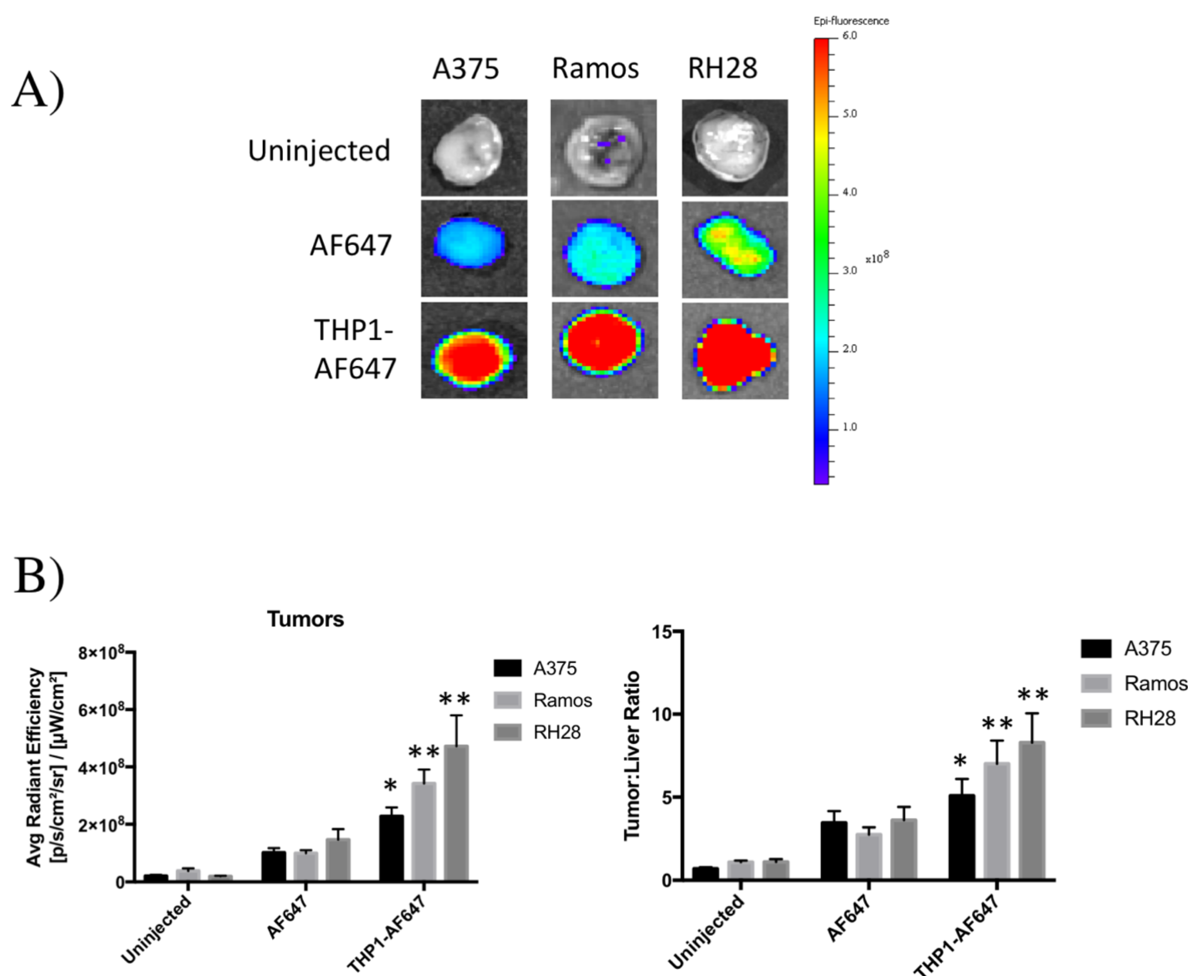


Figure 2.3: THP1-AF647 accumulation in multiple tumor types A) Images of representative tumors comparing the A375, Ramos, and RH28 tumor lines between the three injection groups. B) Quantitation of signals in tumor and tumor:liver ratios in each of the tumor types. (*: $p<0.05$; **: $p<0.01$ as compared with AF647), n=3.

To assess the fraction of cells taking up THP1-AF647, Ramos tumors were dissociated and analyzed by flow cytometry. Mice were dosed with vehicle, 10nmol THP1-AF647 or 53nmol THP1-AF647, euthanized after 1 hour, and DAPI(-) dissociated tumor cells were assessed for AF647 by flow cytometry. A positive shift in fluorescence is seen in the 10nmol treated tumor as compared with control while in the 53nmol treated tumor the vast majority of cells are positive (Figure 4). The average signal of the cells was 4-fold over baseline in the 10nmol group and 15-fold over baseline in the 53nmol group. After identifying the increase in fluorescence of tumor cells that accumulated THP1-AF647, we tested to see where the peptide was localized in the cells of the tumor.

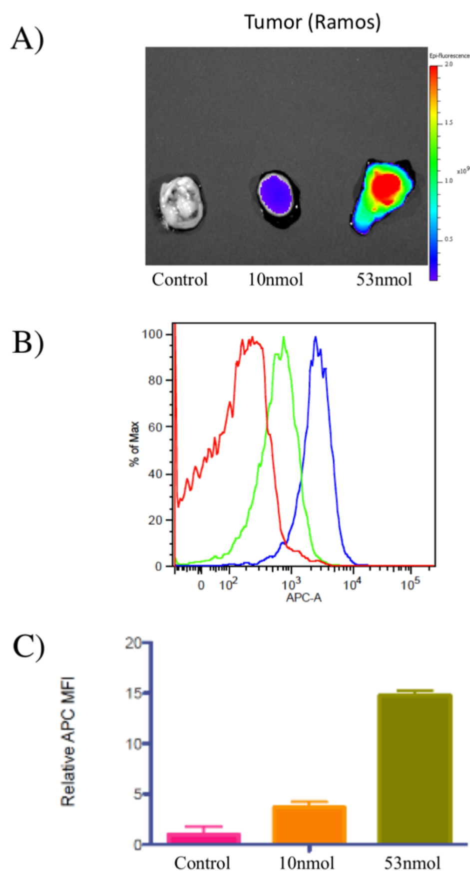


Figure 2.4: THP1-AF647 cellular accumulation in Ramos flank tumors by flow cytometry A) Ramos flank tumors dosed with vehicle, 10nmol THP1-AF647, or 53nmol THP1-AF647 and imaging by IVIS. B) Flow cytometry on dissociated Ramos flank tumors showing intensity of AF647 signal. C) Median fluorescence intensity (MFI) of dissociated tumor cells seen in panel B.

We next determined whether THP1-AF647 is internalized into tumor cells *in vivo*. Mice were dosed with 10 nmol of THP1-AF647, the tumor was harvested 30 min post-injection to maximize tumor signal, and live tissue was sectioned without fixation and imaged *via* confocal microscopy (Figure 5). THP1-AF647 indeed penetrated into RH28 cancer cells and was observed as punctate

staining inside the majority of tumor cells. We next wanted to determine whether the AF647 conjugate changed the biodistribution of THP1.

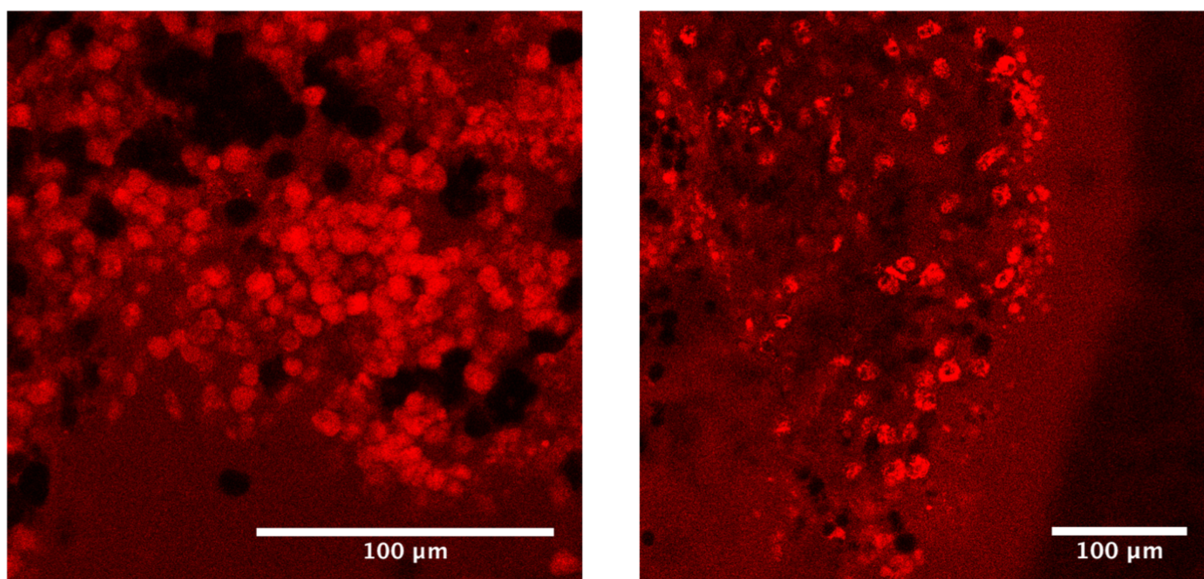


Figure 2.5: THP1-AF647 accumulation in RH28 flank tumors by confocal microscopy Confocal microscopy showing 10 nmol THP1-AF647 internalized in RH28 flank xenografts imaged *ex vivo* 30 min following intravenous (IV) injection *in vivo*.

To investigate the biodistribution of the THP1, both with and without conjugation to AF647, we evaluated a further modified form, termed THP1* and containing a single-lysine (K18), by using whole-body autoradiography (Figure 6). THP1* was prepared with and without a single AF647 conjugate and radiolabeled by reductive-methylation using ^{14}C formaldehyde. Mice bearing RH28 human sarcoma flank tumors were dosed with 2µCi of a radiolabeled form of THP1* and THP1*-AF647. The mice were subsequently embedded, sectioned, and exposed to phosphoimager plates. Qualitatively, the biodistribution does not differ when comparing THP1* to THP1*-AF647 in these mice. This demonstrates tumor delivery of both the peptide and peptide-dye conjugate.

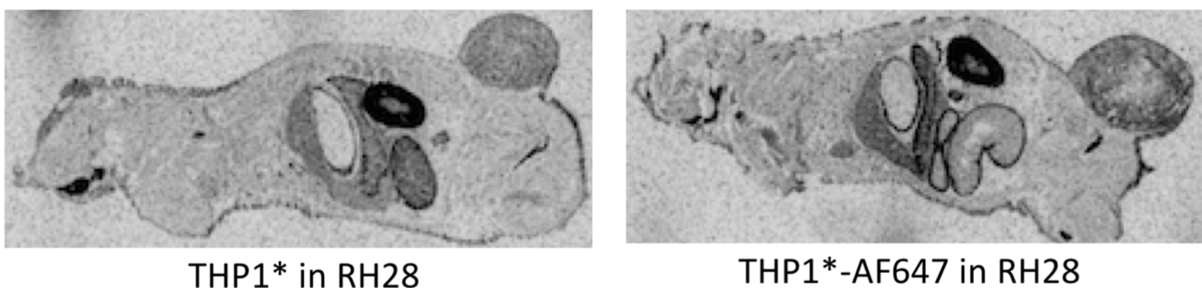


Figure 2.6: Whole body autoradiography of RH28 bearing mice Whole-body autoradiography images comparing the biodistribution of THP1* and THP1*-AF647 at 24 h in mice bearing RH28 flank tumors.

THP1-based peptide-drug conjugate

Peptide internalization into cancer cells is a requirement for any candidate considered for delivery of chemotherapy agents that act on intracellular targets. Given that THP1* accumulated inside of cancer cells *in vivo*, we assessed its ability to deliver chemotherapy as a cleavable peptide-drug conjugate by testing it in a tumor cell line and mouse model. To test whether THP1* would be effective as a drug delivery agent, we designed a peptide-drug conjugate using a Valine-Citrulline-PABA (VCP) dipeptide cleavable linker attached to the microtubule inhibitor monomethyl aurostatin E (MMAE). The VCP linker is cleavable following internalization in lysosomes by the cathepsin B protease (Doronina et al., 2003). This combination of linker-payload has been shown to have clinical utility in hematopoietic tumors as the FDA approved brentuximab vedotin (Younes et al., 2010). MMAE and THP1*-MMAE were tested for their ability to inhibit the growth of Ramos lymphoma cells *in vitro* (Figure 7). Ramos cells have traditionally been tested with MMAE conjugates because of their sensitivity to microtubule inhibitors (Francisco et al., 2003). MMAE inhibited growth of Ramos cells with IC_{50} values between 100 and 1000 pM, similar to prior reports. However, THP1*-MMAE was ~1000-fold less potent than MMAE alone

at growth inhibition of Ramos cells with IC_{50} values from 100-1000 nM. Potency reduction is also seen with brentuximab vedotin, which sees potency losses of up to 30-fold in antigen-positive cells when compared with MMAE alone (Francisco et al., 2003). In order to assess activity in models of chemotherapy resistance, we generated Ramos-250 and Ramos-1000 sublines (resistance to 250 nM and 1000 nM doxorubicin, respectively) by continuously exposing them to increasing concentrations of doxorubicin. Doxorubicin-resistant Ramos sublines (Ramos-250 and Ramos-1000) were resistant to both MMAE and THP1*-MMAE, presumably due to multi-drug resistance mechanisms. While the poor efficacy of THP1*-MMAE did not bode well for *in vivo* efficacy, it was still of interest to learn whether THP1* could deliver a candidate chemotherapeutic agent to tumors *in vivo* or whether targeting activity was impaired by the drug conjugate.

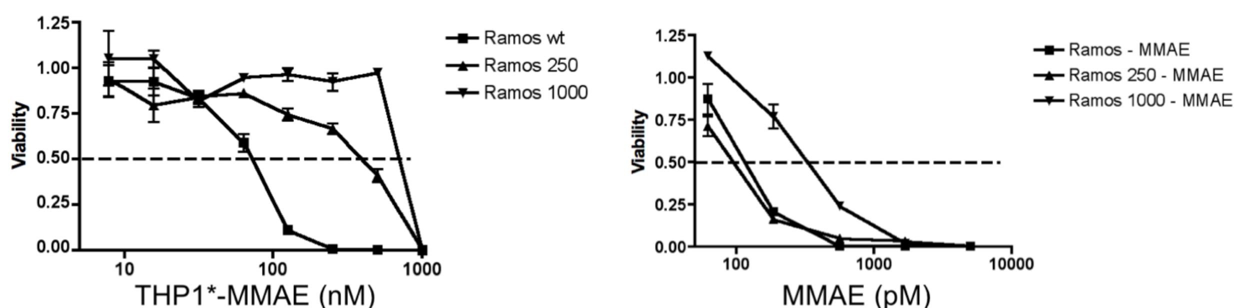
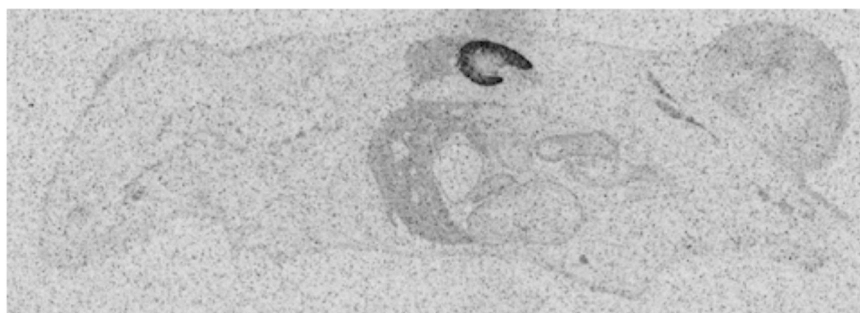


Figure 2.7: Viability of Ramos and dox-resistant Ramos cells *In vitro* cell viability assay quantified using CellTiter Glo. Cells were tested in quadruplicate, treated for 72 h and compared to vehicle treated cells.

To evaluate the biodistribution of THP1*-MMAE, the molecule was ^{14}C methylated, and 2 μCi were administered to mice bearing Ramos flank tumors. After 24 h, the mice were euthanized, sectioned, and exposed to phosphoimager plates. The MMAE bioconjugate accumulation in tumors and normal tissues was similar to radiolabeled THP1 alone; however, the overall signal of the bioconjugate was 3- to 4-fold lower than radiolabeled THP1 in all tissues (Figure 8). We turned

to immunohistochemistry as a readout of cellular response to the peptide-drug conjuagte. Following exposure to THP1*-MMAE, we examined whether the apoptotic pathways (as measured by cleaved caspase 3 signal) were activated in Ramos tumor cells *in vivo*. Mice were treated for 48 h with 100 nmol of THP1*-MMAE before tissues were harvested and assessed for cleaved caspase-3 (CC3) by immunohistochemistry (Figure 9). Not surprisingly, scattered (<10%) CC3 was seen in the tumor of both control and drug-treated mice with little to no (<5%) CC3 seen in liver or kidney. Together, these data indicate that while THP1 is delivered to tumors, THP1-MMAE is minimally active *in vitro* and *in vivo*.



THP1*-MMAE in RH28

Figure 2.8: Whole body autoradiography of THP1*-MMAE peptide-drug conjugate in mice Whole-body autoradiography images demonstrating the biodistribution of THP1*-MMAE at 24 h in mice bearing RH28 flank tumors.

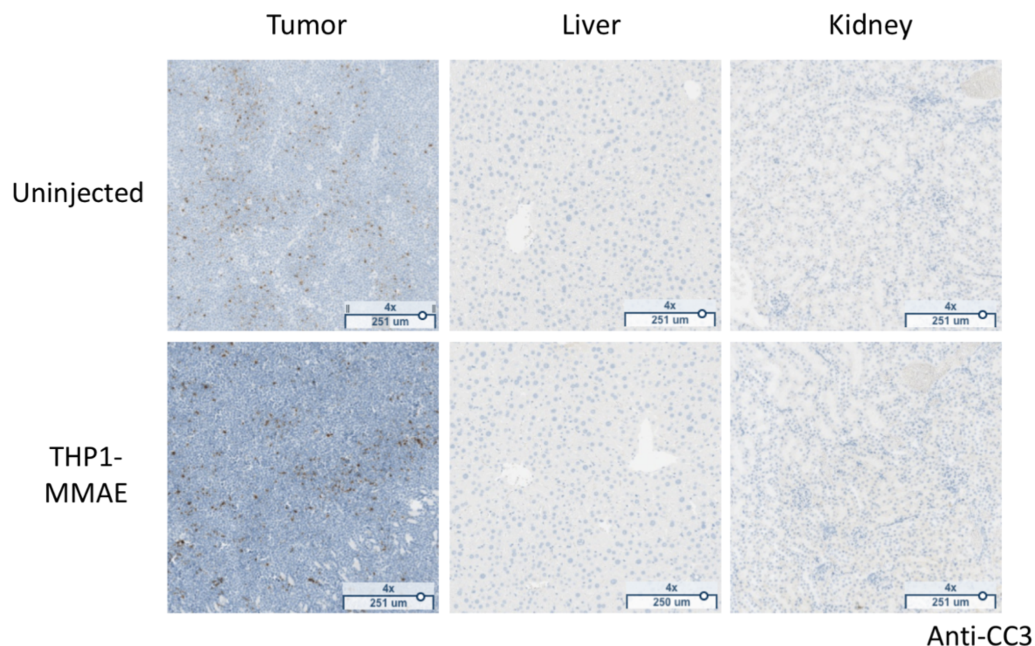


Figure 2.9: Immunohistochemistry showing lack of Ramos tumor response to peptide-drug conjugate Immunohistochemistry showing cleaved caspase-3 (CC3) in select tissues from uninjected mice or mice treated with 100 nmol of THP1*-MMAE conjugate. Mice were treated 48 h prior to necropsy. n=3.

THP1 variants and mechanism of accumulation

To address whether the mechanism of THP1 accumulation in tumors is driven by peptide charge and size, an all D-amino acid form of THP1 was synthesized and termed D-THP1. We then tested for accumulation in RH28 flank tumors. Mice were dosed IV with 10 nmol AF647, THP1-AF647, or D-THP1-AF647, and tissues were imaged *ex vivo* after 1 h, as earlier time points demonstrated a greater ability to detect signal decreases in the tumor (Figure 10). THP1-AF647 had a significant ($p<0.01$) increase in tumor accumulation when compared with D-THP1-AF647. The enhanced tumor accumulation found in THP1-AF647 (in comparison to the D-amino acid form) suggests that accumulation results from the peptide sequence rather than size or charge, and led us to explore sequence variants of THP1.

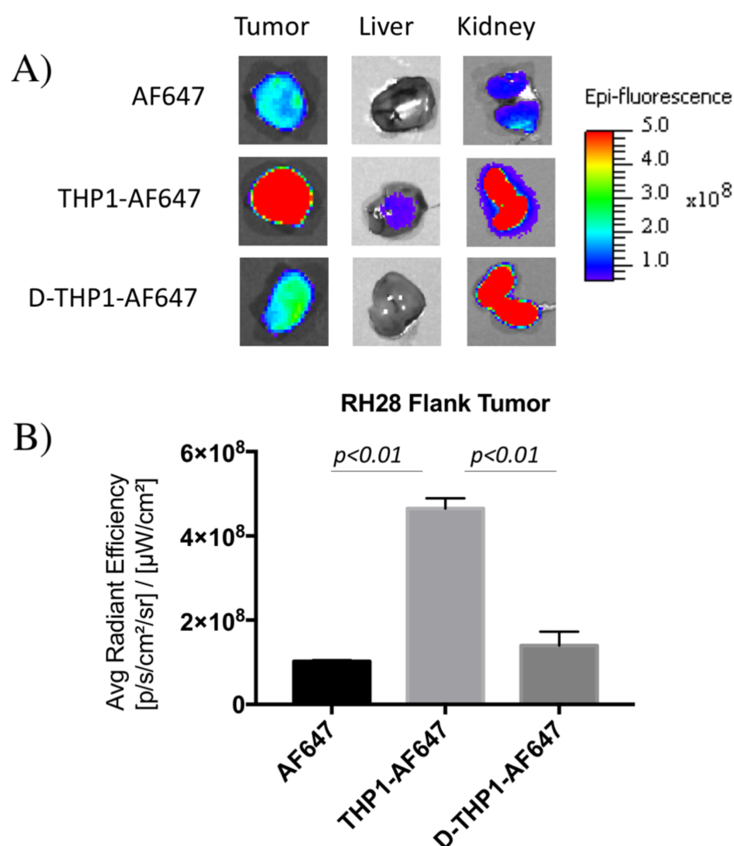


Figure 2.10: THP1-AF647 vs. D-THP1-AF647 accumulation in RH28 flank tumors A) Mice bearing RH28 flank xenografts were dosed with 10 nmol of AF647, THP1-AF647 or D-THP1-AF647 (D-amino acid peptide) and euthanized 1 h following injection. Select tissues were imaged *ex vivo* and quantified by IVIS imaging. B) Quantitation of signals in organs, reported in average radiant efficiency. n=3.

To test the functional relevance of a patch of positively-charged residues found on THP1, we attempted to produce a neutral and negatively charged form of the positively-charged patch on the peptide (Figure 11). Looking at the native PMP-C/LCMI-II structure, a cluster of positively charged residues is seen opposite the N-terminus of the peptide. These peptides produced soluble peptide but following cleavage from their carrier peptide it was observed that there was a mixture of products by non-reduced HPLC. This suggests that these mutations disrupted folding of the THP1 peptide.

THP1 : GSSCEPGRTFRDRCNTCRCGADGRSAACTLRACPNQ (parent)
THP1 RRR->EEE : GSSCEPGRTFEDECNTCRCGADGRSAACTLEACPNQ (negative)
THP1 RRR->AAA : GSSCEPGRTFADACNTCRCGADGRSAACTLAACPNQ (neutral)

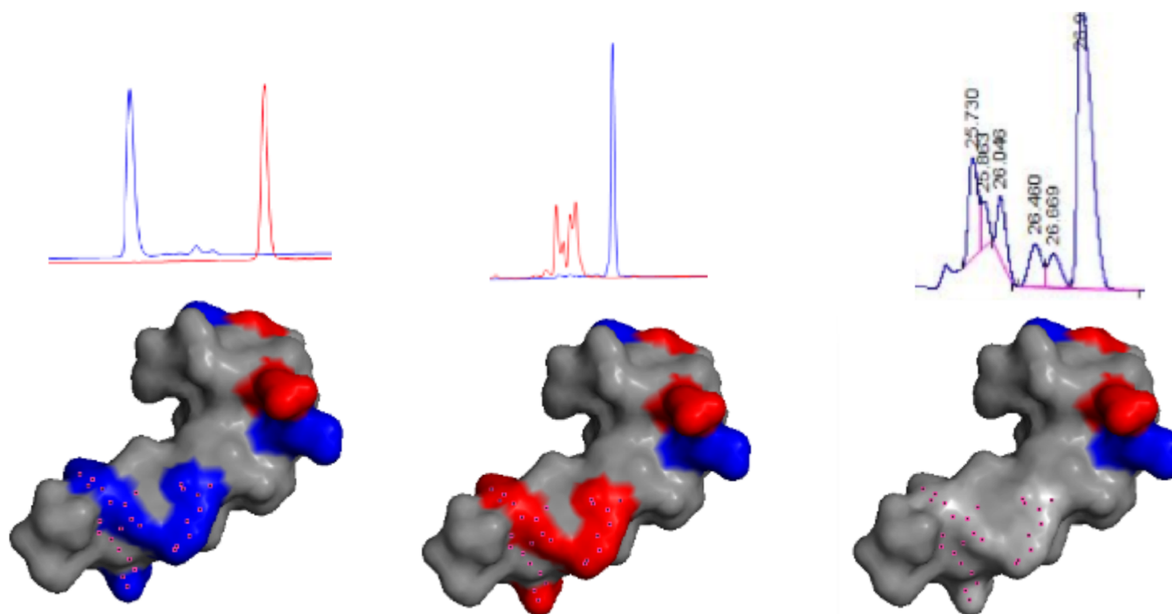


Figure 2.11: Charged-patch mutation of THP1 Sequence alignment, models, and HPLC (non-reduced (blue) and reduced (red)) tracings of THP1 with a mutation in a positively charged patch.

A panel of 27 variants were generated to characterize the ability of THP1 to act as a scaffold for peptide engineering. Mutations focused on: 1) charge-reversal point mutations; 2) mutation of an asparagine residue critical for folding; and 3) using peptide sequences from close homologs identified by BLAST. This panel was assessed for by HPLC under non-reduced and reduced conditions and characterized as either a “well-folded” or “mixed” product (Figure 12). Sixteen of the 27 tested peptides were well-folded and formed single products by HPLC, which shifted upon reduction; 7 of the 16 were based on homologous peptides and 9 were point mutations. We tested the 7 well-folded homologous peptides, with 50-90% amino acid sequence homology when

compared to THP, for their ability to enhance tumor accumulation in RH28 human sarcoma flank tumors. Mice were dosed IV with 10 nmol of peptide-AF647 conjugate and tissues were imaged *ex vivo* after 4 h (Figure 13). Tumor accumulation of THP7-AF647 increased 2.1-fold ($p<0.05$) in comparison to THP1-AF647. Additionally, tumor-to-liver ratios were elevated 1.9-fold ($p<0.05$) and 3.1-fold ($p<0.01$) compared to THP1-AF647 for THP6-AF647 and THP7-AF647, respectively. To test whether these peptides hit the same molecular target in a competitive manner, mice were dosed with AF647, THP1-AF647, or both THP1-AF647 and a 50-fold excess of unlabeled THP7 (Figure 14). Tissues were imaged *ex vivo* and tumor signal was decreased 2.2-fold ($p<0.05$) following the addition of the unlabeled competitor, THP7. This suggests that the yet to be identified molecular target is shared and binds to these peptides in a competitive manner. We next explored if modifying the cell signaling in the tumor cells could affect uptake of THP1-AF647.

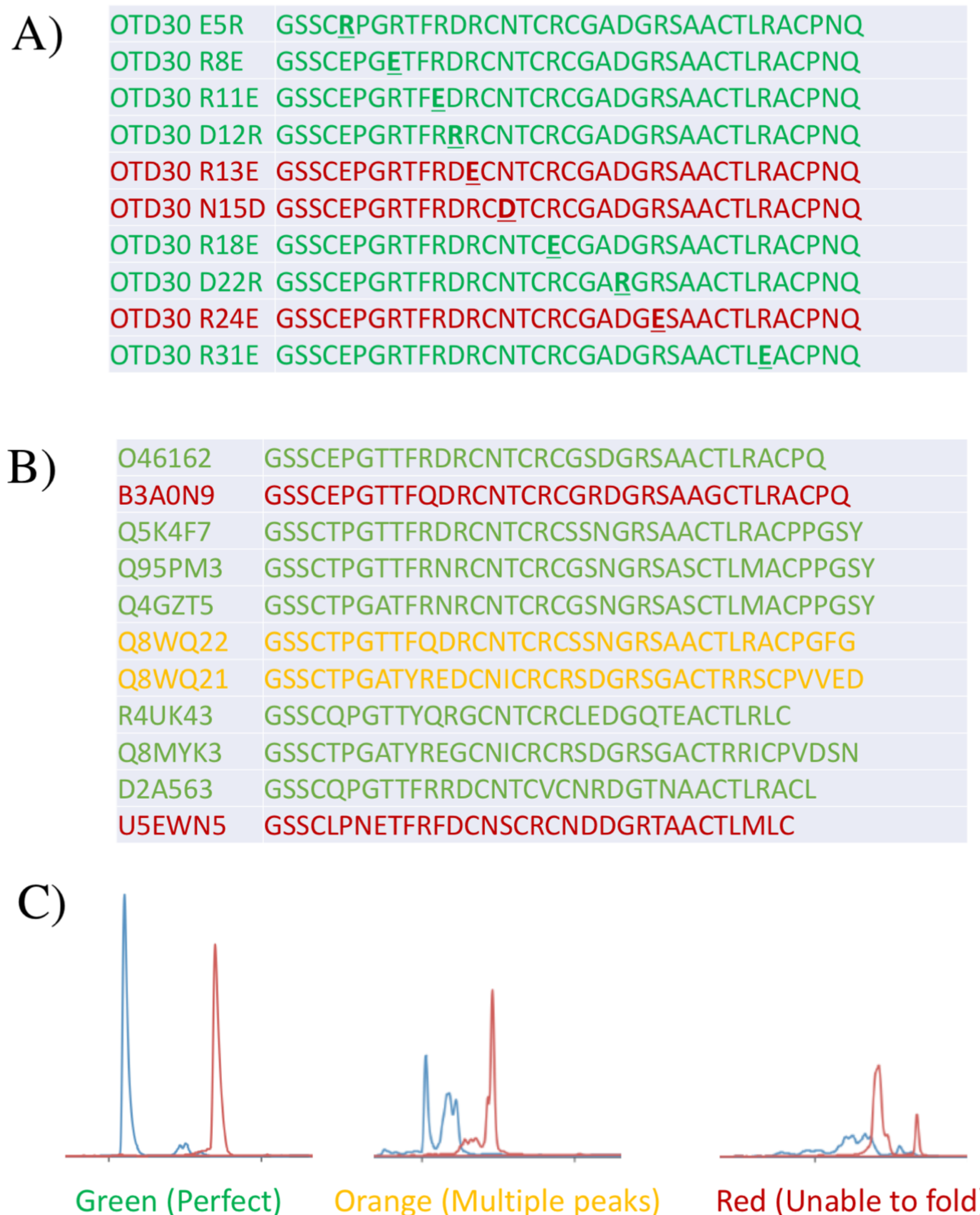


Figure 2.12: Panel of THP1 variants tested for protein expression and folding A) THP1 point mutants scored for their ability to fold properly following expression and cleavage in mammalian protein expression system. B) THP1 homologs with Uniprot ID of parental peptides scored for their ability to fold properly following expression and cleavage in mammalian protein expression system. C) Representative HPLC traces (non-reduced (blue) and reduced (red)) of perfect, multiple peak, and unable to fold categories.

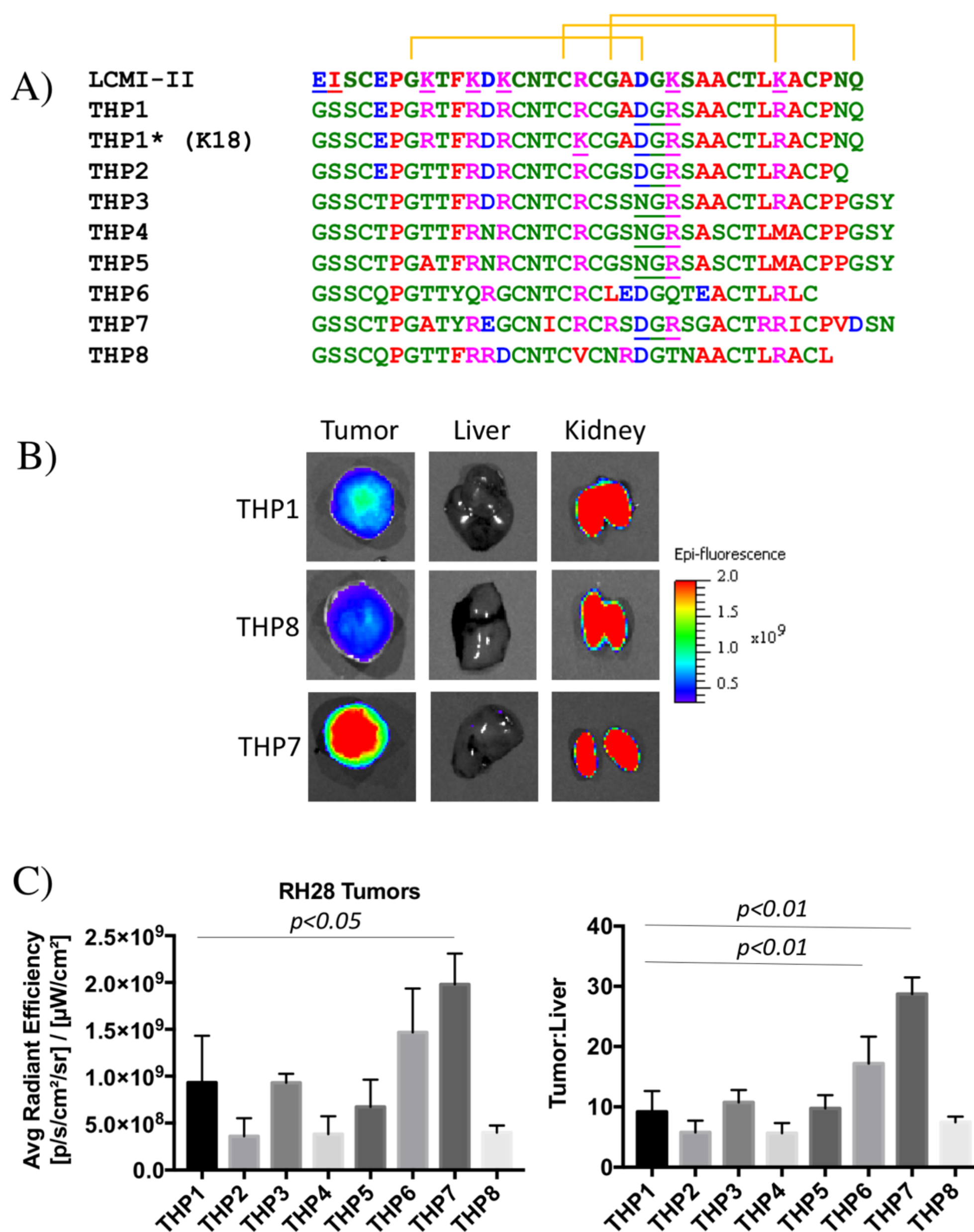


Figure 2.13: Comparison of tumor accumulation of panel of THP1 variants A) Sequences of the native cystine-knotted peptide LCMI-II, modified LCMI-II and variants are shown. Disulfide bonding pattern is illustrated at the top as well as known functional sequence motifs. B) Mice bearing RH28 flank xenografts were dosed with 10 nmol of THP1-AF647 or 10 nmol of THP1 variant-AF647 and euthanized 4 h following injection. Select tissues were imaged *ex vivo* and quantified by IVIS imaging. C) Quantitation of signals in tumor and tumor:liver ratios for each of the variant peptides. n=3-6.

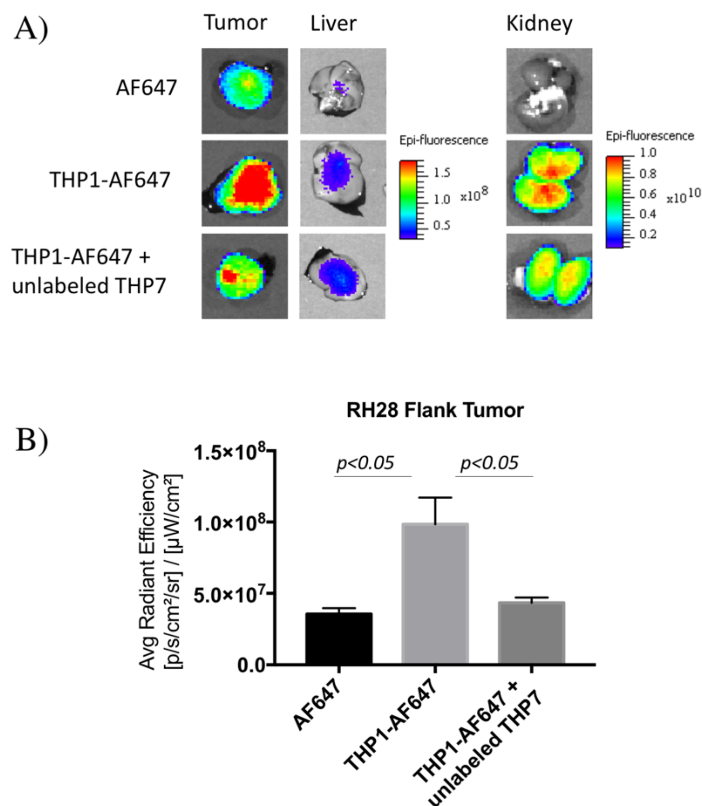


Figure 2.14: Competition between THP7 and THP1-AF647 in RH28 bearing flank tumors A) Mice bearing RH28 flank xenografts were dosed with 2 nmol of AF647, 2 nmol of THP1-AF647, or 2 nmol of THP1-AF647 with 98 nmol of unlabeled THP7 and euthanized 1 h following injection. Select tissues were imaged *ex vivo*. B) Quantitation of signals in organs, reported in average radiant efficiency. n=3.

We sought to modify the uptake of THP1 into cells *in vitro* in the hopes of understanding the mechanism of uptake as well as identifying potential adjuvants for uptake. An *in vitro* assay was established where cells were incubated with drug for 16 hours, 100nmol of THP1-AF647 was added, and incubated for another 4 hours before extensive washing and flow cytometry. We tested the ability of 128 FDA approved cancer drugs to modify the uptake of THP1-AF647 and found a number with elevated or reduced fluorescence (Figure 15). Specifically, HDAC inhibitors appeared to enhance fluorescence while kinase inhibitors appeared to decrease uptake of fluorescent peptide conjugate. These hits suggest that these the induced transcriptional changes

and kinase pathway changes induced by drugs may begin to point to the target and uptake mechanism of THP1-conjugates into cancer cells.

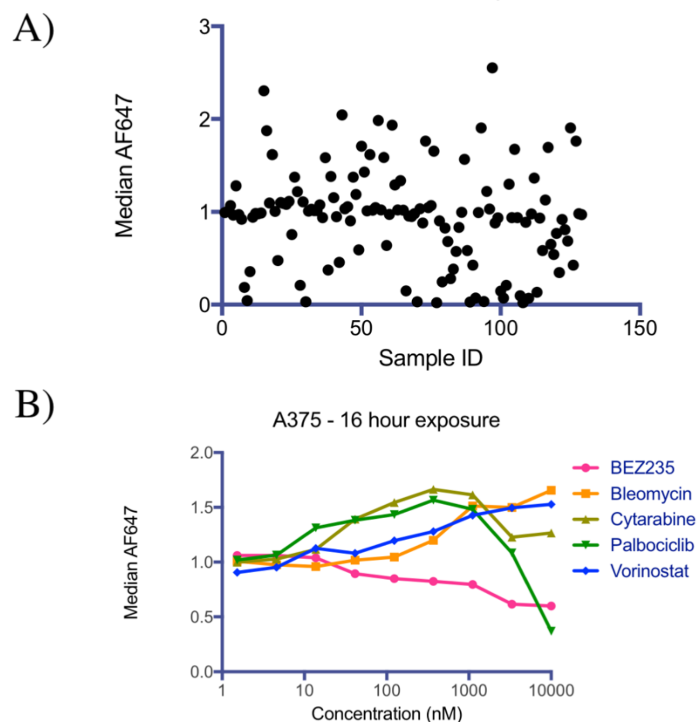


Figure 2.15: Drug-modified uptake of THP1-AF647 *in vitro* A) *In vitro* assay of cells incubate with 10uM of drug for 16 hours, followed by 4 hour incubation with THP1-AF647 in A375 cells showing median THP1-AF647 signal relative to DMSO treated control. B) *In vitro* dose-response of a number of drugs seen as hits in the *in vitro* uptake assay.

Chapter 3. CONCLUSIONS & FUTURE DIRECTIONS

In this study, we identified a novel peptide-dye conjugate derived from the LCMI-II protease inhibitor that accumulates in multiple cancer types. Whole-body autoradiography showed qualitatively similar distribution of THP1* with or without a dye conjugate which suggests that the peptide plays a predominant role in biodistribution and that the payload, in this case AF647, does not interfere with tumor localization. We determined that the accumulation of the THP1-AF647 bioconjugate is both stereospecific and saturable. We identified a modified form that accumulates in RH28 tumors with a more favorable ratio of tumor-to-normal tissue (*e.g*. liver tissue).

LCMI-II is highly tolerant to mutation, as has been seen in cystine-knotted peptides such as EETI, MCoTI, AgRP, and others (Kimura et al., 2009; Quimbar et al., 2013; Silverman et al., 2009), with some residues more tolerant of mutation than others. We have identified the sensitivity to mutagenesis of a number of residues in the THP1 scaffold. Six properly-folded variants of THP1 were assessed for tumor accumulation, and THP7 was identified as a variant with significantly enhanced tumor accumulation. Additionally, THP7 showed a tumor:liver ratio of 30:1, demonstrating high specificity. Tumor accumulation of the variants and the observed competition between THP1 and THP7 demonstrated that certain residues of LCMI-II-related peptides are important for tumor accumulation.

The accumulation of THP1 in the tumor occurs *via* a competitive and stereospecific process, suggesting that THP1 is targeting a specific protein receptor on these cells. The specific cellular receptor remains to be identified and efforts to characterize binding to integrins, serine proteases, and membrane lipids have been inconclusive or limited by technical challenges. Of the

candidate protein receptors, the most intriguing is the potential for pacifastins to bind to cancer-associated serine proteases. The native sequence of LCMI-II is a high affinity inhibitor of both chymotrypsin and elastase (Kellenberger et al., 1995). The structure of chymotrypsin in complex with LCMI-II has a highly-conserved backbone and binding site in comparison to cancer-associated serine proteases (Roussel et al., 2001). Specifically, the type II transmembrane serine proteases matriptase, hepsin, TMPRSS2, and TMPRSS4 have been found to be overexpressed in many cancers, predominantly studied in prostate, breast, and ovarian cancers (Murray et al., 2016). Binding to these transmembrane proteins has the potential to trigger endocytosis and internalization of peptide-dye and peptide-drug conjugates and deliver a therapeutic payload. While beyond the scope of the current work described here, in the event that THP1 is developed for imaging or therapeutic purposes, it will be necessary to distinguish between these possible targets.

We have demonstrated internalization of the peptide-dye conjugate at early time points in human tumor xenografts. The specific subcellular compartment where the conjugate localizes remains to be determined but would be useful for the development of peptide-drug conjugates based on this peptide family. Future work would set out to further characterize the identity of the cells internalizing the conjugate. This would include characterizing whether intensity of cellular staining bears any relation to cell cycle phase, hypoxia, or other feature of the cancer cell. Additionally, it would be of value to characterize the relative uptake of THP1- or THP7-derived agents to that of other peptide-based imaging agents.

We conducted proof-of-concept experiments with THP1-MMAE bioconjugates to assess whether the peptide-drug conjugate is able to release free drug and trigger cell death. We observe cell death *in* vitro; however, at a greatly reduced potency when compared with free drug. When

we look *in vivo* the fraction of apoptotic cells in THP1-MMAE-treated tumors was no different than vehicle controls. The lack of efficacy observed with the delivery of MMAE or other potent drugs may be a result of: 1) short serum half-life of the peptide-drug conjugate; 2) delivery of an insufficient number of MMAE molecules to be therapeutically effective (THP1* carries only one payload, whereas antibody-drug conjugates typically carry 4-8 payloads); 3) premature release of the drug in serum during distribution; 4) reduced potency of the bioconjugate (*e.g.,* incomplete linker cleavage); or 5) intracellular trafficking routes, such as endosomal trapping, that prevent MMAE from reaching microtubules. An example of this trafficking is apoE being retained in recycling endosomes following binding to the LDL receptor-related protein 1 (LRP1) preventing apoE from being trafficked into lysosomes (Laatsch et al., 2012). The reduced potency of THP1-MMAE compared to unconjugated MMAE *in vitro* suggests that drug release is also an issue. Future efforts toward therapeutic peptide conjugates will require significant work on pharmacokinetic optimization (*e.g*., extending serum half-life) and potency (*e.g*., providing a carrier of multiple MMAE or therapeutic payloads). Additional work on intracellular trafficking will also be necessary to understand whether the payload has access to its target.

We showed in an *in vitro* assay that certain small molecules are capable of modifying the uptake of THP1-AF647 into cancer cells. While beyond the scope of the current work, characterizing this finding using *in vivo* tumor uptake could lead to the development of adjuvant agents for this or other peptide conjugates. Alternatively, since many cancer patients are undergoing concurrent therapy with chemotherapy, radiation, molecularly targeted therapy, or immunotherapy, it is important to know the optimal timing of treatments for patients planning to utilize peptide-based cancer therapies or imaging agents.

In our study, our goal was to identify a new cystine-knotted peptide that accumulates in tumors. In future experiments, we plan on identifying ways of extending the half-life of peptide-drug conjugates to increase their exposure to tumors. Additionally, we plan to utilize computational design to generate high-affinity ligands for the cancer-associated proteases based on our starting scaffold THP1. In our work described here, we have shown the potential for using LCMI-II-derived cystine-knotted peptide to deliver imaging agents to tumors, and we have established the foundation for the development of peptide-drug conjugates.

Chapter 4. MATERIALS & METHODS

1. *Materials, cell lines and reagents* - RH28 (MD Anderson, Houston, TX, USA) and Ramos, A375, A204, and A673 cells (ATCC, Manassas, VA, USA) were cultured in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

2. *Peptide production* - All L-amino acid peptides were produced using a lentivirus-based mammalian protein expression system as described elsewhere (Bandaranayake et al., 2011). Purified peptides were assessed under non-reduced and reduced conditions by RP-HPLC and SDS-PAGE gel for purity and stored lyophilized at -20°C. The D-amino acid form of THP1 was ordered from Bachem and synthesized according to published protocols (21). AF647 conjugation reactions were done in 50 mM sodium bicarbonate at pH 7.6. Alexa Fluor 647 NHS Ester (Thermo, Waltham, MA, USA) was resuspended at 10 mg/ml in DMSO and mixed with an equimolar amount of peptide at 2 mg/ml. This reaction proceeded at room temperature for 4 h, additional peptide was added if free dye remained and/or purification occurred *via* RP-HPLC. Concentration was determined by using AF647 absorption at 650 nm with the extinction coefficient 270,000 cm^{-1} M^{-1}.

3. *Animal studies and xenografts* - All studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). Xenografts were established in athymic nu/nu mice (Harlan) by subcutaneous injection on 5 million cells suspended in 100 µl of a 1:1 mixture of serum-free media and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Tumors were then allowed to grow to 250-500 mm^3 before testing for tumor accumulation.

4. *In vivo and ex vivo optical imaging* - Mice were injected by tail vein with 100 µl of peptide solution with 10 nmol of fluorescently-labeled sample. In the competition experiment, mice were injected with 2 nmol of fluorescently-tagged peptide and 98 nmol of untagged peptide. Mice were euthanized at indicated time points and imaged on an IVIS (Caliper

Life Sciences, Hopkinton, MA, USA) with excitation at 640 nm and emission measured at 680 nm. Confocal microscopy was done using the Zeiss LSM 780 LMO microscope on sections cut from live, unfixed RH28 flank tumors 30 min after IV injection of with 10 nmol THP1-AF647.

5. *Tumor Dissociation Flow Cytometry* – Mice bearing Ramos flank tumors were dosed with 10nmol THP1-AF647, after 1 hour, mice were euthanized and tumors were isolated. Tumors were processed using scalpels, trypsin, and trituration. After 5 minutes, trypsin was neutralized with media containing 10% FBS and DAPI at 1ug/ml. Cells were filtered through a 100um and 40um filter. Samples were loaded on a Novocyte Flow Cytometer (ACEA Biosciences) and FSC, SSC, DAPI, and AF647 were measured. Gating was done for single cells that were DAPI negative and median AF647 was plotted.

6. *Small Molecule Flow Assay* – Cells were seeded in a 96-well plate at 5,000 cells per well to adhere and grown overnight at 37°C in 5% CO_2. Cells were treated for 16 hours with 10uM of the 129 compound Approved Oncology Drug Set VII (NCI Developmental Therapeutics Program). Cells were then treated with 100nmol of THP1-AF647 for 4 hours. Following incubation, cells were washed 3 times with 100ul of Flow Buffer, trypsinized, neutralized with flow buffer containing 1ug/ml DAPI, and transferred to U-bottom 96 well plate for flow cytometry. Cells were assayed on Novocyte Flow Cytometer (ACEA Biosciences) and FSC, SSC, DAPI, and AF647 were measured. Gating was done for single cells that were DAPI negative and median AF647 was plotted.

7. *Synthesis of ^{14}C methylated peptide* - Peptides were labeled with ^{14}C methyl groups *via* reductive amination using ^{14}C labeled formaldehyde (Jentoft and Dearborn, 1979). Briefly, peptides were resuspended in water and 10x PBS was added followed by 1% ^{14}C formaldehyde (1mCi ^{14}C formaldehyde per 6 mg of peptide). Sodium cyanoborohydride was added, mixed, and incubated for 4-48 h. Purification was performed using StrataX columns (Phenomenex, Torrance, CA, USA). Labeled peptide was eluted with 2% formic acid in methanol. The solvent was then evaporated with a blowdown evaporator, and

assessed analytically using liquid scintillation counting and HPLC equipped with an in-line scintillation detector.

8. *Whole body autoradiography* - Frozen carcasses were allowed to off gas hexane overnight at -20°C and then embedded in chilled 2% carboxymethylcellulose (Sigma Aldrich, St. Louis, MO, USA) and sectioned sagittally at a thickness of 40 µm on a H/I Bright 8250 Cryostat (Hacker Instruments). Radioactive control guides were drilled into each block to verify even cutting depth along the length of the section. The radioactive guide consisted of ^{14}C-glycine (American Radiolabeled Chemicals, St. Louis, MO, USA) at 0.5 µCi/ml in 0.5% BSA (Sigma Aldrich, St. Louis, MO, USA) in PBS. Sections were collected onto 4-inch wide tape (Scotch 821, ULINE) at 2-4 depths to sample approximately 30 tissues. Collected sections were freeze dried in the cryostat for 48-72 h then mounted on sturdy paper and covered with a single protective layer of cellophane (Reynolds Food Service Film). Mounted sections were exposed to phosphor imager plates (Raytest) along with a radioactive standard Curve (146S-PL, ARC) for 7 days, scanned on a Raytest CR-35 at "25 µm sensitive" setting.

9. *Immunohistochemistry (IHC)* – The expression of cleaved caspase-3 (CC3) was done by IHC on RH28 tumors, liver, and kidney from mice treated with vehicle or 100nmol THP1-VCP-MMAE. Mice were treated and euthanized after 48 hours. Samples were formalin-fixed, paraffin-embedded and sectioned at four microns. Antigen retrieval was performed using pH 9.0 Tris-EDTA based solution (CC1, Roche Diagnostics). Sections were incubated with 1:400 primary antibody against CC3 (Cell Signalling Technologies #9664) for 60 minutes. Sections were then incubated with 1:300 goat anti-rabbit secondary antibody (Vector Laboratories, BA-1000). Staining was developed by 3,3′-diaminobenzidine (DABMap kit, Roche Diagnostics) and sections counterstained with hematoxylin, bluing and then cleared and mounted with MM24 synthetic media.

10. *Cell viability* - Cells were seeded in a 96-well plate at 5,000 cells per well and grown overnight at 37°C in 5% CO_2. Cells were then treated in triplicate with 100µl of DMEM+10%FBS containing varying concentration of MMAE or THP1*-MMAE and

incubated for 3 days at 37°C in 5%. Cell viability was measured using the CellTiter Glo Assay (Promega, Madison, WI, USA) by adding 100µl of reconstituted reagent to each well and incubating with shaking for 10 min. Fluorescence was then measured using a Synergy H4 plate reader (BioTek Instruments, Winooski, VT, USA). Cell viability was expressed as a percentage relative to untreated cells. We subtracted the background by determining the fluorescence of wells containing no cells but only media and CellTiter Glo reagent. Percent viability was reported as (sample-background)/(untreated control-background) with error bars representing standard deviation.

11. *Statistical analysis* - All data were presented as the average value ± the SD of n independent measurements. Graphs were made using GraphPad Prism. Two-tailed Student's t-tests were used to evaluate and statistical significance was assigned for p values of <0.05.

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