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Design, Synthesis, And Characterization Of Dimeric Lysosomal Inhibitors And Their Effect On Cancer Biology

Michael Nicastri
University of Pennsylvania, nicastri@sas.upenn.edu

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Design, Synthesis, And Characterization Of Dimeric Lysosomal Inhibitors And Their Effect On Cancer Biology

Abstract
When detected early and surgically removed, melanoma has a cure rate greater than 90%. However, when the cancer is either unresectable or goes undetected until late-stage metastases develop, the five-year survival rate drops to 20%. Unfortunately, the current standard of care suffers from chemotherapeutic resistance, where the outcome results in a high rate of patient mortality. Recent clinical studies have demonstrated hydroxychloroquine-mediated inhibition of autophagy presents a promising strategy as an adjuvant therapy for melanoma treatment. Regrettably, in clinical trials hydroxychloroquine has not demonstrated reliable potency, at the maximum tolerated doses, in producing autophagy inhibition.

Work by the Winkler and Amaravadi Laboratories has focused on developing a series of dimeric chloroquine-based autophagy inhibitors. Our findings encouraged us to develop a library of dimeric inhibitors, based on hydroxychloroquine and its congener, quinacrine. These not only inhibit autophagy, but also possess single agent anti-cancer activity previously unobserved with small-molecule autophagy inhibitors. Through our efforts we have designed DQ661, a dimeric quinacrine autophagy inhibitor which simultaneously inhibits key drivers of oncogenesis: autophagy, mTOR kinase signaling, and macropinocytosis. We have also discovered an analogous dimeric chloroquine inhibitor, DC661, which is a potent inhibitor of autophagy and cancer cell proliferation. Utilizing these compounds, we have identified a molecular binding partner for our dimeric inhibitors: protein palmitoyl thioesterase 1 (PPT1), a key regulator of palmitoylation within the lysosome. We are currently developing chemical tools to study the role of PPT1 in cancer biology.

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Jeffrey D. Winkler

Keywords
Autophagy, Cancer, Chloroquine, Quinacrine

Subject Categories
Chemistry

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DESIGN, SYNTHESIS, AND CHARACTERIZATION OF DIMERIC LYSOSOMAL
INHIBITORS AND THEIR EFFECT ON CANCER BIOLOGY

Michael C. Nicastri
A DISSERTATION
in
Chemistry
Presented to the Faculties of the University of Pennsylvania
in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
2018

Supervisor of Dissertation
____________________________________
Dr. Jeffrey D. Winkler
Merriam Professor of Chemistry

Graduate Group Chairperson
____________________________________
Dr. Gary A. Molander
Hirschmann-Makineni Professor of Chemistry

Dissertation Committee
Dr. Amos B. Smith III, Rhodes-Thompson Professor of Chemistry
Dr. William P. Dailey, Associate Professor of Chemistry
Dr. E. James Petersson, Associate Professor of Chemistry
DEDICATION

This work is dedicated to my parents and grandparents. Through their support I have been able to pursue a life and career in science.
ACKNOWLEDGMENT

First, I would like to thank my research advisor, Professor Jeffrey D. Winkler, for his support and training. In my time at the University of Pennsylvania, I have grown significantly as a scientist and as a professional. Without his mentorship, this growth would not have been possible.

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Third, I would like to thank my dissertation committee, Professor Amos B. Smith, Professor William P. Dailey, and Professor E. James Petersson, for their advice at annual meetings and during their own office hours. Their advice was particularly important in maintaining the focus of my research in a highly collaborative endeavor.

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Fifth, I would like to thank my friends and colleagues in the Winkler Laboratory and chemistry department at the University of Pennsylvania. Their support and friendship made the process of dissertation research enjoyable. Specifically, I would like to thank
Jack Ferrie, Stan Najmr, and Ben Partridge, three friends from my graduate student class who have helped me grow as a person and as a chemist. Next, I would like to thank Tyler Higgins, a member of the Winkler Laboratory who is both a supportive colleague and friend. Particularly, I would like to thank Tyler for listening to all my chemistry hypotheses on a daily basis, regardless of their impulsiveness.

Finally, I would like to thank my family for supporting me through my five years at the University of Pennsylvania. Through their support, I was able to succeed in growing as a chemist and as a person through my time in graduate school. I would like to thank my father Chris Nicastri and my mother Jamie Nicastri for inspiring me to become the adult I am today. Particularly, I would like to thank my fiancée Bianca Llaneza for her support. She is my best friend and the one who helped me bounce back from unsuccessful research, always giving me the motivation to continue with a new experiment.
ABSTRACT
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Jeffrey D. Winkler

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<th>Description</th>
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<tbody>
<tr>
<td>1205Lu</td>
<td>Cell line of lung metastases of melanoma</td>
</tr>
<tr>
<td>4EBP1</td>
<td>4 E binding protein 1</td>
</tr>
<tr>
<td>4-Mu</td>
<td>4-methylumbelliferone</td>
</tr>
<tr>
<td>A375P</td>
<td>Cell line of BRAF mutant melanoma</td>
</tr>
<tr>
<td>ABPP</td>
<td>Activity based protein profiling</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption distribution metabolism excretion</td>
</tr>
<tr>
<td>APT1</td>
<td>Acyl protein thioesterase 1</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy gene</td>
</tr>
<tr>
<td>Baf</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td>BHQ3</td>
<td>Black hole quencher 3</td>
</tr>
<tr>
<td>BINAP</td>
<td>(2,2'-bis(diphenylphosphino)-1,1'-binapthyl)</td>
</tr>
<tr>
<td>Boc</td>
<td>Tert-butoxy carbonyl</td>
</tr>
<tr>
<td>Cbz</td>
<td>Carboxy benzyl</td>
</tr>
<tr>
<td>CLogP</td>
<td>Computed partition coefficient</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CTSD</td>
<td>Cathepsin D</td>
</tr>
<tr>
<td>DAGLB</td>
<td>Diacylglycerol lipase beta</td>
</tr>
<tr>
<td>DC</td>
<td>Dimeric chloroquine</td>
</tr>
<tr>
<td>DM</td>
<td>Dimeric mefloquine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DP</td>
<td>Dimeric primaquine</td>
</tr>
<tr>
<td>DQ</td>
<td>Dimeric quinacrine</td>
</tr>
<tr>
<td>EDCI-HCl</td>
<td>N-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
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<td>EtOAc</td>
<td>Ethyl acetate</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FQ</td>
<td>Ferroquine</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
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<tr>
<td>HCOOH</td>
<td>Formic acid</td>
</tr>
<tr>
<td>HCQ</td>
<td>Hydroxychloroquine</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HOBT</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>INCL</td>
<td>Infantile neuronal ceroid lipofuscinosis</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
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</table>
$i$-Pr isopropyl

$K_d$ Dissociation constant

$K_{off}$ Off rate of an enzyme

KOH Potassium hydroxide

LC Liquid chromatography

LC3 Light chain 3

LCMS Liquid chromatography mass spectrometry

LGALS3 Galectin 3

LiAlH$_4$ Lithium aluminum hydride

LMP Lysosomal membrane permeabilization

LogD Distribution coefficient

LUMO Lowest unoccupied molecular orbital

MAGL Monoacylglycerol lipase

MAPK Mitogen activated protein kinase

Me Methyl

MeI Methyl iodide

MEK Mitogen activated protein kinase kinase

MeOH Methanol

MQ Mefloquine

MS Mass spectrometry

mTOR Mammalian target of rapamycin

mTORC1 Mammalian target of rapamycin complex 1
<table>
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<th>Full Form</th>
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<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel nitrilo-triacetic acid</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD.Cg-Prkdscid</td>
<td>A genetic variant of mice which are immunocompromised</td>
</tr>
<tr>
<td>Il2rgtm1Wjl/SzJ</td>
<td>Nonidet-P40 (detergent)</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet-P40 (detergent)</td>
</tr>
<tr>
<td>PANC1</td>
<td>Cancer cell line from human pancreas</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium (0) on carbon support</td>
</tr>
<tr>
<td>PD-1</td>
<td>Program cell death protein 1</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced electron transfer</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPT1</td>
<td>Palmitoyl protein thioesterase 1</td>
</tr>
<tr>
<td>PQ</td>
<td>Primaquine</td>
</tr>
<tr>
<td>pS6</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>QN</td>
<td>Quinacrine</td>
</tr>
<tr>
<td>QY</td>
<td>Quantum yield</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SF9</td>
<td><em>Spodoptera Fugiperda</em> 9</td>
</tr>
<tr>
<td>t-Bu</td>
<td>Tert-butyl</td>
</tr>
<tr>
<td>TBTU</td>
<td>1,1,3,3-Tetramethylaminium tetrafluoroborate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>TM</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMS-I</td>
<td>Trimethylsilyl iodide</td>
</tr>
<tr>
<td>Trt</td>
<td>Trityl</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51 like autophagy kinase</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-high performance liquid chromatography</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>VPS34</td>
<td>Vacuolar protein sorting 34</td>
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CHAPTER 1: STUDY OF AUTOPHAGY INHIBITION IN MELANOMA

Malignant melanoma presents a significant challenge to public health. While early detection and surgical resection lead to a 90% cure rate, malignancies which cannot be removed present a severe health risk. Patients diagnosed with unresectable melanoma have a poor prognosis, with a five-year survival rate under 20%. This population of melanoma patients performs poorly in a clinical setting, experiencing high rates of metastases and acquired chemotherapeutic resistance.¹

Advances in genome sequencing have elucidated specific mutations in regulatory pathways which have been found to be pro-tumorigenic. In melanoma, approximately 52% of malignancies have a mutation in BRAF kinase (V600E).² BRAF is a serine/threonine kinase involved in mitogen activated protein kinase pathway (MAPK) signaling, a key regulator of cell growth and proliferation. Aberrant MAPK signaling is found in a large number of human cancers.³ The prevalence of mutations in BRAF kinase in melanoma has driven multiple small molecule drug discovery efforts aimed at modulating the BRAF/MAPK pathways. Specifically, these efforts have led to the synthesis, validation, clinical trials, and FDA approval of multiple kinase inhibitors which now represent the first course of treatment. MEK, mitogen activated protein kinase, is a second kinase which is hyperactive within cancers with aberrant MAPK signaling. Clinical trials have demonstrated that patients with BRAF-mutant melanoma benefit from treatment with MEK inhibitors.⁴ The current standard of care for melanoma with a BRAF mutation is the combination of dabrafenib and trametinib, kinase inhibitors of BRAF and MEK, respectively.⁵ Unfortunately, while these treatments can
demonstrate immediate efficacy in a clinical setting, they lack durability, with significant patient populations acquiring chemotherapeutic resistance. 6

Immunotherapy has shown significant promise as a new strategy for treating metastatic melanoma. PD-1 checkpoint blockade and adoptive T-cell therapy (ACT) have been shown to elicit a response in approximately a third of treated patients.7 While immunotherapy has shown strong durability in patients with melanoma, only a select group among the patient population exhibits a positive response.8,9 The limited success of immunotherapy underscores the unmet medical need for further therapeutic strategies for melanoma treatment.

Metastatic melanoma relies heavily on the use of cellular autophagy to further cell proliferation and overcome therapeutic induced stress. Autophagy has been found to promote acquired drug resistance in melanoma treated with combined BRAF and MEK inhibition.10 Macroautophagy (hereafter referred to as autophagy) is a vesicular trafficking pathway which maintains cellular homeostasis by facilitating the degradation of marked cargo throughout the cellular milieu. The formal discovery of this pathway in the 1990’s by Yoshinori Ohsumi was subsequently awarded the Nobel Prize in physiology and medicine in 2016. Autophagy is a conserved pathway through eukaryotic cells, affecting both normal functioning cells, and cells under metabolic and oxidative stress. On an organismal level, the pathway is integral to development and homeostasis. On an cellular level, the pathway is specifically charged with the degradation of protein aggregates, long-lived organelles, and long-lived macromolecules.12 Autophagy is marked by the elongation of vesicles (phagophores) which upon maturation become
distinct double-membraned vesicles known as autophagosomes. These autophagosomes are formed around cargo marked for degradation and are trafficked to the lysosome for recycling. Once degraded by the lysosome, the constituent building blocks are released back into the cytoplasm, fueling cellular repairs, cell growth, and cell proliferation.\

In a properly functioning cell, autophagy acts as a cytoprotective and anti-tumorigenic pathway. In nutrient-replete conditions, autophagy plays an active role in cell homeostasis. Specifically, the signaling mechanisms which induce autophagy are directly linked to the amino acid-sensing capabilities of the cell. Mammalian target of rapamycin (mTOR) acts as the master regulator of eukaryotic cell metabolism, acting as both a sensor and a signal relay between the cell’s anabolic and catabolic mechanisms. It is widely accepted that functioning autophagy has a strong correlation with individual cell survival. However, the role of autophagy in tumorigenesis is strongly context-dependent.  

In vitro studies have demonstrated that under nutrient-starved conditions, similar to those found in solid tumors, inhibition of autophagy increased the amount of apoptotic cell death. When pro-apoptotic therapeutics are used against cancer cells, upregulated autphagic activity has been shown to blunt drug efficacy. Further, when autophagy is inhibited, the efficacy of pro-apoptotic therapies is improved.\

Autophagy can be viewed in two halves. The first half is early-stage autophagy where multiple signaling pathways converge on key signaling loci, inducing the elongation of the characteristic double-membraned phagophores. The second half is late-stage autophagy, marked by the trafficking and eventual vesicular fusion of mature
autophagosomes with a lysosome organelle.\textsuperscript{17,18} Multiple drug discovery efforts have tried to inhibit early-stage autophagy. However, these efforts have not led to any promising drug leads in the clinic. The cell signaling pathways which comprise early-stage autophagy contain numerous redundancies which complicate inhibition. Late-stage autophagy lacks the same breadth of redundancies. Lysosomal-autophagosomal vesicular fusion is required for completion of the recycling pathway. Inhibition of this fusion prevents the introduction of lysosomal catabolic enzymes to the substrates within the autophagosomes, thus inhibiting the formal act of recycling. The lack of redundancies which can take the functional role of the degradative enzymes of the lysosome makes this vesicular fusion event a prime target for inhibition of the pathway.

Chloroquine (CQ, compound 1, Aralen\textsuperscript{®}) has emerged as one of the most commonly used inhibitors of late-stage autophagy. Chloroquine is a long standing antimalarial compound which was found to be lysosomotropic in eukaryotic cells.\textsuperscript{19} The accumulation of this compound in the lysosome prevents proper function of the organelle, including the lysosomal-autophagosomal vesicular fusion event. Clinical trials for CQ and hydroxychloroquine (HCQ, compound 4, Plaquenil\textsuperscript{®}), a congener of CQ 1, as autophagy inhibitors have shown compelling but unreliable results. CQ/HCQ-mediated autophagy inhibition, which is beneficial in cellular models, is not efficiently reproduced on an organismal level.\textsuperscript{10,17,20,21} These results highlight the unmet medical need for potent small molecule inhibitors of autophagy.
1.1 Detailed Explanation of Autophagy Pathway

Significant efforts have been made to characterize the autophagy pathway in eukaryotic cells. A family of genes referred to as autophagy related genes (ATG) has been identified through significant efforts in yeast, humans, and other organisms. The distinct steps which bring immature phagophores to autolysosomes can be broken down as follows: initiation, nucleation, maturation of autophagic vesicles, and autophagic-lysosomal vesicle fusion. The signaling pathways begin where receptor tyrosine kinases (RTKs) initiate a phosphorylation cascade through members of the ATG family. The initiation phase involves multiple phosphorylation/dephosphorylation cascades of ATG proteins, including ATG1, ATG13, and Ulk1. These proteins make up the Ulk1 complex, a key signaling locus in the induction of autophagy. Ulk 1 interacts with the phosphoinositide3-kinase (PI3K) class 3 complex of enzymes. This complex of PI3K proteins (PI3K-III) contains both Beclin1 and vacuolar protein sorting 34 (Vps34). Beclin1 is a critical autophagy gene required for autophagosome formation and apoptosis. Vps34 is a kinase which participates in lipid phosphorylation and mTOR signaling. The Ulk1 complex and the PI3K-III complex act sequentially in the induction of autophagy, resulting in the initial formation of the phagophore around cargo marked for degradation. Both the Ulk1 complex and the PI3K-III complex have been studied as targets for autophagy inhibition. However, such inhibitors have not achieved efficacy in organismal models.

The maturation of autophagic vesicles is closely associated with the status of light chain 3 (LC3), a protein which is integrated into the membrane of mature autophagosomes. LC3 is a member of the ATG8 family and is modified via proteolysis.
by ATG4. This proteolysis event marks the formation of LC3-I, a substrate for lipidation with phosphatidylethanolamine. The lipidation of LC3-I forms LC3-II, a protein which can be directly correlated to the number of mature autophagic vesicles.\textsuperscript{18,25} LC3-II directly enables both the elongation of the autophagosomal membranes and the recruitment of cargo to be degraded by acting as a recognition point for effector proteins such as p62.\textsuperscript{26}

Mature autophagic vesicles contain cargo marked for degradation. However, they do not contain the catabolic enzymes required for degradation. The vesicles must first fuse with the lysosome, the cell organelle which contains catabolic enzymes. The vesicular fusion event between the lysosome and a mature autophagosome marks end-stage autophagy, where the fused vesicles become an autolysosome. The cargo within the autolysosome is degraded and redistributed to the cell as building blocks for the biosynthesis of new macromolecules.
Figure 1.1: A. The autophagy Pathway. Signaling cascades which activate the Ulk 1 complex cause a signaling cascade which activates the PI3K-III complex. Activation of PI3K-III complex sets in motion the nucleation of autophagic vesicles, first as an immature phagophore, eventually maturing into an autophagosome. Lysosome-autophagosome vesicle fusion occurs to give an autolysosome, allowing degradation to occur. Eventually the autolysosome releases the degraded cargo as its constituent macromolecular building blocks.

B. Light chain 3 (LC3) is a key membrane protein involved in the formation of autophagic vesicles (below). LC3 is first proteolyzed by ATG4, making it a substrate for lipidation (LC3-I) with phosphatidylethanolamine. Lipidation yields a protein (LC3-II) which is incorporated into the nucleating immature autophagic vesicle.
1.2 Small molecule inhibitors of autophagy signaling

The inhibition of autophagy is an attractive therapeutic strategy against aggressive malignancies because of the ties between autophagy and cancer cell metabolism. The most common approach taken by these drug discovery efforts relies on inhibition of early autophagic signaling. The two major targets which have recently been pursued in the field are Ulk1 and the PI3K-III complex, the inhibition of each of which have been successful in cell models.²³,²⁴,²⁷

Multiple strategies for autophagy inhibition have led to the development of anti-cancer agents with in vitro potency but without comparable effects in organismal models. A struggle for the strategy of autophagy inhibition is the ability to translate in vitro success to organismal models, let alone to the clinic. Spautin-1, a small molecule kinase inhibitor of ubiquitin specific peptidase 10 (USP10) and ubiquitin specific peptidase 13 (USP13), prevents the deubiquitination of Vps34-containing complexes (PI3K-III), resulting in their degradation. The loss of these complexes containing Vps34 results in inhibition of early autophagy in mouse embryonic fibroblasts. Despite autophagy being inhibited, no anti-cancer activity or organismal model efficacy was demonstrated.²⁷ A small molecule inhibitor has been developed for Ulk1, a critical signaling locus for the induction of autophagy. SBI-0206965 blocks kinase activity of Ulk1, suppressing the phosphorylation of Vps34, thus inhibiting autophagy by slowing the induction of the pathway. Egan and coworkers demonstrated that Ulk1 inhibition can be combined with mTOR inhibition to result in anti-cancer activity in cellular models. However, no single-agent anti-cancer activity was reported for SBI-0206965.²³
1.3 Efforts exploring CQ and its derivatives for their anti-cancer properties

CQ 1 is a quinoline-based anti-malarial therapeutic, first synthesized in 1934. The synthesis of this drug was spurred by the need for a synthetic anti-malarial alternative to quinine. At the time of its initial synthesis and biological testing, both its structure and biological activity were compared to quinacrine (QN, Atabrine®) and Primaquine (PQ, Plasmochin®) (Figure 1.2). Before the invention of CQ, QN, and PQ, the front-line anti-malarial drug was quinine. Extracted as a natural product, the distribution was monopolized by the Dutch-East India company. As both political and scientific climates changed in the mid-1900s, the need for a synthetically derived medicine became paramount. This led to CQ 1 becoming the front-line anti-malarial compound both for treatment of the disease and as a prophylactic. Due to its long history of medical use, chloroquine’s safety profile is also well known.

In addition to its anti-malarial properties, CQ and its congener HCQ have immunosuppressive properties. HCQ, as it is better tolerated than CQ in humans, is FDA-approved as a treatment for rheumatoid arthritis and lupus erythematosus, two autoimmune diseases.
Analysis of the structure of chloroquine and its derivatives gives insight as to the important structural features required for potency and its predicted biophysical properties. Like quinine and its other synthetic congeners, chloroquine contains the bicyclic heterocycle, quinoline. Quinolines, by nature of the lone pair of the nitrogen, are weakly basic. In general, the pKa of a protonated quinoline heterocycle is approximately 5.0, similar but slightly higher than its monocyclic congener pyridine. However, unlike quinine, the quinoline in chloroquine contains a 4-amino substitution, which donates electron density to the heterocycle, resulting in a significantly increased pKa. This increase in pKa has a substantial effect on the biophysical properties of chloroquine, because the pKa is raised higher than the pH of the biological milieu (pH 7.2 – 7.6). According to the experimentally-determined pKa of chloroquine (8.6 and 9.8) and the Henderson-Hasselbach equation, it is predicted that greater than 90% of the inhibitor is protonated at physiological pH. The necessity of the C-7 chlorine appended to the quinoline ring is less understood. Halogens attached to aromatic rings are electron-withdrawing. The high electronegativity of chlorine increases the pKa, i.e., decreasing the basicity, of the 4-aminoquinoline compared to a non-halogenated derivative. The 4-amino substituent of CQ is a secondary aniline connected to the C-4 position of a pentyl chain which contains a tertiary amine at the C-1 position. The lone methyl group in chloroquine generates a stereocenter. Studies have shown that the absolute configuration does have an effect on anti-malarial potency. However, the stereochemistry appears to affect host metabolism and drug clearance rather than target binding.
HCQ is a congener of CQ which only differs by the replacement of one ethyl moiety with a hydroxyethyl functionality. The intended purpose of this hydroxyl group is to impart more desirable biophysical properties to the inhibitor itself. The hydroxyl functional group introduces a new handle for mammalian metabolism. It is this increased metabolic lability of HCQ which is thought to impart the decreased toxicity of HCQ when compared to CQ.34

1.4 CQ/HCQ in Clinical Trials for Cancer

The path of CQ 1 and HCQ 4 into clinical trials for anti-cancer therapy is significantly shorter than that of new drugs due to their long-standing use in modern medicine as anti-malarials. Clinical studies have already tested CQ/HCQ 1/4 as adjuvants in multiple therapeutic strategies. As of March 23, 2018, 56 clinical trials have studied or are studying CQ 1 or HCQ 4 as an adjuvant therapy with other anti-cancer
strategies (FDA.gov). A Phase 1 clinical trial of HCQ in patients with newly diagnosed glioblastoma showed two indicators of autophagy inhibition: a dose-dependent change in autophagic vesicle count and an increase in LC3II. When patients were given a daily dose of either 600mg or 800 mg of HCQ, tumor biopsies showed an increase in autophagic vesicle count and an increase in LC3II. However, at a lower daily dose of 400 mg, only 20% of patients showed a similar response. 36 Currently, there are no clinical trials of non-chloroquine/hydroxychloroquine-mediated autophagy inhibition.

1.5 Derivatives of CQ/HCQ

The prevalence of CQ/HCQ use as a primary treatment and prophylactic treatment for malaria has led to the evolution of many drug-resistant strains of the parasite. One of the many challenges in the field of malaria is countering acquired drug resistance. 37 As a method of combating the acquired drug resistance, medicinal chemistry efforts have produced multitudes of second- and even third-generation chloroquine-derived anti-malarial therapeutics.38 While most efforts have been unsuccessful, a few exceptional analogs have been utilized as frontline therapeutics in certain malaria populations.

Piperaquine 5 is a chloroquine derivative which contains two units of the 7-chloro-4-aminoquinoline heterocycle (Figure 1.4). However, unlike chloroquine, the resulting aniline is tertiary, and is restricted in flexibility by incorporation into a piperazine ring. Piperaquine was developed for its use in chloroquine-resistant P. falciparum and is currently approved with artemisinin as a combination therapy.39
Ferroquine 6 is an analog of chloroquine which has perhaps the most extensive structural changes from the parent compound (Figure 1.4). The compound’s name is derived from the replacement of two methylenes within the chloroquine butyl linker with two carbons of one of the cyclopentadienyl ligands of ferrocene. This incorporation of sp² carbons increases the rigidity of the molecule. This rigidity however, is only a biproduct of the incorporation of a stable source of iron into the small molecule therapeutic. Conceptualization of this inhibitor begins with the proposed mechanism of the anti-parasitic activity of CQ. In the malaria parasite, CQ is found to accumulate in the digestive vacuole and prevent heme crystallization. These CQ-heme aggregates are unable to be metabolized by the parasite and produce oxidative stress through Fenton-type oxidation chemistry. Ferroquine incorporates soluble, but indigestible iron to the acidic vacuole of the parasite with the purpose of increasing the anti-malarial activity of CQ.\textsuperscript{41}

Figure 1.4: The structures of Piperaquine (PQ) 5, and Ferroquine (FQ) 6.
Clinical trials utilizing CQ/HCQ have shown some success in utilizing autophagy inhibition to improve a cancer patient’s prognosis. However, the intracellular concentration required for autophagy inhibition cannot be reliably achieved in a patient population being given the maximum tolerated dose. Therefore, the next logical extension of these clinical studies is to test a more potent derivative of chloroquine which inhibits autophagy more efficiently in organismal models.

Over the 50+ years in which CQ has been utilized as a front-line therapeutic for malaria, multiple strains of *p. falciparum* have been discovered to be resistant to CQ treatment. The proposed mechanism of CQ toxicity towards the malaria parasite is the sequestration of heme resulting in oxidative stress within the digestive vacuole of the parasite. CQ-resistance, which has arisen in multiple strains of malaria, has been attributed to the existence of a proteinaceous efflux transporter. To circumvent this efflux, Roche developed a dimeric CQ inhibitor which is based on 1,2 cyclohexane-diamine (Figure 1.5). This molecule was found to have excellent cytotoxicity towards the malaria parasite, regardless of the CQ-resistant character of the strain. The authors conclude that the increased potency against CQ resistant malaria is due to the efflux transporter not removing the dimeric CQ from the digestive vacuole of the parasite. The dimeric compound by Roche has not been pursued in the clinic because it was found to be too toxic for use in humans. The success of this dimeric reporter against CQ-resistant strains of malaria led to multiple studies, particularly those by Vennerstrom *et. al* and Girault *et. al.*, both of which have developed multiple dimeric CQ derivatives.
was observed that micromolar quantities of both CQ and bisaminoquinoline dimers are required to inhibit heme polymerization in the digestive vacuole. In one of their studies, Vennerstrom et al. utilized a wide series of polyamines as scaffolds to synthesize dimeric inhibitors. Notably these dimeric inhibitors gave activity comparable to CQ in \textit{p. falciparum}. Girault et al. later expanded upon this idea, producing dimeric and trimeric CQ inhibitors which demonstrated comparable inhibitory activity to CQ in CQ-resistant \textit{p. falciparum}. In their work, Girault et al. observed linear dimers and rigidified dimers possess similar anti-parasitic activity, but rigidified dimers possessed less host toxicity than the flexible linear dimers.

The identity of the efflux transporter for CQ in the human lysosome is not known. The need of a better lysosome-accumulating CQ analog appears to mirror the problem which dimeric CQ analogs have overcome in CQ-resistant malaria. Inspired by the results of Roche, Vennerstrom \textit{et. al.}, and Girault \textit{et. al.}, the Winkler and Amaravadi Laboratories sought to test the autophagy inhibition potency of dimeric CQ derivatives in comparison to monomeric CQ. It is known that CQ is highly lysosomotropic. Small
molecules which are lipophilic and are weakly basic, like CQ, are known to accumulate in the lysosomal compartment. Multiple studies attribute this phenomenon to the pH gradient between the cytosol and the lysosomal lumen (pH 7.2 vs pH 4.5).\textsuperscript{19,47–50} It was reasoned that this effect could be magnified by increasing the number of basic nitrogen atoms in new derivatives of CQ. The Winkler and Amaravadi Laboratories hypothesized that dimeric CQ derivatives which contain a greater number of basic nitrogen atoms should accumulate in the lysosome to a greater degree than monomeric CQ. Roche reported bisaminoquinoline 7 was photocarcinogenic in cell and mouse models.\textsuperscript{44} The potential carcinogenic properties of bisaminoquinoline 7 make the trans-1,2-diaminohexane scaffold unattractive to utilize in the synthesis of anti-cancer agents. Girault \textit{et. al.} reported that dimeric inhibitors synthesized from linear polyamines were less toxic to human cell cultures than dimers synthesized from rigid cyclic polyamines.\textsuperscript{46} The Winkler and Amaravadi Laboratories ruled out the usage of rigid scaffolds for diamines due to the toxicities which have precluded their use in humans. When designing a dimeric inhibitor, one biophysical factor which was addressed was the increase in hydrophobicity of a dimeric inhibitor when compared to CQ. Compounds which have too low an aqueous solubility will have low bioavailability. Therefore, the smallest triamine-based dimeric inhibitor presented by Vennerstrom and Girault, a bis-aminoquinoline linked by diethylene triamine, was selected (Figure 1.5, Compound 8). To maintain similar functionality to CQ, the internal amine of the linker was methylated, resulting in the structure of Lys-05. This compound was also selected for its synthetic ease, as it is rapidly synthesized from a commercially available triamine and an aryl halide precursor to CQ.
A small library of dimeric analogs was synthesized alongside Lys-05 (Fig 1.7.). The library was designed to test the effects of heterocycle dimerization by comparison of dimeric Lys-05 with a monomerically-arylated diethylene triamine linker, Lys-02 (Figure 1.7, Compound 10). Replacement of the C-7 chlorine with a C-7 methoxy group tested the importance of the halogen to the chloroquine ring (Lys-03) (Figure 1.7, Compound 11). The last analog tested the importance of the tertiary amine and the aliphatic linker, by replacing the triamine with a triethylene glycol derived diamine (Lys-04). While an imperfect control for the presence of a tertiary amine, the diamine precursor was commercially available, and maintained the aqueous solubility of the inhibitor.
The mechanism of action of CQ 1 in malaria is presumed to be the prevention of heme crystallization. While crystallization of heme correlates with parasite survival, a molecular target of CQ 1 has not been established in malaria. The lack of a molecular target for chloroquine-mediated autophagy inhibition makes a target-based medicinal chemistry effort impossible. In lieu of structural biology or target binding information, a phenotypic screening approach was taken, where a cellular or organismal system is used to verify the potency and toxicology of a library of inhibitors. The use of a cellular/organismal phenotypic approach provides an assay output which combines the many factors which contribute to the efficacy of an inhibitor lead. Unlike an in vitro assay where the inhibition of an isolated enzyme is tested, changes made to a molecule can not be directly attributed to the binding affinity of the inhibitor for a target enzyme. Therefore, when applying a phenotypic approach, the assay criteria which is measured to interrogate the effectiveness of an inhibitor is critical for the success of the experiment. In order to design a more potent CQ 1 derivative which has organismal potency, three
assays were critically important: cell proliferation assays, autophagy inhibition assays, and tumor growth suppression in xenograft assays.

The compounds were first tested as anti-proliferative agents against 1205Lu melanoma cells by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay cell proliferation assay. The MTT assay determines whether a cell currently is actively metabolizing. An IC$_{50}$ concentration can then be determined which establishes the dosage at which 50% of the cells have active metabolism or cell proliferation. Lys-05 9 performed significantly better than CQ/HCQ, demonstrating a 6 uM IC$_{50}$ for single agent anticancer activity. While in one cell line it appears that Lys-04 12 also demonstrated a significant increase in potency versus CQ 1, Lys-04 12 never showed greater potency Lys-05 9. The panel of compounds was also assayed for autophagy inhibition by using western blotting to quantify the accumulation of LC3-II, the protein corresponding to accumulated mature autophagic vesicles. Lys-05 9 again was observed to show greater potency than CQ/HCQ 1/4 and the entire dimeric inhibitor library. Lys-05 9 was found to not only be a more potent version of CQ/HCQ 1/4, but importantly was ten-fold more potent. It appeared that barring a drastic difference in biophysical properties between Lys-05 and CQ/HCQ 1/4, the gained increase in potency of Lys-05 9 could be attributed to a phenomenon more powerful than a simple increase, i.e., doubling, in local concentration. These results validate the hypothesis that CQ/HCQ-mediated autophagy inhibition could benefit from dimeric chloroquine inhibitors.

The invention of Lys-05 9 had a significant impact on the field of autophagy inhibition. The inhibitor not only shed light on the structure-activity relationships of
chloroquine-mediated autophagy inhibition, but also demonstrated autophagy inhibition in an organismal study. Tumor xenografts implanted on the flanks of mice grew at a slower rate when treated with Lys-05 when compared to HCQ-treated and untreated controls. Further, Western blotting of tumor lysate demonstrated greater autophagy inhibition in the Lys-05-treated mice when compared to HCQ treated mice. Mice treated with Lys-05 suffered from bowel obstruction when compared to the untreated control. The source of the bowel obstruction was found to be Paneth cell dysfunction, a symptom previously reported in autophagy deficient mice. Further, this dysfunction is observed in Crohn’s disease patients with a mutation in the autophagy gene ATG16L1. This result is consistent with autophagy inhibition in an organismal model. While it is encouraging to see autophagy inhibition manifest as an organismal phenotype, this result also highlights a potential dosing limit, decreasing potential therapeutic windows.
CHAPTER 2: THE SECOND GENERATION OF DIMERIC CHLOROQUINE INHIBITORS

The small pilot library which was tested during the discovery of Lys-05 gave insight into elements which should be conserved in the design of future CQ derivatives. We became interested in the importance of linker length, the chain length of the aliphatic triamine which forms the scaffold of Lys-05. From a synthetic perspective, the linker length is easily modified by starting from triamines comprised of different aliphatic chain lengths. Buchwald-Hartwig arylation utilizing Pd(OAc)$_2$ and BINAP as the catalyst system is selective for primary amine arylation over secondary amine arylation (Figure 2.1). We turned to the literature to identify known triamines which could be used as scaffolds for new dimeric inhibitors. Spermidine 16 is a triamine natural product found in eukaryotic cells and is commercially available. Spermidine 16 differs from diethylene triamine in that it has an unsymmetrical number of carbons between the nitrogen atoms of the triamine, and it is three atoms longer (Figure 2.1). The additional carbons add more hydrophobic character to the structure of the inhibitor, but also increase the flexibility of the molecule. Without a validated cellular target, the active conformation of Lys-05 9 is unknown; it was hypothesized that a more flexible molecule would allow a dimer to sample more potential binding conformations with each CQ heterocycle.
It should be noted that compound 17 had been previously synthesized in malaria studies via SNAr reaction between spermidine and 4,7-dichloroquinoline.\textsuperscript{46} Synthesis of 17 is improved by Buchwald-Hartwig arylation (Figure 2.1). The use of 17 as a dimeric autophagy inhibitor is a novel application of the small molecule.\textsuperscript{53}

In a head-to-head comparison, 17, Lys-05 9, and CQ 1 were compared in two different cell viability assays (Figure 2.2). The first, an MTT assay, is a short duration single dose assay, which indicates the effect of the inhibitor on immediate cell proliferation. The second is a long-term colony formation assay, where cancer cells are treated once with the inhibitor, but are allowed to continue proliferating for two weeks post treatment. In MTT assays, both 17 and Lys-05 9 perform significantly better than
CQ but are not significantly different. However, in the long-term colony formation assay, 17 demonstrates significantly better anti-proliferative activity than Lys-05 9. These results sparked a more thorough exploration of the role which the triamine linker plays in the potency of dimeric chloroquine inhibitors (Figure 2.3).

Figure 2.2: Comparison of Lys-05 9 and DC340 17 in two-week colony formation assays and MTT cell proliferation assays (A375P melanoma). Reproduced from Rebecca et. al. 53

A second area that we explored was the role of the substitution of the central nitrogen of the triamine linker. The success of DC340 17 made apparent that secondary amines could be tolerated within triamine linked dimeric chloroquine inhibitors. When
comparing the computed biophysical properties, it became obvious that central nitrogen methylation increased the hydrophobicity of the designed inhibitor. However, perhaps less obvious is the negligible change in the calculated of pKₐ the amine (17 pKₐ = 9.59, 18 pKₐ = 9.54). While it is well accepted that tertiary amines are significantly more basic than their primary amine counterparts, the difference between secondary amines and tertiary amines is not as significant. Tertiary amines do gain electron density from their alkyl substituents; however, they also gain a significant steric penalty when completely substituted. In most acyclic cases, these two effects balance themselves out. Therefore, differences in the properties of secondary amine or tertiary amine linked dimers will more likely reflect the difference in the hydrophobicity of the drug, steric contribution to a binding interaction, and/or potential cell metabolism.

2.1 The second generation of dimeric lysosomal inhibitors – dimeric quinacrine

The invention of Lys-05 9 produced structure activity relationship data which highlighted the importance of the C-7 chlorine on the quinoline.51 However, this study did not comprehensively study the importance of heterocycle identity. In the field of anti-
malarial therapeutics, multiple quinoline based heterocycles exist as substitutes for chloroquine. Quinacrine 2 (QN, Atabrine®), primaquine (PQ, Primaquine®) 3, and mefloquine (MQ, Lariam®) 19 have been used as anti-malarial medicines. Working with Dr. Noel McLaughlin, we derived three new classes of dimeric autophagy inhibitors from these existing anti-malarial therapeutics. We wanted to test the hypothesis that dimeric inhibitors synthesized from these heterocycles could be used as anti-cancer agents. As a later independent effort, I designed and synthesized a series of inhibitors based on piperaquine, the only clinically used dimeric chloroquine anti-malarial (Chapter 2, Synthesis of Piperaquine Derivatives). We also synthesized dimeric forms of ferroquine, a newly emerging anti-malarial compound which has demonstrated anti-proliferative activity in cancer (Chapter 2, Synthesis of Ferroquine Derivatives).54
We designed a specific nomenclature to refer to dimeric inhibitors derived from existing anti-malarial heterocycles. Dimeric inhibitors are described first by a two-letter code, “D” for dimeric, followed by the first letter of the anti-malarial heterocycle. (DC for dimeric chloroquine). Following the two-letter code is a three-digit number, where the first digit corresponds to the length of the first alkyl chain of the triamine, followed by a second digit corresponding to the length of the second alkyl chain of the triamine. The final digit corresponds to whether the central nitrogen of the triamine is a secondary amine (0) or a methylated tertiary amine (1). Utilizing this new nomenclature, Lys-05 9 is
defined as DC221 (Figure 2.5).

**Dimeric Inhibitor Nomenclature**

\[
\text{Ar} = \begin{array}{c}
\text{Cl} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{Cl} \\
\text{OMe} \\
\text{OMe} \\
\text{CF}_3 \\
\text{CF}_3 \\
\text{Ar} \\
\text{Ar}
\end{array}
\]

- **Quinacrine (DQ)**
- **Chloroquine (DC)**
- **Primaquine (DP)**
- **Mefloquine (DM)**

Two letter code where X identifies the heterocycle: 
- DQ = Quinacrine, DC = Chloroquine
- DP = Primaquine, DM = Mefloquine

Followed by three numbers ###
- 1st # = m, 2nd # = n
- 3rd # = 0 / 1 (R=H / Me)

**Figure 2.5: Key for the nomenclature for identifying dimeric inhibitors derived from antimalarial therapeutics.**

Spermidine was selected as a triamine scaffold to test the effect of dimerization on the other heterocycles. The first three aryl halides were synthesized by Dr. Noel McLaughlin according to literature precedent, each corresponding to either QN 2, PQ 3, or MQ 19 (Figure 2.6). The acridine precursor of dimeric quinacrine inhibitors (DQ) was synthesized as a 6,9-dichloro-2-methoxyacridine. Compound 22 was synthesized by an Ullman coupling of p-anisidine 21 (Acros Organics) and 2,4-dichlorobenzoic acid 20 (Acros Organics). Ullman couplings are known to be directed and significantly accelerated by an ortho-carboxylic acid moiety, which enabled the product 22 to be isolated as a single isomer. The benzoic acid 22 derivative was then cyclized by Friedel-Crafts acylation and chlorinated in situ with neat phosphorus(V) oxychloride, heated to reflux, to afford 23. The resulting phenol 23 was chlorinated in situ to afford the 6,9-dichloro-2-methoxyacridine, 24. The primaquine (DP) aryl chloride precursor, 26, was
synthesized according to the method of Childers and coworkers. The heterocyclic precursor of dimeric mefloquine (DM) was synthesized first from the condensation/cyclization reaction of 2-trifluoromethyl aniline (Sigma Aldrich) and ethyl 4,4,4-trifluoro-3-oxobutanoate (Oakwood Chemical) in polyphosphoric acid, resulting in 4-hydroxyquinoline, 29 was chlorinated by phosphorus(V) oxychloride to give compound 30.

Palladium-catalyzed C-N arylation was then used to synthesize dimers from commercially available spermidine as a linker. Spermidine 16 (Acros Organics) was selected as a linker for this second-generation library because of its success in the case of DC340 17. The Pd(OAc)$_2$/BINAP catalyst system is compatible with spermidine, which contains both primary amine and secondary amine functionality, due to the significantly slower rate of the arylation of acyclic secondary amines compared to primary amines.
The Pd(OAc)$_2$/BINAP catalyst in combination with potassium phosphate tribasic as a base yields DC340 17 with excellent chemoselectivity. Dimeric forms of quinacrine 31 (DQ340) and mefloquine (DM340) 33 were synthesized in reasonable yields, demonstrating the toleration of aryl chlorides by this catalyst system. Dimeric primaquine (DP340) 32 was synthesized in only trace quantities using these reaction conditions.

Analysis of the crude reaction mixture by proton nuclear magnetic resonance ($^1$H NMR) showed the starting materials did not decompose. The base utilized in the reaction was switched from potassium phosphate tribasic (K$_3$PO$_4$) anhydrous to anhydrous sodium tert-butoxide (NaO$t$Bu), a stronger base. The source of palladium was also changed to Pd$_2$(dba)$_3$. These new reaction conditions afforded DP340 32 in 83% yield.

The compounds were first subjected to MTT anti-proliferative assays in A375P BRAF mutant melanoma cells. In these assays, the new dimeric inhibitors were compared
to their respective parent monomeric anti-malarial and to Lys-05, the state of the art in
dimeric autophagy inhibitors. As previously observed in MTT assays, cells treated with
DC340 17 exhibited reduced cell proliferation compared to cells treated with either Lys-
05 9 or CQ 1. DM340 33 treated cells exhibited reduced cell proliferation compared to
cells treated with the parent mefloquine 19 (MQ), with activity comparable to Lys-05.
DP340 32 treated cells exhibited reduced cell proliferation compared to cells treated with
the parent primaquine 3 (PQ), with activity comparable to Lys-05 9. Cells treated with
DQ340 31 exhibited reduced cell proliferation when compared to QN 2, and also relative
to Lys-05 9. When compared as a ratio of dimeric/monomeric, cells treated with DQ
exhibited the greatest decrease in cell proliferation. From this result we inferred that
DQ340 31 was the most promising lead molecule tested (Figure 2.8.).

![Diagram of chemical structures](image)

Figure 2.8: MTT assay results for dimeric inhibitors derived from other
commercially available anti-malarial compounds.

Informed by these MTT results, a library of dimeric quinacrine inhibitors was
synthesized in collaboration with Dr. Noel McLaughlin, a postdoctoral researcher in the
Winkler Laboratory. The library was designed to explore two structural features of the
linker region of each dimeric inhibitor: the length of the aliphatic chains linking the nitrogen atoms of the triamine, and the substitution of the nitrogen in the center of the linker. The library was synthesized by arylation of various triamines with 6,9-dichloro-2-methoxyacridine. For each triamine linker, K$_3$PO$_4$ was found to be an adequate base in combination with the Pd(OAc)$_2$/BINAP catalyst system, suggesting that the previously observed difference in reactivity between 8-chloro-6-methoxyquinoline and 6,9-dichloro-2-methoxyacridine lies in the substitution of the aryl halide. Many of the triamines utilized in this library were commercially available (2/2, 3/3, 3/4, and 6/6) or were previously synthesized (4/4, 5/5). However, the remaining triamines (7/7, 8/8, and 11/11) were not known and required the development of a synthetic strategy.

2.2 Synthesis of a DQ library

Triamines based on heptyl and octyl aliphatic chains were prepared from commercially available dibromoalkanes (1,7-dibromoheptane 34 and 1,8-dibromooctane 35). Sodium azide was selected as a protected nitrogen source because of its greater reactivity as a S$_\text{N}2$ nucleophile when compared to potassium phthalimide. Both 1,7-dibromoheptane 34 and 1,8-dibromooctane 35 were subjected to S$_\text{N}2$ reaction with sodium azide in dimethylsulfoxide (DMSO) at room temperature (23 °C). Use of the dibromoalkanes in excess (2.2 equiv.) allowed for minimization of bis-S$_\text{N}2$ product and afforded the mono-substitution product 37 in reasonable yield. Benzylamine was selected as a divalent nitrogen source to link the protected triamine. S$_\text{N}2$ alkylation of benzylamine with 37 in n-butanol afforded product 39 in serviceable yields. The azides can be converted to primary amines 41 with simultaneous debenzylation of the interior
tertiary amine via hydrogenolysis catalyzed by 10 mol% palladium (0) on carbon support (Pd/C) in methanol/ethyl acetate. The triamine products were then utilized in the palladium catalyzed arylation reactions. (Figure 2.9)

The 11/11 linker was synthesized from 11-aminoundecanoic acid 42, a natural product isolated from castor oil (Arkema). First, 11-aminoundecanoic acid 42 was converted into 11-aminoundecanol 43 by lithium aluminum hydride reduction in tetrahydrofuran (THF), upon heating the reaction to reflux. The resulting aminoalcohol 43 was protected by acylation with benzyl chloroformate to yield the Cbz-protected aminoalcohol 44. Previous results demonstrated alkylation of benzylamine with alkyl bromides was poor yielding. Fortunately, the existing alcohol functionality makes oxidation followed by reductive alkylation of benzylamine to be step economical.

Therefore, alcohol 44 was oxidized under Swern conditions to yield 45. Benzylamine was then reductively alkylated twice with the aldehyde, using sodium triacetoxyborohydride as a reducing agent, to synthesize compound 46. We attributed the improved efficiency of this transformation to the elimination of over alkylation. In contrast, S_n2 alkylation of primary amines often results in the production of tetraalkylammonium species in addition to or to the exclusion of tertiary amines. Unlike sodium borohydride, the three acetoxy substituents of this borohydride derivative reduce the hydric character of the boron-
hydrogen bonds, making the reduction selective for iminium ion intermediates formed in the reductive alkylation reaction. (Figure 2.10)
The resulting protected triamine precursor, 46, was then deprotected in one step utilizing hydrogenolysis in the presence of catalytic Pd/C. The reaction required 30 mol% of catalyst for any consumption of starting material to occur. No consumption of starting material was observed when using 10 mol% of Pd/C, even with prolonged reaction times. Additionally, 1.2 equivalents of hydrochloric acid (HCl) were added in an attempt to activate the benzylic amine toward hydrogenolysis. However still no starting material was consumed in 24 hours. The protected triamine precursor was successfully deprotected upon hydrogenolysis in the presence of 30 mol% Pd/C, rapidly affording the product in good yield 47 (Figure 2.10). With the library of triamine precursors in hand, palladium catalyzed arylation was utilized to synthesize the library of dimeric quinacrine compounds (Figure 2.11).
In Lys-05 9 the amine comprising the center of the linker is a methylated tertiary amine. DC340 17 established the dimeric inhibitors containing secondary amines can also be potent anti-cancer agents. A side-by-side comparison of secondary versus tertiary amine-containing inhibitors was not yet available to establish the role of the alkylation of this nitrogen in the potency of the inhibitors. Both previous work in our laboratory and other studies have established that the aminoquinoline nitrogen in CQ 1 and our dimeric lysosomal inhibitors to be a much weaker nucleophile than the linker central secondary amine. Despite the presence of more than ten-fold molar excess of formaldehyde and sodium triacetoxyborohydride, only the central linker amine is methylated at room temperature (23 °C). This sequence allowed for the synthesis of both secondary and tertiary amine linked dimers to proceed through the same common intermediates.
Reactions of aqueous formaldehyde, dimeric quinacrine inhibitor, and sodium triacetoxyborohydride produced a heterogeneous mixture and required long reaction times. This heterogeneous mixture prevented complete consumption of starting material, thereby complicating product purification. As an alternative, Eschweiler-Clarke methylation conditions were explored for the synthesis of tertiary amine-linked dimeric quinacrine compounds. Secondary amine-linked dimeric quinacrine compounds were soluble in aqueous formic acid. The methylation reaction using aqueous formaldehyde in formic acid, which was heated to reflux, furnished tertiary amine linked compounds in a shorter and more consistent reaction time (approximately 4 hours). Reductive methylation of dimeric inhibitors afforded good yields of the tertiary amine products and allowed for gram-scale synthesis of DQ661 64.
### Table 1: Synthesis Yields

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>DQ331</th>
<th>DQ341</th>
<th>DQ441</th>
<th>DQ551</th>
<th>DQ661</th>
<th>DQ771</th>
<th>DQ881</th>
<th>DQ11111</th>
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<tr>
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<td>61</td>
<td>62</td>
<td>63</td>
<td>64</td>
<td>65</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td>Yield</td>
<td>86%B</td>
<td>94%A</td>
<td>83%A</td>
<td>50%A</td>
<td>91%B</td>
<td>74%B</td>
<td>72%B</td>
<td>75%B</td>
</tr>
</tbody>
</table>

#### Method A:

![Method A reaction](image)

#### Method B:

![Method B reaction](image)

**Figure 2.12:** Synthesis of N-methylated DQ inhibitors by sodium triacetoxyborohydride reductive alkylation, or Eschweiler-Clarke reaction. Superscript on yield indicates the synthesis method used.

### 2.3 Assessment of DQ Biological Activity

We worked in collaboration with Dr. Vito Rebecca of the Amaravadi Laboratory to explore the biological activity of DQ compounds. The dimeric inhibitors were first assessed for their anti-proliferative activity by MTT assay in two cell lines known to have upregulated autophagy: A375P melanoma cells and PANC1 pancreatic cancer cells. These inhibitors were compared to early-stage autophagy inhibitors Spautin-1 and SBI-0206965. In melanoma, compounds with linkers containing aliphatic regions between
three and six carbons in length were significantly more potent than the parent monomeric inhibitor QN 2 and Lys-05 9 (Figure: 2.13). Longer linked dimeric inhibitors, with linkers containing aliphatic regions seven carbons and longer, were significantly less potent. We hypothesized that the compounds lost potency due to increased hydrophobic properties imparted by the longer linker. We anticipated that hydrophobicity would reach a threshold which was detrimental to subcellular localization, aqueous solubility, and/or cell penetration of the inhibitor. The utilization of in cell assays rather than enzymatic \textit{in vitro} assays for developing a structure activity relationship allowed us to combine biophysical properties, drug transport, subcellular localization, and potential target binding into one output.

One result which caught our attention was the modest potency of DQ221 52 in MTT cell proliferation assays. Despite being a dimeric inhibitor, and the quinacrine

![Cell Viability Assays for Dimeric Quinacrine Inhibitors](image)

Figure 2.13: MTT assay results for the DQ inhibitor library compared to QN, CQ, Lys-05, and known early autophagy inhibitors, Spautin-1 (Vps-34 inhibitor) and SBI-0206965 (ULK1 Inhibitor).8,9
congener of the potent dimeric inhibitor Lys-05, DQ221 52 was found not to be more potent than its monomeric parent anti-malarial QN 2. Structurally, DQ221 52 and Lys-05 9 contain the same exact triamine linker, only differing in the structures of the heterocycles. The success of Lys-05 9 establishes that 4-N-Methyl-diethylenetriamine 14 is a linker capable of generating potent dimeric inhibitors from CQ 1. However, the result for DQ221 52 suggested that the alteration of the heterocyclic ring system has changed the requirements of the triamine linker region for effective biological activity. Acridines are approximately 50% larger than quinoline rings, by nature of being tricyclic rather than bicyclic aromatic systems. This fact, coupled to the performance of DQ221 52 in MTT assays, indicates that longer linker lengths are necessary for the more sterically demanding tricyclic acridine-based dimers. Increasing the linker length to a three-carbon linker, dipropylentriamine, produced the expected result, a dramatic increase in inhibitor potency when compared to the parent monomeric compound, QN 2. DQ221 52, DQ330 53, and DQ331 60 do not appear to differ significantly in their aqueous solubility. A fragment-based approach utilized to compute theoretical pKₐ values for the internal nitrogen indicates that Lys-05 9 and DQ221 52 should have reduced basicity in comparison to CQ 1, QN 2, DQ330 53, and DQ331 60. However, structurally it is not obvious why this effect would affect the observed potency of DQ221 52 more than that of Lys-05 9 (Figure 2.14).
Pancreatic cancer is an example of another type of cancer which is highly reliant on the autophagy pathway. In a pancreatic cancer cell line, PANC1, MTT assays revealed smaller derivatives DQ330, DQ331, DQ340, and DQ341 are the most potent compounds in the dimeric quinacrine library. PANC1 cells were also utilized in a two-week colony formation assay to assess the long-term effect of a subset of the DQ library on PANC1 cell proliferation (Figure 2.14). Fascinatingly, some of the dimeric...
quinacridones possess potency in suppression of long term cell proliferation at doses as low as 30 nM. As in melanoma, DQ221 52 again lacks the same degree of potency in MTT and long-term colony formation assays for cell proliferation, suggesting the lack of potency is due to the structure of DQ221 52.

Apoptosis is a form of programmed cell death which is induced in response to cellular stress in multi-cellular organisms.61,62 In properly functioning apoptosis, cells which experience stress undergo programmed cell death by the activation of proteolytic enzymes which recycle the contents of the cell. In cancer, however, apoptosis is often functioning incorrectly, leading to further cancer cell proliferation. Anti-cancer therapeutics which induce apoptosis can be used as a means of both stopping cancer cell proliferation and shrinking tumors.62 PANC1 cells treated with the DQ library were assayed for apoptosis, where DQ660 56 and DQ661 64 were the best performing pro-apoptotic compounds at a dose of 3 uM in a 24-hour assay (Figure 2.16). DQ compounds produced significantly larger amounts of apoptosis than Lys-05 9, marking a significant

![MTT Cell Proliferation](image1.png) ![Long-term Colony Formation](image2.png)

Figure 2.15: MTT and long-term colony formation assays for DQ inhibitors in the PANC1 pancreatic cancer cell line.53
improvement over the first-generation autophagy inhibitors. Early-stage autophagy inhibitors Spautin-1 and SBI-0206965, do not induce significant amounts of apoptosis compared to dimeric quinacrine compounds. The result underscores the inability of early-stage autophagy inhibition to prevent cancer cell proliferation.

Next, the DQ library was assayed for its effects on the autophagy pathway. Western blotting for LC3 allowed for a relative quantification of the accumulation of its two forms, LC3-I and LC3-II. The relative accumulation of LC3-II to LC3-I is directly correlated to the accumulation of mature autophagosomes. A subset of the DQ library was assayed, and a peculiar pattern emerged. The compounds containing a methylated tertiary amine performed as hypothesized, where treatment of melanoma cells with inhibitors resulted in substantial autophagy inhibition. However, when cells were treated with the secondary amine linked dimers, LC3 was found to accumulate to a significantly smaller degree. (Figure 2.17)
In autophagy, inhibition of lysosomal-autophagosomal vesicle fusion creates the expected increase of the LC3-II to LC3-I ratio. Unfortunately, induction of the autophagy pathway will also cause an increase in autophagosomes and thus both LC3-I and LC3-II, which can complicate a semi-quantitative technique such as Western blotting. The bafilomycin clamp assay has been developed which eliminates vesicle fusion by inhibition of the lysosome prior to treatment with an autophagy inhibitor. The name of the assay is derived from Bafilomycin A1 (Baf), an inhibitor of the vacuolar ATPase, the main proton pump maintaining the lysosomal pH gradient. Treatment of a cell with Baf causes lysosomes to deacidify and halts lysosomal vesicle fusion. In the Baf-clamp assay, cells are pre-treated with Baf, then treated with the inhibitor of interest. Any subsequent increase in the production of either LC3-I or LC3-II must be attributed to the induction of autophagy. When assayed by Baf-clamp, the unmethylated secondary amines increased the production of LC3-B, indicating the induction of autophagy, while the methylated tertiary amines do not induce autophagy.
Another marker for autophagic activity is the adaptor protein p62. In autophagy, the adaptor protein p62 marks cargo for degradation and facilitates the recruitment of the cargo to the forming autophagosomes. After lysosomal vesical fusion with the autophagosome, p62 itself becomes degraded. In cases where autophagy is inhibited, the...
p62 adaptor protein accumulates because the degradative machinery is sequestered from the autophagosomes containing the p62. PANC1 cells showed a striking difference in levels of p62 when treated with DQ inhibitors. Western blotting of treated cells showed unmethylated DQ compounds allowed p62 degradation, whereas methylated DQ inhibitors caused the accumulation of p62. These experiments further support the idea that methylated DQ compounds inhibit autophagy, while unmethylated compounds induce autophagy (Figure 2.18). NBR1 is a receptor protein which is associated with recruitment of cargo for autophagic degradation. NBR1 is degraded upon lysosomal-autophagosomal vesicle fusion. Following the same trend as p62, NBR1 was found to be degraded in cells treated with secondary amine linked DQ compounds, indicating active autophagy (Figure 2.18).

**P62 & NBR1 Accumulation- Autophagy**

Figure 2.18: A. Western blot analysis for p62 and NBR1 accumulation in cells treated with DQ compounds. B. The average accumulation of p62 in methylated and unmethylated DQ. Accumulated p62 or NBR1 indicates inhibited autophagy, where lack of these proteins indicates active autophagy.  

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53
To understand the striking contrast between the DQ inhibitors, we attempted to identify potential stressors which could be inducing autophagy. Dr. Vito Rebecca recognized that DNA damage is an inducer of autophagy, and therefore cells treated with DQ compounds were assayed for DNA damage. DNA damage is known to induce autophagy through a signaling cascade which engages two key autophagy signaling loci, the Ulk1 complex and the beclin1-Vps34 complex. Through these complexes, autophagy is induced which can lead to DNA damage repair, autophagic cell death, or senescence followed by an immune response. 63 QN is known as a potent DNA intercalator. Therefore it is a reasonable hypothesis that DQ compounds are potent intercalators as well.64 DNA intercalation is known to cause the DNA processing machinery to stall when scanning the phosphate backbone of DNA, resulting in double stranded nicks in the DNA backbone. These breaks are identified by DNA damage response (DDR) machinery of the cell and labeled by histone phosphorylation. When small molecules are intercalated into DNA, topoisomerase inhibition causes the phosphorylation of histone 2A (pH2AX), a marker which can be observed by Western blotting or by immunofluorescence. 65

Immunofluorescence (IF) imaging of pH2AX was performed on A375P melanoma cells. Cells treated with unmethylated inhibitors were found to have significantly more DNA damage than their methylated counterparts (Figure 2.19). Further, as the linker lengths of the DQ compounds increased, the differential amount of DNA damage between methylated and unmethylated inhibitors increased. This result is consistent with the hypothesis that DQ compounds which induce autophagy do so through induction of the cellular DDR.
The subcellular localization of the DQ compounds was assessed using spectroscopic measurements. Acridines, like quinolines, can absorb a photon of light. When the correct wavelength of light is absorbed, the resulting excited molecule can relax back to the ground state by the emission of a photon. Most quinoline based molecules fluoresce when they absorb UV irradiation. Fortunately, with the increased size of the aromatic system comes the red shifting of both absorption and fluorescence emission of the molecule. Quinacrine has long been known to absorb light at approximately 425 nm and fluoresce at approximately 500 nm. One of the first uses of quinacrine was the fluorescent staining of DNA chromosomes for karyotyping. We
investigated the spectroscopic properties of the DQ library by UV-Vis absorbance spectroscopy and fluorescence spectroscopy. The compounds were found to absorb strongly at 424 nm and 440 nm. Each compound had a fluorescence emission maximum at approximately 500 nm. Unsurprisingly, the spectroscopic properties of the DQ compounds varied with the pH of the aqueous medium. Lowering of the pH of the medium (1N HCl) caused a partial quenching of the fluorescence emission, and, surprisingly, the appearance of a secondary fluorescence emission peak. Compounds which have longer linkers were found to have slightly higher fluorescence emission intensities in both peaks.

Using fluorescence microscopy, DQ compounds can be seen to enter the cell and localize primarily in two places: the nucleus and the lysosome. The subcellular localization of the drug was not entirely surprising due to the knowledge of the DNA intercalating ability of quinacrine, and the propensity of weakly basic molecules, including CQ, to localize to the acidic lysosomal compartment.\textsuperscript{19,47,50,64}

Differential subcellular localization was hypothesized as a potential factor for the observed DNA damage or autophagy inhibition, respectively. Microscopy shows a more pronounced lysosomal presence for the methylated DQ inhibitors. However, the experiments done were not quantitative. Therefore, a concerted effort was made to understand where the different classes of DQ compounds were localizing sub-cellularly. A375P melanoma cells were treated with DQ660 \textsuperscript{56} or DQ661 \textsuperscript{64}. The cells were then fractionated into lysosomal and whole cell fractions. Fluorescence spectroscopy was used to quantify the amount of each DQ compound in the respective compartment after the
fractions were normalized for protein concentration. We elected to compare DQ660 56 accumulation versus DQ661 64 accumulation as a ratiometric measurement. DQ661 64 was found to significantly accumulate in the lysosome (36 uM) relative to the whole cell (16 uM). In contrast, DQ660 56 barely accumulates in the lysosome (10 uM) versus the whole cell (7 uM).

The same experiment was performed on isolated nuclear compartments from A375P melanoma cells. When compared to the remainder of the cell, both drugs are trafficked to the nucleus to the same extent. DQ660 56 treated cells exhibit significantly greater DNA damage compared to DQ661 treated cells. However, DQ660 56 and DQ661 64 localized to the nucleus to the same extent. Therefore, subcellular localization cannot be the direct cause for the difference in DNA damage induction between the methylated and unmethylated DQ inhibitors. Further study into the DNA damaging ability of DQ660 56 is ongoing in collaboration with the Maity Laboratory in the Radiology Department at the University of Pennsylvania Medical School. In preliminary organismal studies, DQ660 56 induced DNA damage potentiates radiation therapy in cancer xenograft experiments.

One proposed mechanism of lysosomal inhibition is the induction of lysosomal membrane permeabilization (LMP). Recently, a marker for this phenomenon has been discovered. Lectin galactoside binding soluble 3 (LGAL3) or galectin-3 has been found to swiftly localize to damaged lysosomal membranes. IF microscopy can be used to observe the localization of galectin-3 into puncta when LMP is induced. Melanoma A375P cells were treated with DQ compound or positive LMP control leucine
methylester hydrobromide (LloMe) and compared to an untreated control. IF imaging revealed a diffuse staining of galectin-3 in cells treated with the unmethylated inhibitors, QN, and the non-potent DQ221 52. Small puncta were imaged in cells treated with the positive control LloMe and the methylated DQ compounds with linkers greater in size than DQ221 52. DQ661 64 was found to most significantly induce LMP in melanoma cells. Notably, DQ221 52 is the lone methylated DQ inhibitor tested which does not induce LMP (Figure 2.20).
Lysosomal Membrane Permeabilization

Figure 2.20: IF microscopy which assays the propensity for DQ inhibitors to cause LMP. Antibodies for galectin-3 image the enzyme in the cytosol. When LMP is induced galectin-3 localized to the damaged membrane. In methylated inhibitors which are longer in length than DQ221, significant LMP is observed by the appearance of puncta within the cell. 53
To further understand the degree of DQ661 64-induced LMP, Western blotting was performed to observe the subcellular localization of the lysosomal protease cathepsin D (CTSD). Upon maturation of the enzyme, it is transported to the lysosomal compartment, where it acts as one of the main degradative enzymes within the lysosome.68 Cells treated with either DQ660 56 or DQ661 64 were fractionated into a lysosome enriched fragment and a whole cell fragment. The degree of lysosomal enrichment is measured by Western blotting for lysosomal membrane associated protein 2 (LAMP2), a lysosomal membrane protein. Western blotting for CTSD shows cells treated with DQ661 64 have CTSD in both the whole cell and the lysosome containing fractions, despite having efficiently separated the lysosome containing fraction from the whole cell fraction. This result demonstrates that the lysosomal membrane integrity is being damaged by DQ661 64. Both untreated and DQ660 56 treated cells have CTSD only in the lysosome-enriched fragment (Figure 2.21).
The most potent dimeric autophagy inhibitor DQ661 was also one of the most potent anti-proliferative agents, having caused the largest amount of apoptosis and the largest accumulation of LC3B and p62. These qualities made DQ661 the best candidate for studying the mechanism of action of DQ inhibitors. To elucidate the mechanism, high-throughput experimentation was utilized in a reverse phase protein array (RPPA). Through a collaboration with MD Anderson Cancer Center at the University of Texas, melanoma cells treated with DQ661 were subjected to RPPA. This assay screens the expression levels of >290 proteins involved in signaling pathways commonly misregulated in cancer. Treatment with DQ661 had a strong, reproducible impact on protein levels of autophagy-related proteins, apoptosis-related proteins, and mTOR-signaling proteins. Western blotting for protein levels present in the mTOR
pathway confirmed that DQ661 was indeed causing mTOR inhibition, a phenotype previously not observed in cells treated with Lys-05 9.

MTORC1 is a master regulator of cellular metabolism, extensively regulating autophagy and macropinocytosis.11,69 The mTORC1-signaling pathway involves multiple enzymes which sense the levels of specific amino acids. When amino acids are present, the regulatory proteins, Rag GTPases, recruit mTORC1 to the surface of the lysosome. Residing on the surface of the lysosome are the proteins which comprise the Ragulator complex and Rheb, the master activator of mTOR kinase activity. When mTORC1 is recruited to the lysosomal surface, it binds to the Ragulator, which allows for the localization of Rheb on the lysosomal membrane to the Ragulator complex. When Rheb encounters mTOR bound to the Ragulator complex, mTORC1 is phosphorylated, activating its kinase activity. MTORC1 kinase activity is a suppressor of autophagy and micropinocytosis, two compensatory pathways which a cell can utilize to scavenge nutrients.

Three cell lines (BRAF mutant, NRAS mutant, and WT/WT) were treated with DQ661 64, and then analyzed by Western blot to determine the extent of the mTOR inhibition. DQ661 64 demonstrated the ability to inhibit mTOR signaling in all three cell lines. S6K is a protein which is phosphorylated by mTOR signaling. Treatment with DQ661 64 reduced SK6 phosphorylation, indicating mTOR was inhibited. 4E_BP1 is also a protein which is phosphorylated by mTOR signaling. Again, treatment of A375P melanoma cells with DQ661 64 significantly reduces the phosphorylation of 4E_BP1. S6 is another phosphorylated protein in the mTOR signaling pathway. The phosphorylation
of S6, another protein that is phosphorylated in the mTOR signaling pathway, is also inhibited on treatment of A375P melanoma cells with DQ661 64. Finally, the three cell lines were also blotted for the apoptosis marker caspase-7, a protein which is cleaved when a cell undergoes apoptosis. Cleaved caspase-7 was observed in all cell lines. However, the amount of cleaved caspase7 in both mutant melanomas was significantly greater than in WM3918 (WT) melanoma (Figure 2.22, A).
For each of these markers, a kinetic experiment was performed in BRAF mutant melanoma, where time points were taken between zero and twenty-four hours. In cells treated with 3 uM DQ661, accumulation of LC3B began at approximately one hour. In two hours, the amount of phosphorylated S6K and phosphorylated 4E-BP1 decreased. In six hours, significant amounts of p62 were observed to accumulate, indicating autophagy.

Figure 2.22: A. Western blotting for mTOR kinase substrates (S6K, 4E-BP1, and S6), LC3B, and a marker of apoptosis (caspase-7) in mutants of melanoma. Increased phosphorylation of mTOR kinase substrates indicates mTOR activity. Cleavage of caspase 7 indicates the induction of apoptosis. B. A 24-hour time course measuring mTOR kinase substrates (S6K, 4E-BP1, and S6), LC3B, and caspase-7 in A375P melanoma.
was inhibited. In six hours, when both autophagy and mTOR are inhibited, the cells began to undergo a significant degree of apoptosis, marked by cleaved caspase-7 (Figure 2.22, B).

These results raised the interesting question of whether this type of mTOR signaling inhibition is present when melanoma is treated with other lysosomal inhibitors. Untreated melanoma cells were compared to cells treated with a panel of lysosomal inhibitors, utilizing serum starvation as a positive control for mTOR signaling suppression. Pepstatin A is an aspartyl protease inhibitor which inhibits cathepsin D activity in the lysosome. E64 is a covalent cysteine protease inhibitor, containing an epoxide warhead. Combinations of pepstatin A and E64 can be used to significantly suppress lysosomal degradation. Siramesine, a small molecule inhibitor of sigma-2 receptor, was originally developed as an anti-depressant. Recent studies of siramesine as an anti-cancer therapeutic have established that it leads to inhibition of lysosomal function through inhibition of acid sphingomyelinase and the induction of LMP. PET and PES are two inhibitors of HSP90 designed by the Murphy Laboratory at the Wistar Institute. Hsp90 is known to aid in protein folding and the maintenance of protein homeostasis. One function of autophagy is the degradation of protein aggregates, and therefore we were interested in whether these compounds altered mTOR kinase activity autophagy. These inhibitors alone and in combination were tested in melanoma cells to determine whether they could also impact the mTOR signaling pathway in a similar fashion to DQ661 64. DQ661 64 was found to be the only compound which inhibited lysosomal function and prevented mTOR signaling, as indicated by the phosphorylation
of S6K and 4E-BP1. Only serum starvation, the removal of required nutrients, was able to induce a comparable suppression in mTOR activity. Further, RPPA analysis of DQ660 and QN 2 treated melanoma cells showed the impact on mTOR signaling to be unique to DQ661 (Figure 2.23).

To become activated, mTOR must localize to the lysosomal surface guided by RagGTPases. These enzymes allow mTOR to bind the Ragulator complex on the surface of the lysosome, fixing the location of mTOR. Rheb, the master activator of mTOR, is a kinase which is present on the lysosomal surface. When Rheb encounters mTOR bound

Figure 2.23: A 375P melanoma cells were treated with a panel of compounds. Western blot analysis of mTOR kinase substrates and LC3B. Phosphorylation of mTOR substrates indicates functioning mTOR signaling. Accumulation of LC3-II when compared to LC3-I indicates inhibited autophagy.53
to the Ragulator complex, Rheb phosphorylates mTOR, turning on mTOR kinase activity. In collaboration with the Zoncu Laboratory at UC Berkeley, we explored the localization of mTOR and its recruiting enzymes through IF imaging. Fascinatingly, DQ661 treatment inhibits the lysosomal localization of RagC, a recruiting GTPase, and mTORC1. A proximity ligation assay was utilized to determine the degree of colocalization of mTORC1 and RagC. Utilization of specially modified antibody-DNA conjugates allows for the fluorescent labeling of a protein-protein interaction. When A375P melanoma cells were treated with DQ661, the Rheb and the Ragulator complex showed nearly zero co-localization, as observed by IF microscopy, unlike the untreated control. The same assay was conducted utilizing antibodies for mTORC1 and Rheb, the master activator for mTOR, and imaged by IF microscopy. Untreated melanoma cells showed significant colocalization of these two enzymes. Rapamycin, an mTOR inhibitor, Torin, an mTOR kinase inhibitor, Bafilomycin A1, lysosomal VATPase inhibitor, and siramesine, an inducer of LMP, were tested in the proximity ligation assay. In each case, mTOR and Rheb were found to have a degree of interaction similar to the control, suggesting the inhibitors do not affect the activation of mTOR by Rheb. Further, siramesine, which causes LMP, did not inhibit mTOR in the same manner. These results demonstrate that loss of mTOR localization to the surface was not merely an artifact of disrupting membrane integrity. Treatment of A375P cells with DQ661 completely abolished the interaction between mTOR and Rheb, thus inhibiting mTOR activation.

MTOR inhibition has not led to significant success in clinical studies. MTOR suppression activates the Ulk1 complex, which upregulates autophagy. It has been
hypothesized that this is an effort by the cells to recycle internal cargo and make amino acid building blocks available, which in cancer cells allows for the continuation of cell proliferation. MTOR suppression is also known to increase cellular macropinocytosis which allows for Ras-driven cancers to continue their cell proliferation.\textsuperscript{11}

Macropinocytosis is an endocytic process where cells envelop extracellular proteins which are trafficked to the lysosome. In the lysosome, the proteins are broken down to their constituent amino acids and released into the cell as metabolic building blocks. Thompson and coworkers have demonstrated that tumor regions which are under metabolic stress by growing beyond their amino acid supply can activate macropinocytosis when mTOR is inhibited and continue further cell proliferation.\textsuperscript{73} The engagement of macropinocytosis allows cancer cells to paradoxically continue cell proliferation even when faced with mTOR suppression. The engagement of macropinocytosis is another hypothesis which could explain the lack of efficacy of mTOR suppression in clinical trials. To explore the effect of DQ661\textsuperscript{64} on macropinocytosis, pancreatic cancer cells were grown under nutrient starved conditions with a fluorescently labeled bovine serum albumin derivative added to the medium. The BSA is labeled with such a high density of dye that fluorescence is significantly quenched. When the protein is degraded, fluorescence increases due to dissociation of the labeled amino acids. When macropinocytosis is engaged, uptake and degradation of the labeled BSA is observed. Cells treated with DQ661\textsuperscript{64} or torin-1, an mTOR kinase inhibitor, both engaged in the macropinocytotic uptake of labeled BSA. Cells treated with torin-1 showed significant degradation of the labeled BSA. In contrast, while the labeled BSA was taken up by cells treated with DQ661\textsuperscript{64}, the protein was not degraded by the
lysosome, suggesting DQ661-mediated lysosomal impairment prevents macropinocytosis from circumventing mTOR inhibition. DQ661 therefore represents a new strategy which suppresses mTOR signaling in cancer cells but is not circumvented by either the autophagy pathway or macropinocytosis.

Collaboration with the Rabinowitz Laboratory at Princeton University allowed for the quantification of the effect of DQ661 on extracellular protein uptake. In this experiment, cancer cells are grown in an isotopically labeled medium containing $^{13}$C enriched amino acids. The isotopically-labeled cells are then grown in amino acid starved media with standard BSA supplemented as the only protein source. If BSA is taken up and broken down via macropinocytosis, the incorporation of the light, $^{12}$C only, amino acids into cellular proteins can be quantified versus an untreated control. Due to the sequence specificity of BSA, the amino acid profile which would be incorporated provides a unique signature, and therefore is easily quantified as the source of scavenged amino acids. When comparing cells treated with DQ661 to an untreated control, the amount of amino acid scavenging was significantly suppressed. This result confirms the ability of DQ661 to inhibit macropinocytosis.

In vivo study of DQ compounds in mouse tumor xenograft models produced positive anti-cancer results. BRAF mutant and HCQ resistant melanoma cells, 1205Lu, were implanted in the flank of NOD. Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NSG) mice. These tumors were allowed to grow for a period of one to two weeks, until the tumors were palpable. Treatment, either vehicle control, DQ661, DQ660, or QN, was given to the mouse through an intraperitoneal (IP) injection. The mice were treated with a regimen

61
which did not drastically decrease the weight of the subject. Subjects treated with QN 2 were found not to differ from control at a dose of 8mg/kg. However, at this dose DQ660 demonstrated significant potency, along with DQ661 64. When comparing the two DQ inhibitors, DQ661 64 performed significantly better than its unmethylated counterpart, yielding further evidence for the importance of a tertiary amine within the linker of DQ inhibitors (Figure 2.24, A). When the rate of growth was calculated for the xenograft experiment, only DQ661 64 was able to produce a 0% growth rate at 8 mg/kg (Figure 2.24, B).

Figure 2.24: A. Mice were treated first at 8mg/kg with QC 2, DQ660 56, or DQ661 62. Mice were euthanized at 16 days due to lethargy when treated with DQ661. B. The tumor growth rate plotted for each drug treatment. C. A second experiment was run where mice were given 2mg/kg DQ661, and tumor growth rate was suppressed over the course of 22 days. D. The tumor growth rate plotted for each drug treatment condition.53
To understand the mechanism of action of these inhibitors in the xenograft experiment, tumor biopsies were taken. The biopsies were analyzed by immunohistochemistry for pH2AX and it was found that only DQ660 produced significant histone phosphorylation, indicating DNA damage occurred. When examined for autophagic vesicle accumulation, DQ661-treated tumors showed significant vesicle accumulation, whereas DQ660-treated tumors did not. In our first experiments, doses of DQ661 at 8mg/kg caused significant weight loss (greater than 10% of body weight) in mouse subjects. After one week, mice treated with these large doses experienced lethargy and distended bowels. Autopsy and subsequent histological analysis revealed significant Paneth cell dysfunction akin to that reported in the \textit{in vivo} investigation of Lys-05. The Paneth cell dysfunction is a hallmark of autophagy dysfunction, confirming again that DQ661 is inhibiting autophagy \textit{in vivo}.

\textbf{2.4 Second Generation of DC Inhibitors}

Encouraged by the successful study of the DQ library, we sought to synthesize the analogous DC library analogs. In a combined effort with Dr. Noel McLaughlin, the analogous library was created to mirror the DQ library. Dr. McLaughlin synthesized DC440, DC441, DC550, DC551, in addition to a number of other dimeric compounds which are not linked via simple triamine linkers. Dr. McLaughlin prepared the first samples of DC660 and DC661, and I led subsequent efforts to translate these preparations to multi-gram syntheses for animal studies. We were also interested in determining whether DC inhibitors lost potency at linker lengths greater than 6/6, and therefore
synthesized the following new dimers: DC770, DC771, DC880, DC881, DC11110, and DC11111.

We next wanted to understand whether the bis(hexamethylene triamine linker of DQ661 64 imparted the same biological activity to DC inhibitors. DC661 69 was synthesized by Buchwald-Hartwig C-N arylation of bis(hexamethylene triamine 51, followed by Eschweiler-Clarke reaction to methylate the internal nitrogen of the linker (Figure 2.25). DQ661 64 was tested for its anti-proliferative activity by two-week colony formation assay and autophagy inhibition activity by Western blotting. DC661 69 possesses superior potency in both autophagy inhibition and anti-cancer activity when compared to HCQ 4 and Lys-05 9 (Figure 2.26).

![Multi-gram synthesis of DC661](image)

**Figure 2.25:** The gram-scale synthesis of DC661 69.

We next chose to assay DC661 for the induction of LMP via the galectin-3 IF microscopy assay, where galectin-3 localizes in puncta where LMP occurs on the lysosomal membrane. DC661 69 causes significant LMP, where Lys-05 9 and HCQ 4 do not (Figure 2.27). The three compounds were then tested in three cell lines for their
ability to induce apoptosis. Compared to untreated control, dabrafenib (BRAF inhibitor), trametinib (MEK inhibitor), and the combination of dabrafenib and trametinib (standard of care for BRAF mutant melanoma), DC661 produced significantly more apoptosis in all three cell lines.

Figure 2.26: The structures of HCQ, Lys-05, and DQ661. Assays for anti-cancer activity (colony formation), and autophagy inhibition (LC3B accumulation).
DC661 69 was tested in tumor xenograft experiments with 1205Lu melanoma in NSG mice. Tumors were grown on the flanks of mice until palpable, after which DC661 was administered via IP injection at a dose of 3mg/kg. DC661 69 treatment halted tumor growth over the course of a 15-day experiment (Figure 2.28).
2.5 Synthesis of longer linked DC inhibitors

We synthesized DC11110 via Buchwald-Hartwig arylation conditions utilizing our synthesized 11/11 triamine. The resulting inhibitor was methylated using formaldehyde and sodium triacetoxyborohydride in CH$_2$Cl$_2$ to give 72. We began with the synthesis of DC880 from 4-bromo-7-chloroquinoline, and our 8/8 triamine linker using a Pd(OAc)$_2$/BINAP catalyst system with K$_3$PO$_4$ in dioxane. Unfortunately, no product was observed in the reaction. We had previously taken note of the decreased yields in arylation reactions of triamines with increasing linker length when synthesizing the DQ library. This result suggested that altered arylation conditions would be required to synthesize DC880. We screened different reaction conditions where solvent, base, ligand and palladium sources were changed. However, no product was observed in the reactions. In reactions where the catalyst system was altered, only starting material 13
was observed via $^1$H NMR analysis of the reaction mixture. We attempted to utilize the more efficient Buchwald-Hartwig pre-catalyst which is recommended for primary amine arylation, although no product was observed. The base was changed to NaOtBu in combination with our existing catalyst system Pd(OAc)$_2$/BINAP. However, the outcome of the reaction did not improve when compared to K$_3$PO$_4$. When attempting a solvent screen, we noticed that the 8/8 triamine 41 was sparingly soluble in dioxane and most non-alcohol organic solvents. Tert-butanol was screened for its ability to solubilize the 8/8 triamine 41, as it has been utilized with NaOtBu in arylation reactions. However, the compound was still sparingly soluble. We had previously observed that DC and DQ inhibitors with tertiary amines were much more soluble in organic solvent than their secondary amine-linked counterparts.

Simultaneously with our screen of arylation conditions, we explored utilizing the 8/8 triamine with the internal amine protected 73 (Figure 2.29). Staudinger reduction of the 8/8 bisazide 39 yielded a triamine where the central nitrogen of the triamine was a benzylated tertiary amine 73. We next attempted to arylate the triamine utilizing our original arylation conditions: Pd(OAc)$_2$/BINAP and K$_3$PO$_4$ in 1,4-dioxane heated to reflux. The reaction produced good yields of dimeric chloroquine compound where the linker amine was a tertiary benzylamine 74. We next explored methods of removing the benzyl protecting group from the internal nitrogen. However, hydrogenolysis conditions reductively cleaved the chlorines from the quinoline rings of the inhibitor. We next attempted to remove the benzyl group under S$_N$2 conditions with hydrobromic acid. However, this reaction proved to be unsuccessful. Next, we attempted to acylate the
tertiary amine utilizing methyl chloroformate. Previous work by our laboratory demonstrated that chloroformates can be utilized to deprotect benzylic tertiary amines. However, in the presence of the aminoquinoline rings the reaction did not produce any debenzylated product. As a control reaction we proved the benzylation of the 8/8 bisazide 39 could be acylated and debenzylated by methyl chloroformate in good yield to give compound 75. With this compound in hand we elected to reduce the azides via a Staudinger reduction and assess the compatibility of the methyl carbamate protecting group with our arylation conditions. Buchwald-Hartwig arylation of the diamine 76 provided an 8/8 dimeric chloroquine analog 77 where the internal amine was protected with a methyl carbamate. The carbamate was successfully deprotected by reaction with HBr (33% v/v in AcOH) at 100 °C to give DC880 78. The resulting DC880 78 inhibitor was then methylated with formaldehyde and sodium triacetoxyborohydride in CH₂Cl₂ to give DC881 79.
We next set out to synthesize both DC770 85 and DC771 86 (Figure 2.30). However, we wanted to explore whether utilization of a nitrogen source other than benzylamine could improve the synthesis of dimeric inhibitors. We selected 2,4-dimethoxybenzylamine as a nitrogen source to synthesize a protected triamine precursor, because we believed under acidic deprotection conditions the protecting group could be removed. 7-azidoheptanol 80 was oxidized to 7-azidoheptanal via Swern conditions, which was concentrated to a crude dilute solution due to safety concerns regarding low-molecular weight azides. The crude aldehyde-containing solution was then used to reductively alkylate 2,4-dimethoxybenzylamine 81 in serviceable yield over 2
steps to give bis-azide 82. The resulting bis-azide 82 was then reduced via Staudinger reduction to afford a triamine 83 in good yield. The resulting triamine was then arylated with 13 using Pd(OAc)$_2$/BINAP and K$_3$PO$_4$ in 1,4-dioxane heated to reflux. The resulting dimethoxybenzyl protected DC77 inhibitor 84 was then deprotected in neat trifluoracetic acid with water and triisopropylsilane by heating to 130 °C under microwave irradiation to yield DC770 85. The internal linker nitrogen of 85 was then methylated using formaldehyde and sodium triacetoxyborohydride in CH$_2$Cl$_2$ to yield DC771 86.
We compared the anti-cancer activity of DC770 and DC771 to their 6/6 congeners, DC660 and DC661. Interestingly, both DC770 and DC771 appear to be comparably potent in anti-cancer activity (Figure 2.31). This result contrasts with the DQ series of inhibitors. We had previously hypothesized that longer linked dimers eventually become hydrophobic to an extent that is detrimental to their anti-cancer activity. While DQ inhibitors 57, 58, 59, 65, 66, and 67 lost potency, DC770 and DC771 appear to inhibit cell proliferation to a greater extent than DC660 and DC661. We had hypothesized that linkers longer than 7 carbons in the aliphatic chain were too hydrophobic to utilize as dimeric inhibitors. However, in the
case of DC compounds, it appears that 41 is not too hydrophobic to generate a potent inhibitor. Further study into the activity of longer linked DC inhibitors is required.

2.6 Preparation for the Third Generation of Dimeric Chloroquine Inhibitors

Our studies of the second generation of dimeric lysosomal inhibitors demonstrated the importance of the identity of the triamine linker from which the inhibitor is synthesized. In the absence of a target, we became interested in how the distance between heterocycles effects the potency of a dimeric inhibitor. Unfortunately, increasing the length of the aliphatic chains which comprise the triamine linker also results in a significant increase in hydrophobicity. To separate hydrophobicity and distance as factors, we elected to synthesize dimeric inhibitors derived from less hydrophobic linkers. We wanted to determine whether making single heteroatom substitutions in the aliphatic regions of dimeric inhibitors could improve their potency as

![Figure 2.31: MTT and colony formation assay comparing DC66 inhibitors to DC77](image)
autophagy inhibitors by improving their biophysical properties. We identified PEG linked
dimers to be a sterically conservative modification of the aliphatic linked dimers, while
dramatically reducing the inhibitors hydrophobicity.

We planned to use the strategy applied to the synthesis of DC880 and DC881 to
synthesize PEG linked dimeric inhibitors. We selected two lengths of PEG, diethylene
glycol and tetraethylene glycol, as scaffolds to mimic DC inhibitors with triamine linkers
of length 5/5 and 11/11 (Figure 2.32). The resulting azides, 87 and 89, were converted to
the corresponding alkyl bromides by Appel reaction, 88 and 90. The alkyl bromides
were used to alkylate benzylamine via S_N2 yielding 91 and 92. In the case of PEG-based
alkyl bromides, we observed an increase in yield of the S_N2 reaction when compared to
the aliphatic alkyl bromides. Further, the significant weight added by the bromine atom to
the diethylene glycol chains gave us confidence in our ability to isolate pure azide-
containing material in a safe manner. The resulting bisazide 91 was then acylated with
methyl chloroformate and deprotected in toluene at 85 °C to yield 93. Compound 93 was
reduced via a Staudinger reduction to afford compound 95. The resulting diamine 95 was
then arylated with 13 utilizing Pd(OAc)_2/BINAP and K_3PO_4 in 1,4-dioxane heated to
reflux to yield compound 97. The resulting carbamate-containing derivatives were then
deprotected utilizing either KOH in ethanol (compound 99), heated to reflux, or
trimethylsilyl iodide in CHCl_3 (compound 100). The resulting secondary amine-
containing inhibitors were methylated utilizing CH_2O and sodium triacetoxyborohydride
in CH_2Cl_2 to afford compounds 101 and 102.
We next wanted to determine whether the addition of amines to the linker would improve the potency of dimeric chloroquine inhibitors. We began our synthesis from a protected pentaamine, 103, reported by Jahromi and coworkers, where the three internal nitrogen atoms were Boc protected. The primary amines are arylated with 13 utilizing Pd(OAc)₂/BINAP and K₃PO₄ in 1,4-dioxane heated to reflux to yield a DC inhibitor of 5/5 in length with three Boc-protected amines, compound 104. The Boc protecting groups are then removed by reaction with trifluoroacetic acid in CH₂Cl₂. The resulting DC inhibitor, 105, was methylated with Eschweiler-Clarke conditions in reasonable yield to afford compound 106. We were pleased to observe product without observation of any cyclic aminal intermediates (Figure 2.32).
Figure 2.32: Synthesis of PEG2 linked DC inhibitors and pentaamine linked DC inhibitors
2.7 Synthesis of Piperaquine Derivatives

Piperaquine (PQ) 5 is a commercially available anti-malarial derivative of CQ 1 (Figure 2.33). The inhibitor is the only commercially available dimeric anti-malarial CQ derivative. Currently, a combination therapy of artusunate (artemisinin methyl ester) and piperaquine is one of the standards of care for malaria in east Asia. PQ 5 differs from CQ 1 in that the aminoquinoline moiety is a tertiary amine rather than a secondary amine and is constrained into a piperazine ring. The dimer is formed by linking two CQ-piperazine halves of PQ by a propyl linker. We hypothesized that perhaps PQ could possess unique anti-cancer activity akin to Lys-05 9 by nature of its dimeric structure. We also hypothesized that if the propyl linker were extended, by spacing the CQ rings an equivalent distance to DC661 69, that the anti-cancer properties of the two molecules would be similar in nature.

We began our synthesis by reacting 4,7-dichloroquinoline with an excess of piperazine in ethanol at 135 °C under microwave irradiation. The resulting SNAr reaction produces a CQ-piperazine, 107, in good yield. Three derivatives of piperaquine were derived from three dibromoalkanes: 1,2-dibromoethane 108, 1,7-dibromoheptane 77
34, and 1,8-dibromooctane 35. The S\textsubscript{N}2 reactions were performed by heating in DMF to 120 °C in a sealed vessel (Figure 2.34). The resulting dimers, 109, 110, and 111, will be tested for their anti-cancer activity by MTT proliferation assay.

2.8 Synthesis of Ferroquine Derivatives

Recently, a new chloroquine analog has demonstrated significant success in antimalarial studies.\textsuperscript{79,80} Ferroquine 6 is a chloroquine-based antimalarial which utilizes ferrocene as a stable iron source in place of two methylenes of the linker between the

Figure 2.34: Synthesis of dimeric PQ analogs of various linker lengths
heterocycle and the tertiary amine (Figure 2.35). In malaria it is hypothesized that CQ resistance can be overcome by compounds which accumulate to a greater extent in the digestive vacuole. Work by Biot et. al. has demonstrated that FQ 6 is potent in CQ-resistant malaria. A recent study by Kondratskyi and coworkers has observed ferroquine to be potent when assayed for its anti-cancer activity.

We hypothesized that perhaps the addition of a second CQ heterocycle to the ferroquine skeleton could improve the potency of ferroquine as an anti-cancer agent, just as Lys-05 improved the potency of CQ. We started by purchasing commercially available ferroquine and synthesized the tetraalkyl ammonium salt following the studies of Li and coworkers. We synthesized two secondary amine containing monomeric chloroquine analogs and for the derivatization of FQ. First, piperazine was reacted in excess with 4,7-dichloroquinoline in ethanol at 135 °C under microwave irradiation. The resulting secondary amine, was utilized to synthesize compound . We next synthesized 7-chloro-N-(piperidin-4-yl)quinolin-4-amine, previously synthesized by Madrid et. al. We again used the tetraalkyl FQ salt as an electrophile in a S_N2 reaction with excess to afford dimeric FQ2, (Figure 2.35) These reactions proceeded in modest yield at 40 °C over four days. We believe the yield could be improved by screening reaction conditions.
In a pilot biological experiment, the MTT antiproliferation potency of our two new ferroquine derivatives (FQ1 and FQ2) were assayed in comparison to commercial FQ (6). Interestingly, the FQ2 (115) was more potent than FQ (6) and FQ1 (113), indicating that the formation of a dimeric species did improve the anti-proliferative activity of FQ (6). FQ1 (113) which has a tertiary amino-quinoline instead of a secondary aminoquinoline performed worse than FQ (6). These results could be attributed to the importance of the flexibility of CQ heterocycles in dimeric inhibitors to populate different conformations and the requirement for the CQ heterocycle to contain a secondary aniline. (Figure 2.36)

Figure 2.35: The synthesis of dimeric FQ derivatives, FQ1 113 and FQ2 115.
Currently, further study of the biological activity of dimeric ferroquine compounds is underway.
Chapter 3: Determination of the mechanism of action of dimeric chloroquine and quinacrine lysosomal inhibitors.

In modern day medicinal chemistry, most drug discovery efforts start with a biological target. In most cases, a medicinal chemistry effort will target a specific enzyme either directly involved in an enzymatic transformation, an enzymatic process upstream of the target transformation which will regulate the target event, or an enzymatic process downstream which will induce a feedback loop effecting the target enzymatic process. Often a high-throughput screen is used to identify chemical structures which are most effective at inhibition of the target enzyme. If the enzymatic target responsible for a biological transformation is unknown, the process of intelligent inhibitor design becomes significantly more empirical.

CQ was designed as an anti-malarial therapeutic. However, due to multiple toxicities and resistant malaria strains, the use of CQ as a standard of care has been reduced. An off-target effect of CQ is the inhibition of lysosomal vesicle fusion in the autophagy pathway. The use of CQ as an inhibitor of late stage autophagy is the result of a serendipitous observation rather than rational target-focused design (Chapter 1).

3.1 The Justification of a Protein Target for DQ and DC Inhibitors.

The lysosomotropic nature of CQ is directly correlated to autophagy inhibition, where inhibition of the lysosome prevents lysosomal-autophagosomal vesicle fusion. CQ is a dibasic inhibitor (pKₐ 8.6 and 9.8), causing the molecule to be protonated in acidic compartments of the cell. Dating back to the work of de Duve studying the lysosome,
CQ was observed to significantly accumulate in the lysosome and other acidic vesicles.\textsuperscript{19} It is well known that the lysosome requires an acidic pH to maintain proper function. Therefore, it has been widely hypothesized that the ability of CQ to deacidify the lysosome is the mechanism of action of CQ-mediated autophagy inhibition. However, no direct evidence is available that this lysosomal buffering capacity is solely responsible for CQ-mediated autophagy inhibition. Treatment of eukaryotic cells with millimolar quantities of ammonium chloride will deacidify the lysosome as well.\textsuperscript{83} A simple analysis of the pK\textsubscript{a} of CQ and HCQ would suggest the inhibitors have a similar buffering capacity to that of ammonium chloride (pK\textsubscript{a} ~9). However, CQ and HCQ are both more potent autophagy inhibitors than ammonium chloride.

CQ has also been documented to accumulate to a greater extent than a simple analysis of pK\textsubscript{a} would infer. In a scenario where only the pH gradient governs the accumulation of CQ within a vesicle, it is expected that the ability of the lysosome to accumulate CQ would be directly proportional to the pH gradient between the lysosomal lumen and the cytosol. As the pH gradient approaches zero from accumulated compound buffering, the rate at which CQ accumulates should approach zero as well. Previous workers have postulated that the lipophilicity of CQ allows for more efficient accumulation via partitioning with hydrophobic lipids and proteins within the lysosome.\textsuperscript{19,84} This partitioning could allow for a reservoir of CQ and thus a buffering capacity beyond what an initial analysis of pK\textsubscript{a} would allow. The ability of a hydrophobic molecule to better pass through a membrane compared to ammonium chloride may be an explanation for the observed differences in potency.
Our studies of Lys-05 9 suggest that dimeric chloroquine (DC) inhibitors are acting not only as a lipophilic weak bases. Lys-05 is more than an order of magnitude more potent than CQ as an anti-proliferative agent and as an inhibitor of autophagy. More importantly, Lys-05 derivatives (Lys-02 10, Lys-03 11, and Lys-04 12), which are similarly hydrophobic as Lys-05 9, are no more potent than CQ 1 (Figure 3.1). One could argue the increase in potency is derived from the tribasic nature of Lys-05 9 compared to the dibasic nature of CQ 1. However, this is not consistent with our studies. Other tribasic analogs 10 & 11 of Lys-05 9 were tested and were found to be equipotent with CQ 1 in autophagy inhibition assays. Replacement of the C-7 chlorine on the aminoquinoline ring with a methoxy substituent also decreases activity of the inhibitor 11. The SAR study during the development of Lys-05 indicated small structural changes which do not greatly alter the biophysical properties of the Lys-05 derivatives do have a significant impact on the potency of the resulting inhibitor (Chapter 2). The exchange of C-7 chlorine in compound 9 for a C-7 methoxy substituent should produce an inhibitor with a more basic aminoquinoline ring. It is a reasonable hypothesis that increasing the pKa of the aminoquinoline would improve the efficacy of the inhibitor, however this is not reflected in the experiments reported by our Laboratory.85
The SAR observed in our study of DQ lysosomal inhibitors is in accord with this hypothesis. DQ221 \textsuperscript{52} is significantly less potent than other DQ analogs in MTT anti-proliferation assays. A375P melanoma cells treated with DQ221 \textsuperscript{52} exhibited cell proliferation comparable to melanoma cells treated with monomeric QN. DQ331 \textsuperscript{60}, the linker of which is two atoms longer, was among the most potent anti-proliferative DQ compounds (Chapter 2). This small structural difference between DQ221 \textsuperscript{52} and DQ331 \textsuperscript{60} supports the hypothesis of a mechanism more complicated than these compounds simply acting as lipophilic buffering agents. The tricyclic fused ring system of the dimeric quinacrines is \textasciitilde 50\% larger than the aminoquinoline of CQ and DC inhibitors.

We hypothesized that the increased steric demand of the QN heterocycles necessitated an increase in linker length of the dimeric inhibitors in order to enjoy an increase in potency from dimerization relative to the monomeric heterocycle. The distance between the heterocycles and the tertiary amine of the linker could influence the pK\textsubscript{a} of the linker.
tertiary amine through inductive effects. Lys-05 9 should have been equally influenced by inductive effects as DQ221 52. However, Lys-05 was more potent than the parent monomeric CQ (Figure 3.2). This SAR data set suggests that inductive effects of the heterocycle on the linker tertiary amine pKₐ were not the cause of the lack of potency of DQ221 52.
One plausible hypothesis which would explain the differences in activity across monomeric CQ 1, monomeric QN 2, DC, and DQ compounds is the existence of a protein binding partner. If these molecules were able to inhibit an enzymatic reaction within the lysosome, perhaps an increase in enzyme binding affinity correlating with anti-
proliferative activity could explain the observed differences in the biological activity of the dimeric inhibitors.

3.2 Determining a protein target of a small molecule inhibitor

The sequencing of the human genome in the early 2000s has catalyzed a new approach towards understanding and managing disease pathology. Following the central dogma of biology, where the DNA in a cell contains the sequences of the constituent enzymes of an organism, a non-infectious disease phenotype must be the result of the improper regulation or improper expression of a specific enzyme or enzymes. Therefore, if a gene can be identified in a disease phenotype, one can design and target the enzyme resulting from this gene with a small molecule therapeutic. In parallel with the advances in genome sequencing technology, advancements in mass spectrometry (MS) have led to a rapidly expanding field of proteomics, where differentially expressed or modified proteins can be identified from exceedingly complex biological mixtures. These advances have led to the rapidly expanding field of chemical proteomics, where the molecular target of a small molecule can now be identified using a phenotypic screening approach.86–89

The rapid advancement in MS technology has helped birth the ever-growing field of proteomics. While full length proteins can be difficult to observe by electrospray ionization mass spectrometry, improvements in both liquid chromatography (LC) and MS detection have allowed for the rapid identification of peptides in a complex mixture. In general, proteases, enzymes which cleave the peptide backbone of a protein, have
exquisite selectivity and cleave the protein after specific amino acid residues in polypeptide sequence. Therefore, the peptide products of enzyme-catalyzed proteolysis can be predicted accurately with prior knowledge of the sequence of a protein. These predicted peptides can be thought of as a fingerprint for a specific protein. Sequencing of the human genome has made obtaining sequence information for human proteins significantly more accessible. Combining genome sequencing data, with mass spectrometry identification of peptide mixtures, one can accurately pick out the constituent proteins which comprise a complex mixture.

Often, when a small molecule is biologically active it binds an enzyme or another bio-macromolecule. If the binding interaction of a small molecule to the target protein is tight enough (usually nM affinity), then fixing the small molecule to a solid support would allow for affinity chromatography purification of the target protein. This concept was first employed to isolate proteins from their natural sources by Leonard Lerhman in the early 1950s. Later, this approach was utilized by the McCormick Laboratory in 1964 to purify liver flavokinase. This approach was also utilized by the Anfinsen Laboratory in 1968 to purify staphylococcal nuclease, α-carboxypeptidase, and chymotrypsin. In theory, this idea of small molecule affinity chromatography could be utilized to purify and identify the binding partner for a drug-like small molecule inhibitor. Two of the most well-known examples come from the Schreiber Laboratory which utilized this technique to identify the targets of previously discovered drug-like natural products in their study of the binding partners for immunosuppressants and histone deacetylases. This strategy should be applicable with any small molecule inhibitor.
However, the effectiveness of this approach is significantly affected by the relative abundance of the target protein, the background noise of non-specific binding, and the off-rate of the inhibitor ($k_{off}$, time at which half of the protein-ligand complex has dissociated). In cases where a non-covalent adduct does not bind its target tightly enough to employ affinity chromatography, the introduction of a covalent linkage could negate the issue of inhibitor off-rate and low binding affinity.

A second strategy for identifying binding partners for biologically active molecules is utilization of a covalent inhibitor for an enzyme or enzyme class, and the subsequent MS identification of the bioconjugate or purification of the complex through a known affinity-tag, such as biotin. The earliest example was demonstrated by Glynn and coworkers in their identification of a new serine protease using biotinylated organophosphorus compounds. More recent work by the Cravatt Laboratory at the Scripps Research Institute has expanded on this idea to discover binding partners of drug-like small molecules and to discover new proteins within a class of enzymes. This field, now referred to as activity-based protein profiling (ABPP) is defined as a methodology which broadly profiles the activity of an enzyme in a biological organism through the utilization of a reactive functional group. This reactive functional group forms a covalent linkage between the protein and small molecule ligand, allowing for isolation of a bioconjugate. A phenotypic screen can be interrogated by ABPP through the design and synthesis of probe analogs of a lead molecule. At the cornerstone of a chemocentric ABPP approach is the utilization of a reactive functional group which is specifically reactive towards a class of enzymes, or the utilization of a small molecule warhead which
binds a protein tightly and specifically enough to confer specificity to the reactive functional group. This covalent adduct can be visualized by fluorescent labeling or enriched with affinity chromatography. Analysis of these protein profiles identifies the binding partners of the inhibitor in question, allowing for biological validation of the mechanistically relevant binding partner. Unfortunately, most drug-like small molecule inhibitors are not covalent inhibitors of enzymes, preventing the utilization of this approach to binding target identification. Photoaffinity labeling (PAL) is a technique which has been used in biochemistry and chemical biology dating back to the 1960s. The Westheimer Laboratory introduced this concept in a fundamental contribution to the intersecting fields of organic photochemistry and biochemistry.

The seminal work by Westheimer and coworkers outlined a strategy of forming small molecule-protein covalent adducts through photolysis of a small molecule. Their studies utilized the reactivity of chymotrypsin towards p-nitrophenolate esters to covalently modify the enzyme active site. Acetylation of chymotrypsin prevents the native activity of the enzyme. Reaction of acetylchymotrypsin with hydroxylamine cleaves the acetylation site, recovering the native activity of the enzyme. Unlike previous acetylations of chymotrypsin, Westheimer and coworkers modified the active site with a p-nitrophenyl diazoester. Photoirradiation of the protein conjugate reduced the activity of the enzyme by 25%. This reactivity loss was not recovered by the reaction of the protein conjugate with hydroxylamine, indicating that a new non-acyl bond must have been formed with chymotrypsin. (Figure 3.3)
This example of the compatibility of organic photochemistry with a biological macromolecule has set the foundation of many contributions to modern chemical biology. In their work, Westheimer and coworkers postulated that if the chromophore of the small molecule absorbs at a longer wavelength than the protein in question, organic photochemical reactions can be used to map the molecular contacts of the active site of a protein.\textsuperscript{100} Today, the concepts put forth by Westheimer and coworkers have been extrapolated to form a field known as photocrosslinking, where two molecules in a biological context can be chemically linked utilizing photoirradiation.

As stated by the original Westheimer hypothesis, photolysis leads to a reactive intermediate which enables bioconjugation. The tremendous reactivity of the photolyzed intermediate comes with a complete lack of specificity in what the intermediate reacts
with. In the case of diazoesters, a reactive carbene is formed. When the carbene is solvent exposed, reaction occurs to a substantial degree with bulk solvent, a non-productive side product of the bioconjugation reaction. To bias the photoinduced bioconjugation reaction towards productive reactivity with chymotrypsin, Westheimer and coworkers attached the photolabel covalently to an active site residue of the protein prior to photolysis. This covalent adduct makes the resulting photolabeling reaction intramolecular; improving the selectivity of the carbene insertion for the target protein. This strategy is often not applicable in the realm of medicinal chemistry. The vast majority of medicinal chemistry compounds are non-covalent inhibitors of enzymatic activity. However, because of the kinetics of photoexcitation and reactivity, covalently linking a photolyzable functional group and a non-covalent inhibitor imparts specificity to the reactivity of the typically non-specific photoreactive functional group.

After the introduction of photoaffinity labeling by Westheimer and coworkers, work by the Knowles Laboratory published pioneering work utilizing PAL to map active site residues of proteins. With the advances in proteomic capabilities, proteins can now be identified from PAL reactions after isolation from the biological milieu and MS analysis. Advances in these techniques have led to the rapid expansion of the field. Taunton and coworker published an excellent summary which details a protocol for the selection of a PAL reactive group, and the subsequent techniques which allow for target identification. In modern chemical biology, three photolyzable functional groups dominate the field: aryl ketones, aryl azides, and diazirines (Figure 3.4). Each photolyzable group differs in its excited state reactivity, imparting slight differences
in photolabeling outcomes. Excitation of aryl ketones 121 promotes the molecule to the first singlet excited state. Intersystem crossing to the triplet state results in the ketone having more diradical character than carbonyl character 122. This diradical 122 can relax back to the ground state 121, making the photoexcitation of aryl ketones reversible, or the diradical can abstract a hydrogen atom from a biological molecule resulting in radical recombination and carbon-carbon bond formation 123. This reversibility sets the reactivity of aryl ketones apart from the other photolyzable functional groups. The high homolytic bond dissociation of water makes this functional group unreactive towards the bulk solvent, conferring high selectivity to the labeling reaction. Light excitation of aryl azides 124 causes the loss of nitrogen gas, yielding a nitrene intermediate 125. The aryl nitrene 125 can react with organic molecules in several ways: aziridine formation with alkenes, C-H insertion reactions 126, C-N insertion reactions, or ring expansion followed by conjugation to a nucleophile. The last crosslinking functional group is the excitation of diazirines 127. These functional groups represent the most synthetically complex photoaffinity labels. Once excited, these molecules lose nitrogen gas yielding a carbene intermediate 128. The carbene is then able to either react with solvent and act as an electrophile, undergo H-heteroatom insertion reactions, or undergo C-H insertion reactions 129.
In an ideal scenario, the photolabeling of a protein could be identified through the use of a positive control and a negative control for photoirradiation, where MS analysis could observe a new photoproduct mass for Target + Probe. However, the sheer complexity of the biological milieu results in tremendous background signal making this approach quite challenging. Instead, if an investigator considers that their photo-affinity probe is now a covalent inhibitor of the protein binding partner, then the principles of competitive ABPP can be applied. Small lipophilic molecules have an intrinsic affinity for protein when compared to the bulk solvent, water. Thus, an investigator would expect a certain level of non-specific covalent bonding of the photoprobe, but the expected level and identity of the background will be unknown. In a PAL pulldown, comparing experiments with and without competition answers this question. A specific binding
partner of the parent drug should be enriched through chemical reactivity of the photoprobe. However, in the presence of the non-covalent competition, the signal should be significantly decreased or not observed. This result indicates the protein is making a specific interaction with the inhibitor warhead from which the pulldown probe was derived.

3.3 Design and synthesis of a photoaffinity analog from an inhibitor lead

To perform a photo-affinity pulldown experiment, the photo-affinity analog of the parent inhibitor must be designed with significant preexisting SAR knowledge. The point of attachment for the photocrosslinker and the affinity handle must conserve the selectivity of the parent molecule for the target. To achieve this, we designed a photoaffinity probe by the addition of a photoaffinity label and a biotin handle to our most potent inhibitors. The SAR of dimeric chloroquine (DC) and dimeric quinacrine (DQ) inhibitors indicated that inhibitors containing a 6/6 triamine linker are the most potent inhibitors of autophagy and the most potent anti-cancer agents. SAR studies of both classes molecules identified the importance of the basic nitrogen atom within the linker of the dimeric compound. The linker size and the presence of a tertiary amine was to be maintained in the photoaffinity analog. Studies of DQ inhibitors established the central nitrogen atom alkylation governs whether a molecule induces or inhibits autophagy. We identified that alkylation of the central nitrogen of our triamine linker could serve as an attachment point for the required PAL functional groups. We used these criteria to design a series of photoaffinity probes to identify the target of tertiary amine containing DQ and DC inhibitors.
A linear synthetic strategy was chosen (Fig 3.5, A), where a photocrosslinker could be attached to the DC660 68 (Figure 3.5, B) inhibitor through a flexible linker via alkylation of the central linker nitrogen. The attached photoaffinity moiety would contain an alkyne handle where click chemistry could be used either synthetically to attach an affinity handle or in situ utilizing biorthogonal click chemistry. Benzophenone was selected as the first choice for a photoreactive functional group because of the synthetic ease of its incorporation and its specificity in photolabeling reactions (Figure 3.5, C).

Biotin is a natural product ubiquitous in biological systems and is commonly used as a handle in affinity chromatography. Multiple homologs of biotin-binding proteins exist which form extremely tight non-covalent binding interactions. The interaction
between biotin and streptavidin, a common biotin-binding protein, is non-covalent. The tight binding affinity of this interaction allows for solid-supported streptavidin to rapidly bind biotinylated molecules in solution. The non-covalent interaction between biotin and a biotin binding protein is strong enough that the supernatant can be removed from a mixture of lysate and solid support without breaking the interaction. Further, washing the solid support with detergent will not break the biotin-enzyme interaction. Desthiobiotin is an analog of biotin which maintains a significant binding interaction with biotin-binding enzymes. However, additional biotin can be utilized to displace desthiobiotin from biotin binding proteins (Figure 3.5, D). The advantages of using a biotin analog become apparent when separating biotin labeled targets from native biotinylated molecules.

Polyethylene glycol (PEG) was selected as a linker between each component of the photo-affinity probe. With binding conformation of the parent inhibitor unknown, a flexible linker was chosen to maximize the likelihood of crosslinking. PEG specifically was chosen as the linker because of its lower cLogP value when compared to an aliphatic linker of similar length (Fig 3.6). The addition of benzophenone adds a significant amount of hydrophobic surface area to the probe; therefore, the addition of the hydrophilic PEG chain was designed to maintain the aqueous solubility of the molecule. Tetraethylene glycol was selected as the PEG linker for multiple reasons: first, PEG linkers between two and four monomer units are commodity chemicals; second, tetraethylene glycol afforded a significant amount of hydrophilic surface area to help solubilize the large probe molecule; third, the increased flexibility of the extended linker would maximize the chances of photocrosslinking success, even if the inhibitor warhead
was buried deep within the protein of interest; fourth, the longer linker was selected to space the benzophenone far enough away from the parent warhead so as to not influence the binding mode of the probe molecule.

With advances in the sensitivity of mass spectrometry, it is becoming increasingly possible to generate large profiles of data for multiple photocrosslinking probes. If the number and cost of biological experiments were not a factor, each photoreactive functional group would be combined with each length of spacer. A protein pulled down most often among all analogs would be the most attractive putative target. However, in a case where three photoreactive chemistries are available and three linkers are considered, nine biological experiments would be required prior to the reproduction of the results. Each biological experiment consists of one set of experimental conditions and two controls, which would result in 27 proteomic profiles for analysis of each inhibitor warhead. Such efforts were beyond the scope of our capabilities, and therefore we proceeded with inhibitors utilizing one PEG length and one photoaffinity probe per inhibitor warhead.

Figure 3.6: The structures of PEG chains and the corresponding aliphatic diols of equal length.
With the design of a photoaffinity probe in hand, the synthetic effort focused on the synthesis of three separate regions. The dimeric inhibitor was synthesized as the secondary amine-linked analog, which would allow for the attachment of a PEG chain via reductive amination. The benzophenone could be attached to a PEG chain, which could be used to alkylate the inhibitor. The other side of benzophenone could contain a propargyl group which could facilitate the attachment of biotin via click chemistry.

Figure 3.7: A. General structure of a DC photoaffinity pulldown analog. Key synthetic disconnections are noted by dotted lines. B. Retrosynthesis of a photoaffinity analog of DC661.
Tetraethylene glycol was mono-tosylated using $p$-toluene sulfonyl chloride as the limiting reagent in the presence of pyridine to yield compound 143.\textsuperscript{104} 4,4-
dihydroxybenzophenone was monoalkylated with propargyl bromide in ethanol heated to reflux using potassium carbonate as a base to yield 142.\textsuperscript{105} 4-propargyloxy-4-
hydroxybenzophenone 142 was deprotonated by potassium carbonate in ethanol, and the resulting phenolate was then alkylated with tosylate 143 to give 141. Compound 141 was oxidized by Swern conditions yielding an aldehyde, 144. Aldehyde 144 was used in slight excess, without purification, in the subsequent reductive alkylation of the secondary amine containing DC660 68 with sodium triacetoxyborohydride in CH$_2$Cl$_2$ (Figure 3.8).
The affinity handle for the probe was synthesized starting from the bis-mesylation of tetraethylene glycol in methylene chloride, using triethylamine as a base. The crude mesylate was then reacted with sodium azide \textit{in situ} using a mixture of water and ethanol heated to reflux.\textsuperscript{105} Monoreduction of the bisazide was facilitated by Staudinger reduction using triphenylphosphine in a biphasic solvent system of diethyl ether and 0.9 M phosphoric acid (1:1, 50% v/v) to give compound 147.\textsuperscript{105} The resulting primary amine 147 was then coupled to desthiobiotin 131 via a peptide coupling reaction using TBTU and Hünig’s base in DMF to give 140.\textsuperscript{105} (Figure 3.9)
The alkyne-containing photoprobes 139, 145, and 146 were then linked to the desthiobiotin affinity handle 140 by click chemistry using copper (II) sulfate and sodium ascorbate in a methanol and water solvent system. Click chemistry was selected as the final synthetic step due to its broad functional group compatibility. The click reaction yielded photoaffinity compounds 139, 148, and 149 in serviceable yield. (Figure 3.10).
CQ 1 and HCQ 4 have structural differences in the linker region when compared to DC inhibitors, specifically a methyl group and a diethylamine (Figure 3.11). Both CQ 1 and HCQ 4 are similarly effective in inhibiting autophagy, and thus we hypothesized the hydroxyl of HCQ 4 was not necessary and could be utilized as a handle to attach photoaffinity functional groups to commercial HCQ 4. We envisioned the construction of a photoaffinity analog for CQ/HCQ 1/4 would contain a PEG chain linked to the tertiary amine found in the linker of CQ/HCQ 1/4 (Figure 3.12). Multiple attempts were made to utilize the hydroxyl of HCQ 4 as a free nucleophile in the S_n2 alkylation of a tosylated PEG derivative. Each of these attempts proved unsuccessful, where no alkylated product
was observed. We attempted to reverse the polarity of the $S_N2$ reaction by exchanging the hydroxyl of HCQ 4 with a leaving group via synthesis of the mesylate and the bromide. TLC analysis of both reactions indicated a consumption of starting material; however, a baseline spot was observed which was found to be immobile on silica. We hypothesized that either the molecule is undergoing a decomposition reaction with the unprotected aminoquinoline acting as a nucleophile, or that the tertiary amine could be contributing to the premature ejection of the new leaving group via aziridinium formation. Unfortunately, any attempts to trap the electrophile with triethylene glycol proved to be unsuccessful. This reaction should be studied in future derivatizations of chloroquine. However, in the interest of synthetic ease we pivoted our design strategy to synthesize a CQ 1 derivative de novo.
In 1946 Professor Marvin Carmack and coworkers synthesized monoalkylated versions of CQ. In their paper they described a compound which was referred to as desethyl chloroquine, a chloroquine analog containing a secondary amine rather than the standard tertiary amine. This led us to hypothesize that by accessing this chloroquine derivative, we could apply the same chemistry we had utilized to functionalize secondary amine containing DC inhibitors. The route published by Carmack and coworkers contained no spectroscopic data for the compounds and required high pressure reductions.

Figure 3.11: A. A structural comparison of CQ and HCQ. B. Retrosynthesis of a chloroquine photoaffinity analog. The synthesis proceeds through a secondary amine containing CQ analog which is synthesized de novo.
and extremely low-pressure distillations. Therefore, we proposed to improve the route through the utilization of modern organic chemical methods.

We envisioned synthesizing desethyl chloroquine by first synthesizing an amino alcohol and arylating the nitrogen via Buchwald-Hartwig conditions. We elected to arylate prior to synthesizing the diamine because we feared the length of the carbon linker (four) would make any attempts to synthesize the diamine prone to cyclization. We hypothesized that arylation of the primary amine of the amino alcohol 153 with the electron withdrawing chloroquine ring would help prevent the cyclization from occurring.
We began by synthesizing 4-nitropentanoate, 154, from nitroethane and ethyl acrylate, according to literature precedent.107 Ester 154 was then reduced with lithium aluminum hydride to afford the 4-amino-1-pentanol 153 in good yield. Buchwald-Hartwig arylation of amino alcohol 153 produced the arylated product 152 in serviceable yield. Alcohol 152 was then oxidized to aldehyde 155 using Swern conditions. Initial experiments which used CH₂Cl₂ as a solvent were unsuccessful. Changing the solvent to THF proved critical to the success of the Swern oxidation. Aldehyde 155 was found to decompose on purification. Therefore, the crude aldehyde was reacted without
purification in a reductive amination reaction with ethylamine and sodium triacetoxyborohydride to yield desethyl chloroquine, 151. Compound 151 was reductively alkylated with aldehyde 144 using sodium triacetoxyborohydride in CH2Cl2. Alkyne 150 analog was then ligated to desthiobiotin azide 140 via click chemistry, yielding the final CQ-P 156.

For a pulldown probe to be successfully utilized in a competitive photocrosslinking experiment, it must mimic the in vivo activity of the inhibitor analog from which it was derived. We assayed A375P melanoma cells treated with each of the photoaffinity pulldown molecules to determine whether autophagy was inhibited similarly to A375P melanoma cells treated with the parent inhibitors. DC661-P, (Figure 3.10, 138) caused the accumulation of LC3-B at doses as low as 0.3 nM. DC221-P, 148, and CQ-P, 156, both lost significant potency in comparison to their parent inhibitors (Figure 3.13). However, inhibition of autophagy at high micromolar doses demonstrated that the molecules were still functional, and that the molecules still caused autophagy
inhibition. This indicated to us that they would be acceptable molecules for a competitive PAL experiment.

![Western blot](image)

**Figure 3.14:** Western blot assaying autophagy inhibition (LC3B) and mTOR signaling (p4E-BP1 and pS6) in DQ661-P 149 treated cells. Decreased p4E-BP1 and pS6 indicates inhibition of mTOR signaling.

### 3.4 Implementation of a photoaffinity pulldown probe

In our studies of DQ inhibitors, we observed inhibited cell proliferation, inhibited autophagy, and blocked mTOR signaling in DQ661 treated melanoma cells. The DQ661-P, 149, treated cells were assayed for autophagy inhibition and mTOR signaling. Treatment of melanoma cells with DQ661-P 149 caused the accumulation of LC3B, which indicated that autophagy was inhibited. Western blotting for 4E-BP1 and S6 demonstrated that phosphorylation was decreased upon treatment of A375P melanoma cells with 149, indicating that mTOR activity was suppressed. Therefore, we reasoned that 149 would be effective in competitive PAL experiments.
Doses for the pulldown assay were determined with maximum target capture in mind, while minimizing background non-specific binding. In a standard model for protein-substrate binding, the affinity of an enzyme for a substrate or inhibitor can be quantified in terms of the dissociation constant \( K_d \) of the ligand, defined by the concentration of free enzyme and free ligand divided the concentration of enzyme-ligand complex. Utilizing this metric, a smaller numerical value indicates a tighter binding interaction. The amount of inhibitor present at concentrations below the \( K_d \) is rapidly complexed with enzyme. Enzymes quickly reach a point of saturation where no further ligand is bound after the dissociation constant is reached (Figure 3.15). Inhibitor added beyond the enzyme saturation point will have a greater contribution to unwanted background signal than increased target binding. We hypothesized that for a phenotype to be induced, significant quantities of the target enzyme must be bound. Therefore, a dose of 200 nM was selected, because at this dose LC3B is observed to accumulate, indicating autophagy inhibition. From this result we hypothesized that a significant quantity of the putative target must be bound to cause the autophagy inhibition phenotype. Our proposed proteomic analysis was used only for protein identification purposes, and therefore we valued background signal reduction greater than increased target binding.
The experimental controls are critical to the successful design of a pulldown experiment. At its core, the goal of a photoaffinity pulldown is to make an unbiased crosslink between the photoreactive functional group and the nearest protein to the probe. In all cases, addition of the probe functionality to parent warhead inherently will add significant non-specific binding, because desthiobiotin will be bound by native biotin binding proteins and benzophenone will make non-specific hydrophobic interactions with proteins. Further, it is expected that all small molecule therapeutics have non-specific protein binding due to the drug partitioning between the aqueous solvent and the more hydrophobic macromolecules of the cell.

The experimental setup becomes a balancing act of generating and isolating as many crosslinked potential targets as possible, while discerning which of those crosslinks are specific and which are non-specific. We utilized three controls for every probe tested.
This first control was a negative control in which cells were treated with the photoaffinity probe but were not exposed to UV irradiation. Proteins identified should not be covalent adducts and were regarded as nonspecific binding. In the next experiment, the cells were treated with the photoaffinity probe and irradiated with UV light. The benzophenone and the affinity handle will generate false targets which bind the photoaffinity probe but would not have significant affinity for the parent inhibitor. In order to separate these false positive results from the true potential targets, a competition control was utilized. We hypothesized that the mechanistic target of dimeric lysosomal inhibitors will bind equally or more potently to the parent inhibitor than the photoaffinity probe. This hypothesis was based on the observation that neither benzophenone nor biotin/desthiobiotin have been reported as inhibitors of autophagy. We hypothesized that the administration of a competition dose of tenfold greater magnitude than the photoaffinity probe would displace the photoaffinity probe from the putative protein target. This decrease in signal for proteins would indicate that their crosslinking was conferred by a specific binding affinity between the inhibitor warhead and the protein. The crosslinking of proteins which non-specifically bind the benzophenone or affinity handle should not be significantly altered by the competition dose.

Once cells were either irradiated or not irradiated, they were immediately harvested and lysed. The cell lysate was frozen at -80 °C in the presence of broad spectrum protease inhibitors until affinity purification. To facilitate affinity chromatography, a streptavidin protein conjugated to polymer support was required. Neutravidin (Thermo Scientific), a bioengineered homolog of avidin and streptavidin,
was chosen for our experiment. Neutravidin binds biotin as tightly as both avidin and streptavidin, but the surface of the protein is mutated to maximally reduce non-specific protein binding to the resin. The protein is conjugated to agarose as a solid support. The neutravidin resin was equilibrated with cell lysis buffer containing protease inhibitors, and was mixed with cell lysate for a period of 12-16 hours at 4 °C. The resin was then separated via centrifugation and washed with a series of NP-40-containing buffers to remove cell debris and non-specifically bound proteins. The washes were analyzed via SDS-PAGE to determine whether further washing was required.

The use of desthiobiotin as an affinity handle facilitated a non-denaturing elution step, yielding a cleaner elution from the resin. Desthiobiotin 131 (Figure 3.5) is an analog of biotin 130 (Figure 3.5), where the sulfur atom is removed from the tetrahydrothiophene ring, leaving only the cyclic urea of the fused ring system. Multiple orthogonal elution methods based on chemical linkers which are labile to bioorthogonal chemistry have been invented.108 We elected to utilize desthiobiotin because of synthetic ease and the predefined protocols for elution from avidin resins.

Desthiobiotin binds avidin/streptavidin with a $K_d$ of $\sim 10^{-13}$ versus the $K_d$ of biotin $10^{-15}$. The difference of two order of magnitude allows for elution of desthiobiotinylated molecules via incubation with a biotin-containing buffer. A 15-minute incubation of the solid supported neutravidin resin in a 4 mM biotin buffer produced proteomic profiles which were analyzed by trypsin digest LC-MS/MS to identify the proteins which were pulled down.
3.5 Selection and Validation of the Putative Protein Targets

The proteomic profiles were analyzed with two metrics. First, the abundance of a protein was scored by viewing the positive experimental condition (+UV) divided by the negative control (-UV). A higher score in this metric demonstrates an enrichment via the UV-irradiation induced crosslinking process, separating a pulled down protein from background noise. Second, the protein abundance was scored by viewing the positive experimental condition (+UV) divided by the competition control (+UV+Comp). A higher score in this metric is indicative of the degree of specificity of the interaction of the drug, and its ability to impart specificity to the non-specific crosslinking reaction.

![Proteomic profiles of DC661-P and DQ661-P](image)

Figure 3.16: Proteomic profiles of DC661-P and DQ661-P (best performing candidates were highlighted). CTSD and PPT1 are the best protein candidates which are known lysosomal enzymes.

The protein profile of DQ661-P was analyzed with the two metrics, revealing a number of potential protein candidates. The best proteins judged by these metrics were
then cross-referenced with their association with the lysosome and autophagy. Filtering by protein association revealed three promising lysosomal targets: PPT1, cathepsin D, and LGALS3 (Galectin-3) (Figure 3.16). When interrogating the proteins by their abundance across all photoaffinity probe synthesized, PPT1 and cathepsin D stood out as the two best candidates. LGALS3 is a protein known to localize to the lysosome after permeation of the lysosomal membrane occurs. Therefore, this protein was discounted as a mechanistically relevant target.67

Cathepsin D, while significantly pulled down in every experiment, was not competed off effectively (Figure 3.16). From this result, we concluded that the natural abundance of cathepsin D (CTSD) in the lysosome made the protein a non-specific binder of the dimeric inhibitors. CTSD is an aspartyl protease, acting as one of the major proteolytic enzymes in the lysosome. Therefore, we sought to determine whether CTSD activity was inhibited by DC/DQ in activity assays. However, CTSD activity from neither in vitro nor cell lysate was affected by treatment with DQ or DC inhibitors (Figure 3.17).
The combination of poor competition efficiency in the pulldown assay, inability for DC/DQ inhibitors to affect CTSD activity, and the lack of literature support for CTSD producing the observed phenotype led to the exclusion of CTSD from the likely target list.

Figure 3.18: Western blotting for PPT1 reveals specific binding by the DC, DQ, and CQ inhibitors. A. Western blot for PPT1 in a pulldown experiment (DC661-P, 138), where three conditions were assayed (+UV, -UV, and +UV +competition). B. Western blot for PPT1 in a pulldown experiment (DC221-P, 148), where three conditions were assayed (+UV, -UV, and +UV +competition). C. Western blot for PPT1 in a pulldown experiment (CQ-P, 156), where three conditions were assayed (+UV, -UV, and +UV +competition). D. Silver stain SDS-PAGE of a pulldown experiment for DQ661-P 149 treated A375P melanoma cells. E. Western blot for PPT1 and CTSD in a pulldown experiment (DQ661-P, 149), where three conditions were assayed (+UV, -UV, and +UV +competition).
The next most prominent lysosomal protein pulled down by DQ and DC photoaffinity probes was protein palmitoyl thioesterase 1 (PPT1). PPT1 scored highly in the selectivity metric for proteomic analysis, meaning the competition experiment indicated DQ/DC inhibitors make a specific interaction with PPT1. PPT1 is a member of the serine hydrolase family of enzymes.\textsuperscript{109} The enzyme is known to reside within the lysosome of the cell.\textsuperscript{110} The function of PPT1 is to help maintain dynamic lipid signaling on the surface of proteins, in addition to removing S-acyl fatty acids from substrates it encounters in the lysosomal compartment. PPT1 was originally observed as a protein bound to the kinase H-Ras, an important enzyme in oncogenesis.\textsuperscript{111} Biochemical studies of PPT1 have defined a wide substrate scope for the enzyme including palmitoyl-CoA, myristoyl-CoA, and palmitoylated thio-glucoside.\textsuperscript{111-113} It is hypothesized that PPT1 recognizes the fatty-acid and hydrolyzes the labile acyl bond, regardless of the molecule which is acylated.\textsuperscript{114} It has been proposed that PPT1 is responsible for cleaving palmitate from proteins which have been trafficked to the lysosome for degradation. The activity of PPT1 has been monitored using both small molecule cleavage assays and fatty acid labeled proteins.\textsuperscript{112,113}

![Native Activity of PPT1](image)

*Figure 3.19: Native activity of PPT1 cleaves thioester derivatives of fatty acids in the lysosome of the cell.*
PPT1 is most well-known for the role it plays in infantile neuronal ceroid lipofuscinoses (INCL). INCL is a subset of Batten disease, a fatal lysosomal storage disorder which effects the nervous system of human patients. In these patients, lysosomal activity is significantly reduced, resulting in the build-up of toxic lipidated species. The buildup of these toxic species manifests itself in multiple neurological disorders including retinal toxicity, resulting in the patients’ loss of vision. Perhaps most interesting is the observed retinal toxicity in patients who have taken extended courses of CQ. While this result may be anecdotal, it is consistent with the observation that PPT1 dysfunction can perturb lysosomal functional on an organismal level. We hypothesized that extended CQ usage could result in tremendously high doses accumulated in the lysosomes of patients, resulting in a toxicity similar to that experienced by INCL patients.
To explore whether the catalytic activity of PPT1 was affected by treatment with dimeric lysosomal inhibitors, we adopted a fluorescence assay described by van Diggelen et al.\textsuperscript{112} This assay leverages the agnosticism of PPT1 toward the identity of the palmitoylated substrate by building the reporter 157 from a thioglucose scaffold. The reporter molecule 158 contains a primary thiol which is palmitoylated. At the anomeric position of the sugar is a coumarin, which is non-fluorescent when the oxygen is alkylated. In order to release the fluorescent coumarin, β-glucosidase is required to cleave the anomeric sugar bond. When the sugar is palmitoylated, β-glucosidase is unable to cleave the fluorescent coumarin, resulting in a quenched fluorescence signal. However, when PPT1 is present, it rapidly cleaves the palmitoyl chain from the thioglucose reporter, yielding compound 158. This allows for efficient release of the fluorescent

Figure 3.20: Mechanism of PPT1 reactivity.
coumarin 159 by β-glucosidase. The assay is designed with beta-glucosidase in excess, forcing the rate determining step of the reaction to be the enzymatic activity of PPT1. Thus, the increase in coumarin fluorescence is directly correlated to the activity of PPT1.

We adapted the reported assay by utilizing homogenized melanoma cell cultures treated with different inhibitors. The cell culture conditions were assayed by dose and treatment duration with DC/DQ inhibitors to understand the effects of our inhibitors on PPT1 activity. Once the treatment period had elapsed, the cultures were homogenized using mechanical and osmotic stress to lyse the cells, as per the van Diggelin protocol. The cell homogenates were then normalized for their protein content as a method of maintaining the same amount of PPT1 across the reactions. The normalized homogenates were then mixed with an assay cocktail containing the reporter and exogenous beta-glucosidase and reacted for one hour at 37 °C. The results of DC661-mediated 69 PPT1 inhibition were the most striking (Figure 3.23). Cells treated with escalating doses of

Figure 3.21: The assay described by van Diggilen et. al. for measuring PPT1 activity.
DC661 69 for 3 hours were found to have significantly diminished PPT1 activity.

DQ661-treated melanoma cells were also found to have decreased PPT1 activity. In an effort to validate PPT1 as a target across chloroquine-based molecules, Lys 05 9 and HCQ were also tested for their ability to inhibit PPT1. Gratifyingly, Lys-05 demonstrated inhibitory activity against PPT1. Cells treated with higher doses of HCQ 4 also demonstrated some inhibition of PPT1 (~50% activity), supporting the hypothesis that PPT1 is also a target of the parent monomeric heterocycle chloroquine.
Figure 3.22: A. The dose-dependent inhibition of PPT1 by cells treated with DC661. The activity of PPT1 was measured by a fluorogenic reporter assay. B. HCQ, Lys 05 also inhibit PPT1 in melanoma cell culture when cells were assayed with the same fluorogenic reporter assay.
With cellular data in hand we wanted to determine if PPT1 could be inhibited by the dimeric compounds \textit{in vitro}. The Arrowsmith Laboratory had previously solved the crystal structure of a PPT1 construct which was expressed in insect cells (SF9) via infection with baculovirus (PDB: 3GRO). The construct for the insect cell expression was made available for purchase through Addgene. PPT1 contains post-translational modifications, N-linked glycosylation, which are required for the proper function of the enzyme. Unfortunately, \textit{E. coli}, the standard organism used for recombinant protein expression, does not glycosylate expressed proteins. In collaboration with Dr. Julie Rotenberg-Barber in the Marmorstein Laboratory at the University of Pennsylvania, we acquired and expressed the \textit{in vitro} construct of PPT1 in SF9 insect cells. During the initial purification we identified that the protein was being excreted into the medium, requiring ammonium sulfate precipitation and resuspension in new buffer to facilitate His-tag/nickel-NTA purification.\footnote{53}

To correlate the recombinant enzymatic activity to the fluorescence emission of the van Diggelin assay, the linear activity range of the protein was established.\footnote{112} According to Michaelis-Menten kinetics, when a protein catalyzes a reaction in a range where the correlation between signal and concentration of the substrate is linear, then the rate of the reaction is directly dependent on the binding interaction between the protein and the substrate. We hoped to observe whether DC or DQ compounds function as competitive inhibitors of PPT1 catalytic activity. Recombinant PPT1 was subjected to the van Diggelin assay conditions, where 50 nM PPT1 was found to react linearly over twenty minutes with 640 uM thioglicoside substrate.
Once the linear range of the enzyme was established, the assay was set up by charging each reaction vessel with recombinant protein (50 nM) and inhibitor (20 uM) in a pH 5.0 citric acid/sodium phosphate buffer. The protein and inhibitor samples were incubated for 90 minutes at 37 °C to allow time for any potential binding interactions to occur. A separate reaction cocktail was created containing the standard van Diggelin assay components. The cocktail was then added to the protein-containing reaction vessels and mixed with a pipet. The reactions were incubated at 37 °C and the fluorescence was read via a fluorescent plate reader. Unfortunately, no concentration of DC661 in vitro could replicate the inhibition of PPT1 which was observed in vivo. (Figure 3.24)
To confirm that a binding interaction exists between PPT1 and the DC/DQ inhibitors, we again turned to our collaboration with the Marmorstein Laboratory. Differential scanning calorimetry (DSC) is a method that detects the heat at which a protein begins to melt and unfold. The fold of a protein is the summation of numerous favorable interactions which cooperatively lend stability to the tertiary structure of the protein. When enough heat is added, the protein absorbs enough energy to transition from the folded to the unfolded state. This point is defined as the melting temperature (T_m). Theoretically a molecule which binds a folded protein can either increase the stability of the fold by buttressing a cleft which is normally occupied by solvent and providing favorable bonding interactions, or the binding molecule could lower the activation barrier to unfolding and destabilize the fold of the protein. Dr. Julie Rotenburg-Barber conducted the DSC experiments in the presence of DQ661 64, DC661 69, Lys-05 9, and HCQ 4. Each inhibitor was found to interact with recombinant PPT1. DC661 69, Lys-05 9, HCQ 4, and DQ661 64 all cause a decrease in the melting temperature of the protein by a significant value compared to PPT1 without an inhibitor present. We have concluded from this decrease in melting temperature that PPT1 is bound by each inhibitor and is destabilized by the interaction with the inhibitors.
Work by Dr. Vito Rebecca of the Amaravadi Laboratory monitored the effectiveness of PPT1 depalmitoylation in A375P melanoma cells via the use of a biotin-acyl exchange assay. In this assay, cell lysate is harvested from melanoma cells treated with DQ661\(^64\) or from an untreated control. The free cysteines in the lysate are first alkylated via maleimide chemistry, then the palmitoylations are removed by the addition of hydroxylamine. Maleimide biotin was then added to the lysate to label the proteins which had existing palmitoylations. These palmitoylations prevented reaction with the initial maleimide treatment. The biotinylated proteins were pulled down with avidin resin and analyzed by Western blotting. CD44 is a protein which is well documented to be palmitoylated.\(^{117}\) Western blotting for CD44 in the acyl biotin exchange assay revealed that treatment with DQ661\(^64\) resulted in the increase in palmitoylated CD44, demonstrating PPT1 inhibition \textit{in vivo} (Figure 3.25, A.).
PPT1 was knocked down in melanoma cells utilizing siRNA (siPPT1). Cells which were treated with siPPT1 showed inhibited autophagy by the accumulation of LC3B and p62. Phosphorylation of S6 substrates decreased over time with siPPT1, indicating mTOR inhibition. These results demonstrate that PPT1 suppression mimics DQ661 treatment in melanoma cells (Figure 3.25, B.). N-tert-butyl hydroxylamine (NTBHA) is a derivative of hydroxylamine which is less toxic to human cells. A study by the Mukherjee Laboratory has utilized NTBHA to mimic PPT1 activity in cells with deficient PPT1.118 Our studies found that melanoma cells treated with DQ661 64 and
NTBHA showed blunted autophagy inhibition and blunted mTOR inhibition. (Figure 3.25, C.)

A375P Melanoma cells were treated with DC661 to examine the effect on global palmitoylation. CD44 was enriched via the acyl-biotin exchange assay, indicating that DC661 treatment suppressed the depalmitoylation activity of PPT1. HCQ treatment also resulted in the accumulation of CD44 as observed by the acyl-biotin exchange assay, indicating that PPT1 activity was suppressed. Finally, NTBHA was utilized in combination with HCQ, Lys-05, and DC661 to observe the effect of the combinations on autophagy inhibition. Western blotting for LC3B indicated that autophagy inhibition was blunted by the co-treatment of HCQ and DC inhibitors with NTBHA. This result suggests that compensation for the activity of PPT1 blunts autophagy inhibition via HCQ, Lys-05, and DC661.

Figure 3.26: A. A375P melanoma cells treated with DC inhibitors or HCQ. CD44 palmitoylation was assayed via the acyl-biotin exchange assay and Western blotting for CD44. Cells treated without hydroxylamine were utilized as a control. CD44 observed on the Western blot is palm B. A375P melanoma cells treated with DC inhibitors or HCQ. The cells were treated with or without NTBHA treatment (PPT1 activity mimetic). The autophagy inhibition in cells was measured by Western blotting for LC3-B.
3.6 The effect of PPT1 inhibition on mTOR kinase function

To understand how inhibition of PPT1 could inhibit lysosomal functional, we surveyed the literature for connections between PPT1 and our observed phenotypes. Reports by Bagh et al. recently reported that a specific subunit of the VATPase is palmitoylated. They determined that, when PPT1 is dysfunctional in cells, subunits of the VATPase do not correctly localize to the lysosomal membrane. This in turn prevents proper VATPase function and contributes to the deacidification of the lysosomal compartment. In addition, Zoncu et al. reported that the VATPase is critical for mTOR localization to the lysosome. The VATPase interacts with the Ragulator complex on the surface of the lysosome. When the VATPase is not functional, the Ragulator complex cannot effectively bind mTOR and lead to activation of the kinase. We have proposed a mechanism of action for DQ661-mediated mTOR inhibition, whereby inhibition of PPT1 function and the lysosomal localization of VATPase subunits are disrupted. This in turn results in dysfunctional mTOR signaling, resulting in the inhibition we have observed. Consistent with this hypothesis, Dr. Vito Rebecca observed mislocalization of the VATPase subunit which was reported to require PPT1 function for its correct localization (Figure 3.27).
3.7 Precedent for Aberrant Behavior of Recombinant PPT1

We were interested in why PPT1 in melanoma cells was inhibited by DC/DQ treatment, whereas recombinant PPT1 is not inhibited. We considered that the lysosomal environment could be critical for the inhibition of PPT1 activity. There have been studies of PPT1 which suggest that recombinantly expressed PPT1 does not behave in the same manner as endogenous PPT1. Work by Lyly et al. demonstrated that recombinantly expressed PPT1 was catalytically active. However, when overexpressed within fibroblasts or neuronal cells, the protein was found to localize differently than the endogenously expressed PPT1. In non-neuronal cells, PPT1 is transported to the lysosomal compartment. Overexpression, however, resulted in the improper localization of the enzyme to non-lysosomal vesicle structures within the cell. Further biochemical studies were performed to identify activity differences between endogenous PPT1 and

![Image: Western blot of A375P melanoma cells (wild type and PPT1 siRNA knockout) analyzing the localization of subunits of the VATPase. Loss of signal for V1A indicates that the VATPase subunit required for proper mTOR localization is not present in the membrane fraction compared to the wild type control.](image-url)
recombinant PPT1. In a fascinating experiment, PPT1 was overexpressed as a fusion protein with eGFP as an affinity handle and label for co-immunoprecipitation. This experiment should only pull down PPT1-eGFP fusion. However, when the co-immunoprecipitation fractions were analyzed by Western blot, both eGFP-PPT1 and endogenous PPT1 were detected. This experiment establishes the previously unreported ability of PPT1 to homo-oligomerize. Lysosomes were enriched and separated by size-exclusion chromatography, where PPT1 was observed to elute during the gradient corresponding to proteins with a molecular weight >100 kDa (3-fold the molecular weight for the enzyme). To quantify this oligomerization affinity, PPT1 was assayed by surface plasmon resonance. In this technique, labeled PPT1 was fixed to a surface, and recombinant PPT1 was flowed over the PPT1-containing surface. If a binding interaction occurs, the detector should be reflected by a plasmon resonance in the surface, indicating a binding event. Strikingly, in this experiment using only recombinant PPT1, no binding event was observable by surface plasmon resonance. This result gives biochemical proof that endogenous PPT1 and recombinantly expressed PPT1 behave differently in a series of different assays.

With these results in mind, we propose that studying the extra-lysosomal activity of PPT1 is not an accurate model of DQ/DC-mediated PPT1 inhibition in vivo. Therefore, future efforts will focus on the use of techniques that can better monitor the effect of DQ/DC inhibitors in a live cell context.
Chapter 4: Design, Synthesis, and Application of a Fluorogenic Substrate for Monitoring PPT1 Activity *In Vivo.*

4.1 The Need for a Live Cell Reporter for PPT1 activity

Having identified protein palmitoyl thioesterase 1 (PPT1) as a specific binding partner of the dimeric lysosomal inhibitors, we tested the effect of DC/DQ compounds on the catalytic activity of PPT1. These experiments were conducted both *in vivo,* utilizing cultured melanoma cells, and *in vitro,* with recombinantly expressed PPT1. During these studies we observed that endogenous PPT1 activity was inhibited, while the activity of exogeneous, recombinantly expressed, PPT1 was not inhibited (Chapter 3, Selection and Validation of the Putative Protein Targets).

Previous workers have also observed different activities with recombinant PPT1 and endogenous PPT1 in multiple assays (Chapter 3, Precedent for Aberrant Behavior of Recombinant PPT1).\(^{119}\) We hypothesize that the lysosome is a privileged environment which cannot be adequately recapitulated *in vitro.* It appears that endogenous PPT1 is required to study the interaction of the enzyme with DC/DQ inhibitors. Existing reporters for PPT1 activity require cell lysis. Lysis of the cell necessarily disrupts the lysosomal environment. For these reasons, the development of a live cell probe for PPT1 activity, which represents an unmet need in the study of palmitoylation regulation in cancer biology, became the next goal for our studies.
4.2 Existing Applications of Fluorescence Reporters

The successful application of bioorthogonal fluorescence in living systems has fundamentally changed the biological and life sciences. The discovery and application of green fluorescent protein was recognized with the Nobel Prize in 2008, marking the tremendous impact which bioorthogonal fluorescence has had on biology and the life sciences. In addition to genetically encoded fluorescent reporters, it has become common place to design experiments which utilize fluorescent dyes which have bioorthogonal fluorescence (fluorescence emission red-shifted beyond the fluorescence emission of nucleotides and aromatic amino acids) to monitor biological processes both in vivo and in vitro. Multiple efforts have successfully harnessed the sensitivity of fluorescence emission to design molecules which can be used to sense ionic analytes in human cells.\textsuperscript{120–124} In a similar manner, reactive molecules have been detected in cellular environments.\textsuperscript{125} Small molecule and polypeptide substrates have also been utilized to monitor the activity of different enzymes in vivo and in vitro.\textsuperscript{126–129}

4.3 Fluorescent Properties of Small-Molecule Dyes

Fluorescence emission is extremely sensitive to both the electronic structure of a fluorophore and the environment of the fluorophore. For fluorescence emission to occur, a molecule must first absorb a photon of high energy light. This excitation promotes an electron to the first singlet excited state. In order to relax back to the ground state, the molecule can emit a photon of light corresponding to the gap between the ground state and the first singlet excited state. (Figure 4.1) In many cases, non-radiative relaxations
are also available to molecules in the form of internal conversion, vibrational relaxation, rotational relaxation, electron transfer, and chemical reactivity.\textsuperscript{130,131} Both the intrinsic energetics of a fluorophore and the surrounding environment can shift the balance between radiative and non-radiative relaxations. Therefore, in select cases the bias of a molecule towards fluorescence emission can be correlated to a chemical event.

A chemical understanding of the photophysics of the fluorophore affords the capability to harness fluorescence emission as a sensor by biasing the molecule into either a radiative or non-radiative state through molecular design. When designing a sensor or a probe from a fluorophore, the on-state and off-state can be assigned to either a fluorescence emission (on) or lack thereof (off). These states can be extrapolated and applied to any system where the chemical reactivity being observed will bias a molecule towards either of these two states. By leveraging predictable electronic effects, these environmentally sensitive biases can be engineered into a molecular probe. (Figure 4.2)
A fluorophore has multiple measurable spectroscopic properties that impact the translation of excitation light into fluorescence emission. The absorption of excitation light by the fluorophore is linearly correlated to the intensity of fluorescence emission. The molar extinction coefficient of the fluorophore describes how efficiently a molecule absorbs a photon of light. This efficiency is directly correlated to the ability of the molecule to enter an excited state. The intensity of the fluorescence emission is dependent on another intrinsic quality of the fluorophore known as the quantum yield. This factor is defined in a range between zero and one and can be referred to as the
efficiency in which a fluorophore converts the energy of absorbed light into emitted radiation. The wavelength of the emission corresponds directly to the HOMO-LUMO energy gap. Alteration of electronic effects of a fluorophore pi-system directly affects this energetic gap, and thus the fluorescence emission. Often, the energy of the light absorbed is higher than that of the resulting fluorescence emission. This absorption of a photon promotes a chromophore to a higher state than the first singlet excited state. This energetic difference in absorption versus fluorescence emission is due to differences in torsional energies, vibrational energies, or solvation energies between the ground state and the excited state of the fluorophore. This causes a fluorophore to undergo a non-radiative relaxation to enter the first singlet excited state prior to emitting a photon of light. This difference in the excitation versus the resulting fluorescence emission is known as the Stokes shift of a fluorophore. Each of these characteristics are directly tied to the chemical structure of the fluorophore and can be synthetically engineered.\textsuperscript{130,132}

The design and synthesis of cyanine dyes represents an example of modulating the wavelength to a more efficient absorption maximum of light. The absorption maximum of cyanine dyes is easily tuned by synthetically increasing or decreasing the number of methine bridges between the two nitrogen atoms of the fluorophore. Cyanine 3\textsuperscript{160} has an absorption maximum at approximately 550 nm. Synthetic modifications which insert two methines, or one alkene, to the bridge region yield a dye with an absorption maximum at approximately 650 nm, known as cyanine 5\textsuperscript{161} A near-infrared absorbing, ca. 750 nm, cyanine analog can be synthesized by the addition of one more alkene to the bridge region, to yield cyanine 7\textsuperscript{162,133,134} (Figure 4.3)
The quantum yield of a molecule is also tied to structural features of a fluorophore. While predicting the quantum yield of a dye is quite difficult, many dyes have been developed that use well-known molecular mechanisms which decrease fluorescence emission. In the case of fluorescein, the chemical reactivity of the phenol is directly tied to the quantum yield of the dye. When fluorescein 163 is deprotonated at the phenolic position, the resulting phenolate 164 can collapse to a carbonyl, fully aromatize the xanthene skeleton, and liberate the carboxylate from the tricyclic skeleton of the dye. This causes a dramatic increase in the quantum yield of the dye, effectively turning on its fluorescent properties 165 (Figure 4.4). In acidic media, activation of the carbonyl induces cyclization of the carboxylate onto the xanthene skeleton. This cyclization results in a dramatic decrease in the quantum yield of the dye, effectively turning off the fluorescence emission.
4.4 Translating Fluorescence Spectroscopy to Monitoring a Chemical Event

Correlating the fluorescence properties of a molecule to a chemical event can be abstractly assigned to a two-state problem. These states are most easily assigned to the magnitude of fluorescence emission of a dye, where increased fluorescence emission represents the on-state, and the lack of fluorescence emission represents the off-state. These states are assigned by the investigator through selection of the excitation and emission wavelengths for observation via fluorescence spectroscopy. To apply this two-state system to monitoring a chemical event, the spectroscopic output from the two states must be understood in order to correctly frame the excitation and emission wavelengths.

The previously described spectroscopic properties of a molecule can affect whether an interrogated state appears to be on or off. From the perspective of the
observer, a change in quantum yield will result in a change in fluorescence intensity easily correlating to pushing a switch from one state to the other. (Figure 4.5 C&D).

Often, the making or breaking of a covalent or transient chemical bond induces a shift of the HOMO and LUMO of the fluorophore. This in turn shifts the absorption maxima and emission maxima either higher or lower in energy, presenting a more complex signal to parse into a two-state problem. However, when taking precise single point fluorescence emission measurements, a change in the absorption and emission maxima will fundamentally mimic a change in quantum yield from the perspective of the observer. (Fig 4.5 E & F).
Figure 4.5: A. Typical absorption and fluorescence emission spectra. The gap between the maxima in each spectrum is known as the Stokes shift. B. Jablonski plot which indicates the cause of the stokes shift. C. An absorption spectrum and two fluorescence emission spectra, where turn on of fluorescence is observed. D. Jablonski plot of fluorescence emission, where a radiative and non-radiative pathway are in competition. The equilibrium between these events yields the intensity of the turn-on in fluorescence. E. An absorption spectrum and a fluorescence emission spectrum which depicts a molecule where the HOMO-LUMO gap changes. F. Jablonski diagram which depicts a change in the HOMO-LUMO gap, resulting in a change in the energy difference between $S^0$ and $S^1$. 


4.5 Fluorophore Choice for a Fluorogenic PPT1 Substrate

The most common classes of fluorophore utilized for emitting light in the visible range are: xanthene dyes (fluorescein, rhodamine, rhodol, etc.), cyanine dyes (Cy3, Cy5, Cy7, etc.), coumarin dyes (4-methylumbelliferone 159, and more), and BODIPY dyes 167. Each class of dye has found different applications in chemical biology, depending on the photophysical requirements of the study. (Figure 4.6)

In many fluorogenic substrates dyes which have reactive heteroatoms are utilized. These heteroatoms are used to either form covalent bond which can be cleaved or altered by the targeted enzyme. In the PPT1 fluorogenic assay designed by van Diggelin et. al. the fluorescence emission of 159 is greatly diminished, and blue-shifted, when the phenol oxygen is substituted. In this assay, the 4-MU is linked to the anomeric position of a
glucose derivative 158, which contains a palmitoylated thiol as a substrate for PPT1. This linkage is stable to biological conditions and prevents phenolate formation (Chapter 3, Figure 3.21). Thus, the glycosidic bond biases the 4-MU 159 into the off state. The cleavage of this bond with beta-glucosidase, and subsequent raising the buffer pH causes 4-MU 159 to predominantly exist as the phenolate (the on-state), resulting in a large increase in observed fluorescence emission (Figure 4.7). A second example is the use of acylated fluorescein-type dyes as substrates for esterases.135 Xanthene based dyes, such as rhodol 169, exhibit a very high quantum yield, (Fluorescein QY = 0.95), and a well understood mechanism for internal quenching.132 However, when the phenol oxygen is protonated or substituted by chemical modification 170, such as acetylation, the phenyl ring containing the phenol exists as an aromatic system more closely related to benzene. This causes the free carboxylic acid to attach to the electropositive carbon resulting in a pentacyclic structure which has broken the conjugation of the aromatic xanthene ring. The broken conjugation and resulting orthogonal pi system result in a dramatic decrease in the fluorescence emission of the dye. When the dye is deacylated, the lone pairs of the oxygen can form a carbonyl through resonance, delocalizing across the large xanthene conjugated system and restoring the quantum yield of fluorescence (Figure 4.7).
4.6 Conceptualizing a PPT1 Fluorogenic Substrate

In order for a fluorescent probe to be used to monitor PPT1 activity, it must contain a palmitoylated substrate for PPT1, a reactive linkage which will bias the state of a fluorophore, and it must be capable of traveling to the lysosomal compartment. Conceptually, we envisioned that such a PPT1 probe could be realized by chemically linking each of the different functional groups which serve each listed purpose above. PPT1 is a catabolic enzyme in the class of serine hydrolases. We envisioned using a probe where a bond cleaved by PPT1 either alters the fluorescence of the probe molecule or induces further reactivity which influences the fluorescence of the probe. We hypothesized that a probe can fundamentally be broken down into four parts: the PPT1
substrate, the fluorophore, the lysosome localizing tag, and the labile chemical linkage which biases the state of the fluorophore. (Figure 4.8)

![Probe Scheme]

*Figure 4.8: A scheme of the construction of a PPT1 probe. The green represents a lysosomal localizing functional group, the blue represents a fluorophore, the red is a PPT1 substrate, and the arrow points to a labile linkage between the substrate and the dye. The labile linkage will be used to bias the state of the fluorophore.*

### 4.7 Assigning a PPT1 Substrate for a Fluorogenic Probe

To adapt strategies which are used in modern fluorogenic substrates to design a PPT1 probe, we first considered the native substrates for PPT1. The palmitoylation of proteins most commonly occurs by acylation of a free thiol of cysteine residues. We therefore elected to use cysteine as the scaffold for our fluorogenic probe. Our previous unsuccessful efforts to demonstrate dimeric lysosomal inhibition, *in vitro*, heavily influenced this choice of scaffold. (Chapter 3) We hypothesize that while the identity of the substrate is not important for PPT1 catalytic activity (thioester hydrolysis), perhaps DC/DQ-mediated PPT1 inhibition only occurs when PPT1 encounters specific substrates.
Our inability to recapitulate DC/DQ-mediated PPT1 inhibition in vitro with recombinant PPT1 was a significant cause for concern. We hypothesized that in addition to the lysosome being a privileged environment, the thioglycoside which was utilized as a palmitoylated substrate was too far removed from the intended substrate of the enzyme. Work by the Crews laboratory established that the didemnin series of natural products are uncompetitive inhibitors of PPT1.\textsuperscript{113} (Figure 4.9) An uncompetitive inhibitor is a binder of the enzyme substrate complex, not the unbound enzyme active site. We viewed this work as further evidence that when interrogating inhibition of the enzyme, perhaps substrate binding is also important in defining a context for DC/DQ mediated PPT1 inhibition. We hypothesized that the design of a probe more closely related to the native substrate would give a more accurate representation of \textit{in vivo} activity.

\textit{The Uncompetitive Inhibition of PPT1 by Didemnin Natural Products}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_4_9.png}
\caption{The uncompetitive inhibition of PPT1 by the didemnin series of natural products.}
\end{figure}
4.8 Selection of a Lysosomal Localizing Tag

For the probe to function properly, the molecule must localize to the lysosomal compartment of the cell. Lysosomotropism has been observed in molecules dating back to the discovery of the lysosome as an organelle.\textsuperscript{19} In his contributions to modeling lysosomotropism, de Duve described a scenario where charged molecules were less likely to pass through a membrane than neutral, hydrophobic small molecules. Therefore, molecules which become protonated in the lysosomal compartment, and become positively charged, accumulate at a faster rate than their diffusion out of the lysosome. These molecules accumulate as a function of the pH gradient between the cytosol (pH 7.4) and lysosome (pH 4.5 – 5.5). Since de Duve’s original hypothesis, the lysosomal concentration has been spectroscopically measured for multiple kinase inhibitors which contain weakly basic nitrogen atoms, and the anti-malarial chloroquine.\textsuperscript{50} This work established that molecules with nitrogen atoms possessing a higher pK\textsubscript{a} than the lysosomal lumen pH were able to accumulate in the lysosome preferentially and supports the hypothesis put forth by DeDuve, where basicity is the driving force for localization. However, drug phase partitioning between lipid/protein bound inhibitor and solid precipitation also plays a significant factor in the extent that a small molecule accumulates in the lysosome. We envisioned that a lysosomotropic probe could be synthesized by incorporating basic nitrogen atoms within the fluorogenic probe. Our results with dimeric lysosomal inhibitors supports these observations.\textsuperscript{53}

An added benefit of the incorporation of basic nitrogen atoms is their contribution to added aqueous solubility. Tertiary amine incorporation within the molecule will yield
nitrogen atoms that have a conjugate acid pKa greater than seven, thus making them protonated at physiological pH. Protonation of the molecules will aid in the aqueous solubility of the resulting fluorescent probe. Furthermore, molecules with a significant degree of positive charge are known to associate with the negatively charged cell membrane. This association with the membrane increases the likelihood for hydrophobic molecules to either pass successfully into the cell or be taken up by endocytosis and potentially ending up in the lysosome.

4.9 Selection of a NIR fluorophore: CyOH

For the fluorogenic probe to be used in cells in the presence of dimeric lysosomal inhibitor, we required a fluorophore with minimal spectral overlap with our library of compounds. DQ absorption is strong between 350 nm and 450 nm, yielding a fluorescence emission maximum at 490 nm. However, at high concentrations of inhibitor, excitation of the DQ inhibitors results in a broad emission band that stretches beyond 600 nm. With the degree of concentration of the DQ inhibitor within the lysosome both unknown and uncontrollable, we chose to pursue the application of near-infrared radiation (NIR) dyes (excitation >600 nm and emission >650 nm). (Figure 4.10)
Cyanine, BODIPY, and xanthene derived NIR dyes exist. Cyanine dyes commonly exhibit fluorescence in the red to NIR range. However, by their nature they lack a reactive handle from which to effectively make a standard fluorogenic probe. BODIPY dyes have also been synthesized which extend their fluorescence into the NIR range. However, these dyes similarly lack a reactive handle by which to modulate their fluorescent properties. Xanthene dyes have been synthesized which extend into the far-red to NIR range. However, their fluorescence is quenched by low pH. With the lysosomal pH being between 4.5-5.5, quenching in an acidic environment is likely to be problematic for lysosomal imaging. Further, NIR analogs of xanthene dyes present significant synthetic challenges, including the requirement of a large number of linear synthetic steps.\textsuperscript{138}  

![Normalized Fluorescence Emission Spectrum of DQ661](image)

**Figure 4.10:** The normalized fluorescence emission spectrum of DQ661, excitation at 400 nm.
Work by Yuan and coworkers disclosed a series of NIR dyes which structurally are reminiscent of both cyanine and xanthene dyes. In these molecules the indolenine scaffold is tethered to a xanthene ring through an alkene linkage. The new dye, referred to as CyOH 173, is strongly red-shifted like the parent cyanine. However, more akin to the xanthene class of dyes, a free phenol strongly modifies the fluorescent properties of the dye based on its substitution.

The dyes are derived by the degradation of existing NIR dye, 171, by resorcinol 172 in the presence of a base (Figure 4.12). Proceeding first through an addition elimination mechanism 174, a chlorine from the NIR dye is displaced by resorcinol 175.
In the presence of trace water and heat, the dye undergoes retro-Knovenagel reaction resulting in a free aldehyde 176. Addition of the phenol to the aldehyde 177, followed by rearomatization, results in the CyOH 173 dye scaffold. Cy7 171 is both commercially available on a gram scale and is easily synthesized in two steps. (Figure 4.11)

![Mechanism of CyOH Formation](image)

Figure 4.12: Mechanism of resorcinol degradation of cyanine 7 to yield CyOH.

Upon their initial disclosure of the dye, Yuan and coworkers also investigated the spectroscopic properties of the substitution state of the terminal phenolic oxygen. To prevent phenolate ionization, the authors synthesized two O-alkylated derivatives, and compared these controls to the phenol bearing CyOH 173. The O-alkylated derivative
exhibits a blue-shifted absorption spectrum, with the maximum absorbance shifting from 690 nm to 608 nm. CyOH 173 has two fluorescence emission peaks, 690 nm and 715 nm. O-alkylation of CyOH blue shifts the emission maximum to 675 nm. The reported quantum yield of CyOH 173 drops from 0.36 to 0.006 when the oxygen is alkylated. 139 These results establish that substitution of the phenolic oxygen shifts the fluorophore into the off-state, making CyOH an excellent candidate for a fluorogenic probe. The authors also applied CyOH to two small molecule fluorogenic sensors, a sensor for hydrogen peroxide (H$_2$O$_2$), compound 178, and a sensor for thiol detection (R-SH), compound 179. Encouragingly, both applications showed significant turn-on in fluorescence at 700 nm. (Figure 4.13)

*Reactive CyOH Probes with Turn-on Fluorescence*

Figure 4.13: Hydrogen peroxide and thiol reactive sensor derivatives of CyOH. Both have significant turn on in fluorescence.
4.10 Utilization of a Lysosomotropic Analog of CyOH

On consulting the literature for further applications of this dye scaffold in the context of the lysosome, we found that Wan et. al. prepared a morpholine-containing derivative of CyOH to probe lysosomal pH. Lyso-CyOH 187 maintains its fluorescence even in the acidic environment of the lysosomal compartment (Figure 4.16). Corresponding with the work of Yuan and coworkers, Wan and coworkers also saw significant increase in fluorescence at 708 nm upon increasing the pH from 5.0 to 7.4. This result, where fluorescence emission increases with pH, demonstrates that 187 is not sufficiently quenched by the tertiary amine to not make the dye useful. Further elaborated in the work by Wan and coworkers, 187 maintains an inflection point in its fluorescence emission spectrum at 670 nm, allowing for pH insensitive fluorescence emission measurements. Their work demonstrated 187 can accurately probe the pH of the lysosomal compartment by comparing the emission ratio of 708 nm with 670 nm when excited at 635 nm. These results confirmed that the fluorophore is functional at lysosomal pH, as well as at neutral pH. We therefore hypothesized that utilization of 187 would allow for the incorporation of the two functionalities required of a PPT1 probe, the fluorophore and the lysosome localizing tag, via the incorporation of one molecule.

4.11 State of the Art in Palmitoylation Probes

While we were considering these dye attachment/release methods for our fluorogenic probe, the Dickinson Laboratory at the University of Chicago published an analogous probe 180 for monitoring palmitoylation of acyl protein thioesterase 1 (APT1),
a cytosolic homolog of PPT1.\textsuperscript{141} APT1 is responsible for regulating the palmitoylation of proteins within the cytosolic compartment of the cell.\textsuperscript{110} The technology disclosed in the paper leverages the nucleophilicity of free thiols, where acyl transfer can occur through a 5-membered transition state. Free thiols, under the correct conditions, are able to nucleophilically attack amide, carbamate, and ester carbonyls. Under aqueous conditions, the resultant tetrahedral intermediate can lead to the transfer of the acyl group to the thiol sidechain. Naturally occurring enzymes known as inteins also rely on thiol-mediated peptide-bond cleavage to facilitate protein splicing.\textsuperscript{142,143} Without the aid of enzymatic catalysis, this process is thermodynamically uphill, forcing the equilibrium significantly towards amide, carbamate, or ester. However, in the case of N-carbamoyl cysteine the release of the phenol allows for the dissociation of the molecule into two halves, driving the cleavage reaction to completion. Multiple small molecule probes have utilized a similar system of thiol-mediate acyl cleavage. However, the Dickinson APT1 probe \textsuperscript{180} represents the first translation of this molecular strategy directly to a cysteine residue bearing a N-terminal carbamate\textsuperscript{141,144} (Figure 4.14).
The probe developed by the Dickinson Laboratory utilizes the fluorophore rhodol 169 (Figure 4.14), a derivative of fluorescein. Rhodol contains a reactive phenol and a tertiary amine moiety, the latter which is present in the more red-shifted rhodamine family of xanthene dyes. While the use of the Dickinson fluorescent probe piqued our interest, we hypothesized the probe would be inadequate for our observation of PPT1 activity for two reasons: first, the molecule possesses no driving force for lysosomal localization, making the differentiation of PPT1 activity from APT1 activity a significant challenge; and second, the fluorescence emission of rhodol shares spectral overlap with the DQ library of compounds. Therefore, we elected to take the insights gleaned from the Dickinson fluorogenic probe in designing our own PPT1 targeted fluorogenic probe.
4.12 Design of a CyOH-Fluorogenic PPT1 Probe

We envisioned that a small molecule probe, which represents the union of a CyOH reactive probe and the technology described the Dickinson laboratory, would be ideal for studying PPT1 activity in cells (Figure 4.15). The NIR fluorescence properties of CyOH would allow for studying of PPT1 activity in the context of DQ mediated autophagy inhibition. The technology of thiol-mediated carbamate cleavage would also allow for a substrate that is closer to the native substrate for PPT1 than non-endogenous thio-glucose.

The union of these technologies required that the labile linkage between substrate and dye modify the intrinsic spectroscopic properties of the fluorophore. We reasoned that acylation of the phenolic oxygen would suppress resonance delocalization of the...
phenol oxygen into the keto-form. This in turn would induce the same suppression in fluorescence emission, between 690 and 710 nm, that alkylation of the phenolic oxygen did in the work of Yuan and coworkers.\textsuperscript{139} Therefore, we believed this strategy would accomplish the incorporation of all four required elements for a probe: a lysosomal localizing tag, a PPT1 substrate, a labile linkage of the scaffold and dye, and a DQ and lysosome-compatible fluorophore (Figure 4.15).

**4.13 Chemical Synthesis of a 1st Generation PPT1 Fluorogenic Probe**

We set out initially to take advantage of the lysosomal analog of CyOH \textsuperscript{187} disclosed by Wan et. al (Figure 4.16).\textsuperscript{140} We believed that lysosomal localization of the probe could be accomplished by the presence of the morpholine functionality on \textsuperscript{187}. This construction strategy would also allow for the dye to be constructed in two linear steps from commercial Cyanine 7 dye, maintaining the step economy advantage of CyOH derivatives.

\textsuperscript{187} was synthesized by reacting \textsuperscript{186} with of a commercially available Cy 7 derivative \textsuperscript{171}, as described by Wan \textit{et. al.}\textsuperscript{140} Next, we chose to elaborate a cysteine scaffold, modeled after the work of Dickinson and coworkers, where a N-methyl cysteine derivative will be linked to the dye through a stable tertiary carbamate linkage. Initially we prepared the same cysteine derivative utilized in the Dickinson synthesis (Figure 4.16).\textsuperscript{141}
Next, we chose to couple 187 to 191 in a similar fashion to the work by the Dickinson laboratory. We hypothesized that both 187 and rhodol 169 (Figure 4.14) would have similar reactivity because of their similarly acidic phenols (pKa rhodol 169 = 5.5, pKa CyOH 173 = 5.0). In the work by Dickinson and coworkers, triphosgene is first reacted with the free phenol of rhodol at 0 °C, presumably forming a chloroformate in situ. The crude chloroformate is then used, without purification, to acylate the secondary amine of the cysteine scaffold.

However, when we attempted to repeat the chloroformate synthesis with 187, no product was observed by mass spectrometry analysis of the crude reaction mixture. We then attempted to trap a chloroformate by reaction of an aliquot with excess morpholine. However, no product was observed in this reaction. From these results we hypothesized that the chloroformate intermediate derived from 187 was either not stable under the reaction conditions, or was not forming with triphosgene. The reaction was set up
according to the protocol of Kathayat et. al., where \( 187 \) (pKa = 5.0) is deprotonated first by triethylamine (pKa = ~10) in THF.\textsuperscript{140,141} However, \( 187 \) is only sparingly soluble in THF. Chloroformates have previously been synthesized by the reaction of phenols in CH\(_2\)Cl\(_2\), a solvent in which \( 187 \) exhibited significant solubility. The reactants are mixed by slow addition of the solution of \( 187 \) and triethylamine in CH\(_2\)Cl\(_2\) to the reaction vessel containing a chilled solution of triphosgene in CH\(_2\)Cl\(_2\). Upon addition of a blue solution of \( 187 \) to the triphosgene solution, a purple color evolves but quickly changes back to blue within minutes. This result suggests an intermediate species is forming in the reaction mixture but is being consumed prior to the end of the reaction.

Based on the experimental results from our attempts to acylate \( 187 \), we believed that the ortho benzylic morpholine substituent could lead to decomposition of any dye-triphosgene adduct formed \textit{in situ}. As a control we wanted to react triphosgene with CyOH \( 173 \), a dye lacking the ortho amine functionality. First, CyOH \( 173 \) was synthesized according to the conditions by Yuan et al. and Ma \textit{et al.}\textsuperscript{139,145} Through our efforts we found that the utilization of inorganic bases furnished better yield in the CyOH \( 173 \) forming reaction when compared to the use of organic tertiary amine bases. Gratifyingly, we also found the yield of this reaction to be substantially better than the synthesis of the Lyso-CyOH \( 187 \), enabling multi-gram scale synthesis of CyOH \( 173 \). (Figure 4.17)
With CyOH 173 in hand, we explored the formation of CyOH-chloroformate 192, derived from the reaction of 173 with triphosgene. A solution of deprotonated dye, in CH$_2$Cl$_2$, was introduced to the reaction vessel containing triphosgene in CH$_2$Cl$_2$ at 0 °C, in the same manner as the previous attempts with 187. Slow addition of the 173 solution causes the rapid formation of a purple color which persists over the course of the addition, unlike attempts with 187. TLC analysis of the crude mixture instantaneously showed the appearance of a baseline spot, purple in color, which was not observed in the reaction of 187 with triphosgene.

Encouraged by these results we analyzed the mixture by $^1$H NMR, observing a down-field shift of the methine protons of the alkene, indicating the reaction of 173. An aliquot of the crude reaction was reacted with excess methanol and analyzed by LC-MS,
where the CyOH starting material 173 and two carbonyl containing species were observed: the methyl-CyOH carbonate 193 and the bis-CyOH carbonate 194.

Due to the presence of symmetrical 194, we hypothesized that the rate of addition of 173 was too fast for the reactivity of the derived chloroformate intermediate at 0°C. To test this hypothesis, we chose to analyze the reaction via UV/Vis-LC-MS at 0 °C and after warming to 25 °C. At 0 °C the reaction was significantly retarded, where, after quenching with excess methanol, large quantities of the phenol 173 were present in comparison to a mixture of 193 and 194. Warming of the reaction to room temperature causes the reaction to proceed directly to a mixture of only 193 and 194 as observed by LC-MS.
The reaction was run at ambient temperature (23 °C), and despite slow addition of a solution of 173 in CH2Cl2, over the course of one hour, the reaction still yielded a mixture of the two carbonate species 193 and 194 upon reaction with methanol (Figure 4.18). The crude reaction mixture was analyzed after adding 0.08 molar equivalents of 173 with respect to triphosgene. LC-MS analysis found 194 carbonate was still observed, suggesting that the rate of addition was not causing the formation of the carbonate. We next attempted to see if the mixture composition could have been biased by the change of the reaction solvent from the literature precedent, THF, to CH2Cl2. THF, with either 50% or 10% CH2Cl2 (v/v) as a co-solvent, utilized to solubilize the starting dye, was tested as
a reaction solvent. With a 1:1 mixture of THF and CH$_2$Cl$_2$ as the reaction solvent, the reaction proceeded to completion after 2 hours at 23 °C. Reaction of an aliquot with excess methanol and UV/Vis-LC-MS analysis showed an improved ratio of 193 to the carbonate 194 but did not inhibit the formation of the side product. With 10% CH$_2$Cl$_2$ in THF the reaction never fully consumed the starting CyOH phenol during 24 hours at 23 °C.

In parallel, we tested whether 194 could be used as a CyOH acylation reagent. Due to the extensive delocalization of the resulting phenolate anion, we hypothesized that 173 could be a comparable leaving group to chloride anion in CH$_2$Cl$_2$. Therefore, we hypothesized 194 could be cleaved by an amine to yield the desired carbamate. Reaction of the crude triphosgene reaction mixture with excess N-Boc-piperazine 196 in CH$_2$Cl$_2$ gave the desired product 197 as observed via LC-MS, consuming both the 193 and the 194. (Figure 4.20)
With evidence that 171, could be accessed through reaction with 194 or 192 we concluded that our inability to utilize 187 to synthesize carbamates was due to the presence of the ortho-morpholine substituent. We altered our fluorophore selection from 187 to 173. In doing this, we lost the ability to append our lysosomal localizing tag to the fluorophore itself. The work by the Dickinson laboratory indicates that the enzyme-substrate compatibility is maintained in APT1 when the C-terminal position of the cysteine residue is substituted with multiple R groups, including the amino acid lysine.\textsuperscript{141} This result mirrors similar observations made in the literature about PPT1 substrate flexibility. PPT1 is well documented to hydrolyze palmitate chains from large proteins such as H-Ras.\textsuperscript{114} Alternatively, multiple studies also describe the ability of PPT1 to hydrolyze palmitate thioesters appended to other non-protein substrates.\textsuperscript{112–114}
To test our hypothesis, we altered our probe design to move the lysosomal tag from the dye to the peptide scaffold 198 (Figure 4.21). We determined that the design of a diamine which contains a morpholine and a primary amine could be used as the second residue in our peptide scaffold and as a lysosomal tag. We decided to use the known diamine 204.146 We first synthesized a phthalimide protected aminopropanol 201 by reaction of 3-aminopropan-1-ol 200 and phthalic anhydride 199 neat at elevated temperature.147 Alcohol 201 was oxidized by Swern oxidation to aldehyde 202 and reductively aminated with morpholine to yield 203.148,149 The phthalimide protecting group was removed from 203 by reaction of hydrazine in ethanol, heated to reflux, to give compound 204.146 Primary amine 204 was coupled to 189 (Figure 4.16) utilizing EDC/HOBt in DMF to yield 205. The Boc protecting group was removed from 205 with TFA to yield secondary amine 206 (Figure 4.22).
We elected to complete the synthesis of the first-generation probe utilizing the crude reaction mixture of 192 and 194 to acylate our cysteine scaffold. Secondary amine 206 (Figure 4.22) was dissolved in CH₂Cl₂ with triethylamine. The amine containing solution was reacted with the crude reaction mixture of 192 and 194, with 206 as the limiting reagent. The acylation reaction proceeded to completion as observed by LC-MS. We proceeded to remove the trityl protecting group from the cysteine thiol 207 via reaction with TFA and triisoproxylsilane in CH₂Cl₂. Removal of the solvent followed by acylation with palmitoyl chloride yielded the product, compound 198, which was isolated by reverse phase HPLC in 20% yield with respect to 206 over the 3 steps.
The probe molecule was found to be soluble at concentrations of 100 uM in both PBS (pH 7.4, physiological pH) and acetate buffer (pH 5.0, simulated lysosomal pH). The absorbance and fluorescence spectra of the probe were then measured and compared with those of the starting dye, 173. The UV-Vis absorption spectra of both 173 and the PPT1 probe 198 were measured in PBS at pH 7.4. We observed a blue-shift of the absorption spectrum of the probe relative to free dye 173. We were optimistic about these results, as they were quite similar to those reported by Yuan and coworkers in the O-alkylated and sulfonated CyOH derivatives (Figure 4.13, 178 & 179). \(^{139}\)
Next, the fluorescent properties of the probe 198 and CyOH 173 (100 μM) were examined at pH 7.4. Preliminary results were promising, where substantially increased fluorescence was observed at 708 nm when comparing CyOH 173 to the PPT1 probe 198. After extended periods of time, the probe was found to precipitate irreversibly from pH 7.4 PBS. To address this issue, Triton-X100 was added to the buffers at 0.1% (w/v). Previous fluorescent assays which contain palmitoylated substrates utilize this surfactant.112,141 We hypothesized that the palmitoylated probe could aggregate at high concentrations in solution due to the hydrophobic nature of the palmitate chain. fluorescent molecules which aggregate at high concentrations are known to have intermolecular interactions which quench the fluorescence emission.150 In our case, we hypothesized that the observed turn-on in fluorescence between CyOH 173 and the
palmitoylated probe \textbf{198} could be artificial, and therefore needed to be measured again in the presence of the surfactant.

In the presence of a surfactant, an approximately four-fold turn on in fluorescence between compounds \textbf{173} and \textbf{198} was observed at lysosomal pH. In our previous studies without surfactant compounds \textbf{173} and \textbf{198} exhibited 100-fold turn-on, where \textbf{173} was significantly more fluorescent. Previous studies which reported fluorescence emission differences between O-substituted derivatives of CyOH reported significantly larger increases in fluorescence emission. O-alkylated derivatives presented by Yuan et. al. show a 50-fold difference in fluorescence emission turn-on when the phenol oxygen is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.25.png}
\caption{Fluorescence intensity measured at 690 nm. The 1\textsuperscript{st}-generation probe was assayed in comparison to CyOH (free dye) across a selection of buffers with increasing pH. Triton-X100 was utilized to prevent self-quenching of the probe due to small molecule aggregation \textit{in situ}.}
\end{figure}
unsubstituted. Previous applications of CyOH as a fluorogenic substrate in living systems utilized derivatives which had significantly greater turn on in fluorescence. Further, the probe applied by the Dickinson Laboratory claimed a turn-on of fluorescence significantly greater than 100-fold.

Based on these data we determined that while the first-generation probe could be studied in cells utilizing a four-fold fluorescence turn-on to observe depalmitoylation by PPT, analysis would be greatly simplified by reducing the background noise from the unreacted fluorescent probe. Therefore, we elected to design a second-generation probe which would exhibit a significantly greater turn-on of fluorescence when cleaved by PPT.


One method of increasing the turn-on sensitivity of a fluorescent probe would be reducing the background signal due to imperfect quenching of the unreacted PPT1 probe. Unlike xanthene dyes such as rhodol or fluorescein, CyOH does not have a defined chemical mechanism for fluorescence quenching. Yuan and coworkers reported that alkylation of the phenolic oxygen with methyl or ethyl iodide substantially decreases the quantum yield from 0.36 to 0.007. However, according to our observations acylation of the free phenol with a carbamate did not adequately quench the fluorescence of CyOH. To reduce the quantum yield of fluorescence for the unreacted probe we derived two possible chemical strategies: the first would involve the incorporation of a
quenching functional group on the peptide substrate scaffold, and the second would be the alteration of the carbamate linkage to the dye to incorporate a labile alkylation between the carbamate and the dye. We elected to proceed with the incorporation of a known quencher to the peptide scaffold because we hypothesized this strategy was the most likely to be effective, as well as being the most conservative by not altering the chemistry of the dye release mechanism.

To design a molecule which had lower fluorescence in the uncleaved form, we needed to reduce the intrinsic fluorescence emission of the probe through the incorporation of a quenching functional group. In fluorescence spectroscopy two general strategies are utilized to quench fluorescence: 1) the incorporation of a PET quencher, or 2) the incorporation of a FRET quencher. A PET quencher, or photoinduced electron transfer quencher, is a functional group which can either accept an electron from or donate an electron to the photoexcited fluorophore. This electron transfer then inhibits the

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**Figure 4.26:** A mechanism of photoinduced electron transfer quenching (PET), where an excited state dye can undergo electron transfer to a PET quenching functional group. The electron transfers to a lower energy state and relaxes through a non-radiative transition.
ability of the fluorophore to relax to the ground state by the emission of a photon. A FRET quencher utilizes Forster resonance energy transfer, where a second dye can couple to the excited state of the original fluorophore. This coupling allows for a non-radiative energy transfer between the dyes, where the second dye can either relax in a non-radiative fashion or emit light which is further red-shifted than the original chromophore.

Two examples of PET quenching functional groups are tertiary amines and thioamides. A tertiary amine was not an option as a PET quencher in this case because the first-generation probe contains a tertiary amine as the lysosomal localizing tag and still possesses too strong a fluorescence emission. Initial attempts to induce intermolecular quenching via a PET mechanism proved unsuccessful. Incubation of the first-generation probe in a buffer containing 10 mM thioacetamide produced no change in fluorescence emission when compared to a buffer without thioacetamide. At this juncture
we abandoned the strategy of adding a small functional group which could quench our CyOH fluorophore 173.

We next consulted the literature for the identification of a FRET quencher that would be compatible with CyOH 173. In the NIR range, fewer options exist for quenchers outside the use of the black hole series of quenchers. Comparison of the emission spectrum of the first-generation probe with the absorption spectra of the black hole series of quenchers indicated that black hole quencher 3 (BHQ3) would be the optimal choice for incorporation.

Figure 4.28: The UV-Vis absorbance of the 1st-generation PPT1 probe, the fluorescence emission spectrum resulting from excitation at the absorption maximum for the 1st generation PPT1 probe. Also plotted is the UV-Vis absorption spectrum for black hole quencher 3. Black hole quencher 3 has significant overlap with both the absorption and emission of the 1st generation PPT1 probe.
We envisioned that BHQ3 would be an ideal FRET partner for the first-generation probe because of the documented spectral overlap. With the first-generation probe containing both a labile thioester and the exceptional leaving group capability of CyOH, we sought benign conditions for the incorporation of BHQ3. Fortuitously, an azide containing analog of BHQ3 (Figure 4.32, 219) had been described by Chevalier et. al.\textsuperscript{153}

The incorporation of an alkyne handle into the peptide scaffold for click chemistry required an alteration of the lysosomal tag region of the first-generation probe. We envisioned a linear scheme for the synthesis of the probe where the dye was connected to the peptide substrate in the same manner as the first-generation probe, followed by introduction of a lysosomal tag which contains an alkyne. In a final step this alkyne-containing first generation probe could be clicked to the azide containing BHQ3 yielding the proposed structure 209 (Fig.4.29). This synthetic sequence would add

![Figure 4.29: The design of a second generation of a PPT1 probe. Right, depicts the structure of the second generation PPT1 probe with key synthetic disconnections labeled.](image-url)
potential for chemical diversity in quencher incorporation in the event that a different quenching molecule was required.

The diamine scaffold was redesigned to incorporate a propargyl amine functionality by changing the original morpholine from the first-generation probe to a piperazine ring. Starting from 3-amino-1-propanol and phthalic anhydride, compound 201 was synthesized by heating the reagents neat (Figure 4.22).\textsuperscript{147} The resulting alcohol 201 was then oxidized to the corresponding aldehyde 202 by Swern oxidation (Figure 4.22).\textsuperscript{148} N-propargyl piperazine 211 was synthesized by alkylation of N-Boc-piperazine 196 with propargyl bromide in THF under microwave irradiation, followed by deprotection of the resulting N-propargylated carbamate 210 using a 1:1 mixture of TFA:CH\textsubscript{2}Cl\textsubscript{2}, to yield 211 in good yield.\textsuperscript{154,155} Aldehyde 202 and amine 211 were reductively aminated using sodium triacetoxyborohydride to furnish 212 in good yield. The phthalimide protecting group was removed from 212 by reaction with hydrazine in ethanol heated to reflux to yield 213. Compound 189 was then coupled to triamine 213 via peptide coupling conditions using EDC and HOBT in dimethylformamide.\textsuperscript{141} The resulting peptide 214 was then deprotected using a 1:1 mixture of TFA:CH\textsubscript{2}Cl\textsubscript{2} to afford 215.
CyOH 173 was deprotonated with triethylamine and reacted with triphosgene in a 1:1 mixture of CH$_2$Cl$_2$:THF to synthesize the previously described mixture of the chloroformate 192 and symmetrical carbonate of CyOH 194 (Figure 4.19). The peptide substrate 215 was then added to the CyOH chloroformate 192 (Figure 4.19) to acylate the N-terminus of the cysteine substrate. Once the peptide starting material was consumed, the trityl protecting group was removed \textit{in situ} by first switching the solvent to CH$_2$Cl$_2$, followed by the addition of TFA and triisopropylsilane. The resulting thiol was acylated by reaction in neat palmitoyl chloride, and isolated by preparative HPLC to give 218 in 19 % yield over three steps from the peptide scaffold 215.
Chevalier and coworkers published a two-step synthetic route to an azide containing analog of BHQ3 \(^{221}\).\(^\text{153}\) The derivative of BHQ3 was synthesized through a diazo coupling between a tertiary aniline \(^{220}\) and the diazonium salt of Methylene Violet 3RAX \(^{219}\). The resulting azide can be purified by reverse phase HPLC and isolated as a blue solid. The synthesized azide and alkyne coupling partners were joined through copper catalyzed click chemistry using Cu(II)SO\(_4\) and sodium ascorbate in a solvent system of water and DMSO to give \(^{222}\).
First, the resulting second-generation probe 218 and the probe attached to BHQ3 222 were tested for solubility. Both molecules were found to be soluble at 100 μM in PBS (pH 7.4) and acetate buffer (pH 5.0). Buffered solutions containing Triton-X100 maintain colored solutions for multiple hours with no obvious precipitation. In order to apply the second-generation probe as an effective sensor for PPT1 activity, the spectroscopic properties of the probe must be characterized, followed by its validation as a substrate for recombinant PPT1. First, the alkyne, azide, and final clicked probe were analyzed by UV-Vis absorption spectroscopy to determine the absorption maxima and extinction coefficient of each component (Figure 4.33).
Figure 4.33: A. Structures of probes (198, 218, 221, 173, 222) which were observed by UV-Vis absorption spectroscopy. B. The UV-Vis absorption spectra for both generations of PPT1 probes (Figure 4.23, 198 & Figure 4.31, 218), black hole quencher 3 (BHQ3) (Figure 4.32, 221), CyOH (Figure 4.31, 173), and the second-generation probe attached to black hole quencher 3 (Probe G2/BHQ3) (222)
Next the probe (Figure 4.33, 222) was analyzed by fluorescence spectroscopy utilizing two excitation wavelengths taken from the results of our study of the visible light absorption, 600 nm and 675 nm, of the molecules. Excitation of the molecules at either wavelength produced the same fluorescence emission spectra. This result demonstrated that excitation of these molecules at either wavelength caused similar fluorescence emission spectra. In order to translate these spectroscopic properties to an assay for enzymatic activity, we needed to correlate probe reactivity to fluorescence emission. (Figure 4.34)
Figure 4.34: The fluorescence emission spectra of the PPT1 probe compounds (Structures in Figure 4.33) (150 uM, in pH 5.0 Acetate Buffer). The compounds were excited at 600 nm and 675 nm the two absorbance maxima for CyOH 173. For structures of probes see Figure 4.33.
The probe **222** (Figure 4.33) was designed to degrade under aqueous conditions when the palmitoyl chain is removed from the thiol. The free thiol, through a five-membered transition state, will attack the carbonyl of the tertiary carbamate, and collapse of the tetrahedral intermediate will result in the loss of CyOH **173**. Therefore, the increase in CyOH **173** concentration would be reflected in increased fluorescence emission and correlate to enzymatic activity. With the spectroscopic properties of the free dye and unreacted probe characterized, we next chose to validate the reactivity of the probe towards recombinant PPT1. The probe was dissolved in acetate buffer (pH 5.0, 150 mM NaCl, 0.1% Triton-X100) at a concentration of 100 μM. Recombinantly expressed PPT1 was then added to the reaction vessel to give an enzyme concentration of 50 nM, and the reaction was incubated at 37 °C. After 15 minutes, the appearance of free dye can be observed by LC-MS, along with another compound, the mass of which corresponds to the quencher-cysteine containing fragment of the probe. After 60 minutes, the reaction contains no starting probe, and significant quantities of both CyOH and the quencher-cysteine fragment. These results confirm that PPT1 can catalyze the removal of palmitate from the PPT1 probe, and once depalmitoylated, the thiol-mediate carbamate cleavage occurs at pH 5.0. (Figure 4.35, bottom)
Figure 4.35: Two chromatograms observed by UV-Vis absorbance at 650 nm. The identity of the peaks was determined by mass spectrometry analysis of each peak. The observed molecular ions are indicated on the figure for each species. The top chromatogram is a negative control displaying only the PPT1 2\textsuperscript{nd}-generation fluorogenic probe 222. The bottom chromatogram displays a reaction at 60 min where only the cleaved fragments of the probe are observed.
With these results in hand we chose to monitor the evolution of CyOH 173, as catalyzed by the PPT1-mediated decomposition of the probe molecule 222, by fluorescence emission at 700 nm (excitation at 675 nm). An excitation wavelength of 675 nm was chosen because this is the strongest absorption band for the CyOH at pH5. Further, unreacted probe-G2-BHQ3 222 begins to decrease rapidly in absorptivity beyond 650 nm. Due to the inherent sensitivity of fluorescence spectroscopy, we were able to dramatically decrease the scale and concentration of the substrate in each of these reactions, compared to assays observed via LC-MS (UV-Vis). Each reaction, for monitoring via fluorescence emission, contained 500 nM PPT1, and 10 μM PPT1-probe. The concentration of the enzyme was increased to increase the rate of reaction for efficient measuring of the reactions kinetics. The reactions were run in a fluorescence plate reader warmed to 37 °C, where measurements of the fluorescence emission at 700 nm were observed every three minutes. Gratifyingly, under these conditions we observed rapid consumption of the probe molecule, where the entirety of the substrate was consumed after 20 minutes. (Figure 4.36).
We are currently exploring the cell permeability, cytotoxicity, and PPT-mediated degradation of the 2nd generation probe 222 in live melanoma cell cultures. Future work will describe the reactivity of the probe in A375 melanoma cells and PPT1 CRISPR knockout melanoma cells. Further, HDSF will be used as a positive control to chemically knockdown PPT1 as a positive control.
Biological Methods:

Chapter 2:

All biological assays were performed as described by Rebecca et. al.\textsuperscript{53}

Chapter 3:

MTT assay, and autophagy inhibition assays were performed as described by Rebecca et. al.\textsuperscript{53}

Pulldown Methodology:

A375P melanoma cells were grown to confluency in 15 cm diameter culture dishes (~20 million cells per dish). For each experimental condition, the cultures were grown in quadruplicate. The cell cultures were treated with the photoaffinity compound (400 nm) for 24 hours. For each compound, three conditions were tested: 1) photoaffinity probe without UV irradiation, 2) photoaffinity probe with a 30-min UV irradiation, and 3) photoaffinity probe with 1 hour of parental inhibitor competition, followed by UV irradiation (Spectroline transilluminator 365 nm, 15 watts)(Cultures were placed on a sheet of aluminum foil and the transilluminator was held above the culture dishes).

Inhibitor competition was assayed by treatment of a culture after the 24-hour photoaffinity probe treatment, with the inhibitor warhead for 1 hour at a 10-fold dose compared to the photoaffinity probe. The culture was then irradiated with UV light for 30
min. After irradiation was performed the cell cultures were then lysed using RIPA buffer (4 mL/culture dish). The RIPA buffered lysate was then frozen at -80 °C.

Next, while the cell lysate was thawed on ice, Neutravidin resin (Avidin on agarose, high capacity, Thermo Scientific. Cat. No. 29204) was aliquoted into a 50 mL falcon tube. The resin, packaged in a 1:1 slurry, was aliquoted where 1 mL of packed resin was used per experimental condition. The buffer was exchanged to the cell lysis buffer (RIPA buffer containing protease inhibitor tablets, Roche) by first pelleting the resin via centrifugate 1 minute (1500 x G), discarding the supernatant, and resuspension of the resin in cell lysis buffer. This process was repeated three times to fully exchange the buffer. To each sample of cell lysate, 2 mL of 1:1 resin:buffer slurry, was added. The resin was nutated with cell lysate for 16 hours at 4 °C. The resin-lysate mixture was pelleted for 1 minute via centrifugation (1500 X G). The lysate was removed, with 200 uL of supernatant saved as the flow-through. The packed bed of resin was then rigorously washed with 0.1% NP-40 followed by buffer exchanging into PBS. One wash consists resuspending packed resin in a volume of wash buffer, pelleting the resin via centrifugation (1500 x G), and removal of the supernatant, leaving packed resin. The resin for each condition was washed with 0.1% NP-40 (7 x 30 mL) and exchanged into PBS by washing (3 x 30 mL). The packed resin was transferred into 1.5 mL Eppendorf tubes by repeated resuspension in PBS followed by pelleting via centrifugation (1500 x G), and removal of the PBS supernatant. The pelleted resin was dehydrated with a gel loading pipet tip to remove the retention volume of the resin. The resin was then resuspended in 300 uL of elution buffer (4 mM biotin in PBS) and incubated at 37 °C.
After 5 minutes, then resin was agitated by repeated shaking of the Eppendorf tube. This process was repeated until 15 min has elapsed. The 300 uL elution buffer was removed and placed into an Eppendorf tube. The elution process was repeated twice more, yielding three total elutions.

Proteomic analysis:

Trypsin digest LC-MS/MS analysis was performed by the Speicher Laboratory at the Wistar Institute as described by Rebecca et al.\textsuperscript{53}

Fluorogenic PPT1 assay

Melanoma cell cultures were treated with the specified dose of inhibitor for 3 hours. The cells were lysed by osmotic and mechanical stress in milli-Q water. The cell lysates were adjusted to an equal protein concentration of 0.1 ug/ uL. The cell lysates were used a source of PPT1 and assayed according to the methodology described by Van Diggelin et al.\textsuperscript{112}

Recombinant PPT1 Expression

Recombinant expression of PPT1 was performed in SF9 cells and purified by nickel-NTA/his-tag purification. The protein was expressed and purified by Dr. Julie Rotenburg-Barber as described by Rebecca et al.\textsuperscript{53}

CD-44/Acyl biotin exchange assay

The assay was performed as described by Rebecca et al.\textsuperscript{53}
Differential Scanning Calorimetry

DSC assays were performed by Dr. Julie Rotenburg-Barber as described by Rebecca et al.\textsuperscript{53}

Chapter 4 Assay Methods

Spectroscopic characterization

Small molecule fluorescence and absorption properties were observed on a Tecan M1000 fluorescent plate reader. UV-Vis spectroscopy was collected in triplicate and 420 nm was found to be the average most red shifted absorption across the library of dimeric quinacrine. Emission spectra were collected for each compound at 1 μM solutions, which was confirmed to be in the linear range of fluorescence. Solutions of pH 7.4 and 1 N HCl, were used to bias the degree of protonation of the heterocycles. Emission data was processed with a five-point floating average to reduce instrument noise. The data was then normalized to the fluorescence maximum of the library, DQ 661.

Expression of PPT1 was performed as described above in chapter 3.

LC-MS observation of PPT1-mediated degradation of the second generation PPT1 probe

The probe was dissolved in acetate buffer (pH 5.0, 150 mM NaCl, 0.1% Triton-X100) at a concentration of 100 μM. Recombinantly expressed PPT1 was then added to the reaction vessel to give an enzyme concentration of 50 nM, and the reaction was incubated at 37 °C. The reaction was monitored by injection into a Waters UPLC-MS with UV-Vis absorbance detection. The probe was identified by monitoring absorption at
650 nm. The molecular identity of the reaction products were identified by mass spectrometry.

Fluorogenic Assay for PPT1-mediated degradation of the second generation PPT1 probe

The fluorescence assay was conducted in a Tecan M1000 fluorescent plate reader heated to 37 °C. Each 50 uL reaction contained 500 nM PPT1, and 10 μM PPT1-probe, dissolved in acetate buffer (pH 5.0, 150 mM NaCl, 0.1% Triton-X100). The reactions were monitored by fluorescence emission (ex. 675, em. 700 nm) every three minutes. The data shown is an average of 9 experiments, where the error was calculated as the standard deviation of fluorescence emission.
APPENDIX

Synthetic Methods

General Synthetic Methods

Solvents used for extraction and purification were HPLC grade from Fisher Scientific. Unless otherwise indicated, all reactions were run under an inert atmosphere of argon. Anhydrous tetrahydrofuran, ethyl ether and methylene chloride were obtained via passage through an activated alumina column. Commercial reagents were used as received. VWR pre-coated silica gel plates (250 μm, 60 F254) were used for analytical TLC. Spots were visualized using 254 nm ultraviolet light or with potassium permanganate if no chromophore was available. Chromatographic purifications were performed on Sorbent Technologies silica gel (particle size 32-63 microns). $^1$H and $^{13}$C NMR spectra were recorded at 500 MHz and 125 MHz, respectively in CDCl$_3$, CD$_3$OD, or DMSO-d$_6$, on a Bruker AM-500 or DRX-500 spectrometer. Chemical shifts are reported relative to internal chloroform (δ = 7.26 for $^1$H, δ = 77.00 for $^{13}$C), methanol (δ = 3.31 for $^1$H, δ = 49.00 for $^{13}$C), or DMSO (δ = 2.50 for $^1$H, δ = 39.00 for $^{13}$C). HPLC purification was performed utilizing a Shimadzu HPLC (Water [0.1% TFA v/v, MeCN]) with reverse phase columns from Waters (Analytical X-Select, C$_{18}$, 5 um pore size, column dimensions 4.6 mm x 250 mm; Preparative X-Select, C$_{18}$, 5 um pore size, column dimensions 19 mm x 250 mm). Infrared spectra were recorded on a NaCl plate using a Perkin-Elmer 1600 series Fourier transform spectrometer. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are uncorrected. Accurate mass measurement analyses were conducted on either a Waters GCT Premier, time-of-
flight, GCMS with electron ionization (EI), or an LCT Premier XE, time-of-flight, LCMS with electrospray ionization (ESI). Samples were taken up in a suitable solvent for analysis. The signals were mass measured against an internal lock mass reference of perfluorotributylamine (PFTBA) for EI-GCMS, and leucine enkephalin for ESI-LCMS. Waters software calibrates the instruments, and reports measurements, by use of neutral atomic masses. The mass of the electron is not included. We thank Dr. Charles W. Ross III and Joo Myung Jun for their measurement and analysis of compound accurate mass.
Chapter 2: Synthetic Methods

Synthesis of Dimeric Chloroquine Inhibitors

DC220-3HCl

To a flame-dried round bottom flask, the triamine linker (14), (TCI America) (1.9 mL, 17.6 mmol, 1.0 eq.), 4-bromo-7-chloroquinoline\textsuperscript{156} (13) (9.00 g, 37.0 mmol, 2.2 eq.), Pd(OAc)\textsubscript{2} (Strem Chemicals) (160 mg, 0.71 mmol, 0.04 eq.), (\pm)-2,2′-bis(diphenylphosphino)-1,1′-binaphtalene (BINAP) (Strem Chemicals) (880 mg, 1.41 mmol, 0.08 eq.), K\textsubscript{3}PO\textsubscript{4} (Acros Organics) (11.27 g, 52.9 mmol, 3.0 eq.) were added at 25°C. The reagents were placed under an argon atmosphere and dissolved in 1,4-dioxane (44 mL, 0.4 M). The reaction was then heated to 105 °C under a reflux condenser. The reaction was monitored by \textsuperscript{1}H NMR (CDCl\textsubscript{3}) where the disappearance of the primary amine linker marks consumption of the limiting reagent (peak at δ ~2.7 ppm). The reaction was then cooled to 23 °C and filtered through Celite\textsuperscript{TM} using 300 mL of a 2:1 mixture of CHCl\textsubscript{3}:MeOH. The resulting solution was concentrated under reduced pressure to afford a yellow solid (15.0 g). The solid was triturated three times with boiling CH\textsubscript{2}Cl\textsubscript{2} (100 mL) to afford a crude white solid (9.75 g) which was collected via vacuum filtration. The solid was purified by salt formation (recrystallization in 1N HCl; 700 mL),

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yielding a white solid (15) (5.72g, 61%), the structure of which was confirmed by \(^1\)H NMR. TLC (Rf = 0.2, 10:89:1; MeOH:CH2Cl2:NH4OH). Melting point (mp) (H2O) = >240 °C (decomposition). \(^1\)H NMR (500 MHz, DMSO-\textit{d}_6): δ 9.89 (s, 2H), 9.81 – 9.74 (m, 2H), 8.82 (d, \(J = 9.2\) Hz, 2H), 8.63 (d, \(J = 7.1\) Hz, 2H), 8.08 (d, \(J = 2.1\) Hz, 2H), 7.77 (dd, \(J = 9.1, 2.1\) Hz, 2H), 7.05 (d, \(J = 7.1\) Hz, 2H), 3.97 (q, \(J = 5.8\) Hz, 4H), 3.41 (t, \(J = 6.0\) Hz, 4H). \(^{13}\)C NMR (126 MHz, D\textit{2}O): δ 155.64, 142.67, 139.69, 137.26, 127.73, 123.73, 118.82, 114.77, 98.48, 44.59, 39.15. FTIR (thin film) \(\lambda\) (cm\(^{-1}\)): 3021.73, 2920.36, 2783.11, 1626.95, 1611.13, 1584.16, 1550.07, 1451.45, 1365.71, 1343.21, 1228.39, 1210.89. HRMS (ESI) C\textsubscript{22}H\textsubscript{24}Cl\textsubscript{5}N\textsubscript{5}: Calculated for [M+H-3HCl] C\textsubscript{22}H\textsubscript{22}N\textsubscript{5}Cl\textsubscript{2}, 426.1252; found: 426.1247.

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\begin{align*}
\text{Cl} & \quad \text{Cl} \\
\text{N} & \quad \text{N} \\
\text{H} & \quad \text{H} \\
\downarrow & \quad \downarrow \\
\text{15} & \quad \text{15} \\
\end{align*}
\]

DC220-3HCl (15) (5.00 g, 9.3 mmol, 1.0 eq) was added to a two-neck round bottom flask with a stir bar. A septum was placed in one neck, while a reflux condenser was placed over the other neck. The entire apparatus was placed under an atmosphere of argon. Formic acid (HCOOH) (Acros Organics) (31 mL) was added to the reaction vessel via a syringe, resulting in a 0.3M solution. Formaldehyde (CH\textsubscript{2}O) (Fisher Scientific) (1.2 mL, 13.6 mmol, 1.45 eq.) was added as a solution (37% w/w aqueous) via a syringe to the reaction vessel. The reaction was heated to reflux with an oil bath preset to 105 °C. The reaction was monitored via TLC, whereby consumption of the starting material

Lys 05 (DC221)
DC220 ($R_f = 0.2$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH) and appearance of the product as a UV 254 nm active spot (purple in color, $R_f = 0.45$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH) indicated completion of the reaction. The resulting mixture was then cooled to 23 °C and poured onto 200 mL of ice water. More ice was added to a final volume of 300 mL, and the pH of the solution brought to 12 with concentrated NH$_4$OH (ca. 60 mL) at which point a white solid precipitated. The solid was collected via vacuum filtration (3.5g). Further purification was affected by recrystallization from hot ethyl acetate (EtOAc), which on cooling to -20°C resulted in the formation of small off-white needles whose structure was confirmed by $^1$H NMR. The product was collected via vacuum filtration (2.41g, 59%) and washed with cold EtOAc (-20 °C). The structure of the resulting product was confirmed by $^1$H NMR, and found to match literature precedent (9).$^{85}$ $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.53 (2H, d, $J = 5.5$ Hz) ppm, 7.94 (2H, d, $J = 2.0$ Hz), 7.41 (2H, d, $J = 9.0$ Hz), 6.99 (2H, dd, $J = 9.0$, 2.0 Hz), 6.38 (2H, d, $J = 5.5$ Hz), 5.45 (2H, s(br)), 3.40 (4H, quart., $J = 5.0$ Hz), 2.89 (4H, t, $J = 6.0$ Hz), 2.46 (3H, s).

Lys 05 (Water Soluble Salt)

Synthesis of Lys 05 the water-soluble salt of DC221, was performed according to literature precedent. NMR of the water-soluble salt was found to match the reported literature precedent.$^{85}$ $^1$H NMR (500 MHz, D$_2$O): $\delta$ 8.26 (d, $J = 6.9$ Hz, 2H), 7.86 (d, $J = 9.0$ Hz, 2H), 7.71 (d, $J = 2.2$ Hz, 2H), 7.36 – 7.42 (m, 2H), 6.75 (d, $J = 7.0$ Hz, 2H), 4.01
(s, 4H), 3.76 (s, 4H), 3.23 (s, 3H). $^{13}$C NMR (126 MHz, D$_2$O): δ 155.43, 142.80, 140.01, 136.94, 127.86, 123.86, 118.78, 114.49, 98.68, 52.91, 42.65, 38.15. Purity of the final compound was determined by UPLC-MS analysis. Lys05 was found to be a single peak, with a mass corresponding to the reported literature precedent, $[\text{M+2H-3HCl}]/2 = 220.8$

![Chemical structure](image)

1-Bromo-7-Azidoheptane

1,7-Dibromoheptane 34 (959 mg, 3.81 mmol, 2.0 equiv.) was weighed into a round bottom flask. The flask was placed under an argon atmosphere, and DMSO (4mL) was added via a syringe to the reaction vessel. Sodium azide (124 mg, 1.90 mmol, 1.0 equiv.) was added, and allowed to stir for five hours at room temperature. Upon completion, the reaction was diluted with water (40mL), and extracted with diethyl ether (3x25 mL). The organic layer was washed with brine, and dried over sodium sulfate. Concentration yielded a clear oil (1.00g), which was purified by column chromatography (30mm x 150mm, 10:1 Hexanes: Ethyl Acetate) to yield a clear oil 36 (264mg, 63%), the structure of which was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$) δ 3.41 (t, $J =$ 6.8 Hz, 2H), 3.26 (t, $J =$ 6.9 Hz, 2H), 1.90 – 1.81 (m, 2H), 1.64 – 1.57 (m, 2H), 1.50 – 1.30 (m, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 51.41, 33.96, 32.81, 28.92, 28.47, 28.16, 26.72. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2932, 2857, 2095. HRMS (ESI) C$_7$H$_{14}$N$_3$Br: Calculated for [M+H] C$_7$H$_{14}$N$_3$Br, 220.0449; found: 220.0420.
1 –Bromo-8-Azidooctane

Synthesized by the general procedure used for 1-Bromo-7-Azidoheptane 36. Product (37) isolated as an oil (1.07g, 60%) the structure of which was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$) δ 3.41 (t, $J = 9.8$, 4.1 Hz, 2H), 3.26 (t, $J = 8.1$, 5.8 Hz, 2H), 1.86 (m, 2H), 1.60 (m, 2H), 1.48 – 1.42 (m, 2H), 1.40 – 1.31 (m, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 51.34, 33.78, 32.64, 28.85, 28.70, 28.50, 27.94, 26.51. FTIR (thin film) λ (cm$^{-1}$): 2931, 2857, 2095. HRMS (ESI) C$_7$H$_{14}$N$_3$Br: Calculated for [M-N$_2$] C$_8$H$_{16}$NBr, 206.0544; found: 206.0624.

7-azido-N-(7-azidoheptyl)-N-benzylheptan-1-amine

1 –Bromo-7-Azidoheptane (36) (1.577g, 7.17 mmol, 2.2 equiv.), potassium carbonate (1.348g, 9.77 mmol), and potassium iodide (108 mg, 0.65 mmol, 0.2 equiv.) were added to a round bottom flask. After placing the reaction under argon gas, n-butanol (14 mL) was added and heated to 115 °C. Benzylamine (360 uL, 3.26 mmol, 1.0 equiv.)
was added via a syringe, and the reaction was stirred at reflux for 16 hours. The reaction was dissolved in diethyl ether (60 mL), filtered through Celite™, and concentrated to a yellow oil (1.61g). The crude material was purified by column chromatography (dry loaded onto 2g SiO₂, then eluted on a 40mm x 150 mm column with 5:1 Hexanes:Ethyl Acetate), yielding a yellow oil (38) (435mg, 35%), the structure of which was verified by 1H NMR. 1H NMR (500 MHz, CDCl₃) δ 7.39 – 7.25 (m, 5H), 3.58 (s, 2H), 3.28 (t, J = 7.0 Hz, 4H), 2.47 – 2.40 (m, 4H), 1.61 (q, J = 7.2 Hz, 4H), 1.55 – 1.47 (m, 4H), 1.43 – 1.27 (m, 12H). 13C NMR (126 MHz, CDCl₃) δ 140.16, 128.97, 128.20, 126.81, 58.79, 53.84, 51.58, 29.15, 28.92, 27.35, 27.02, 26.83. FTIR (thin film) λ (cm⁻¹): 2932, 2857, 2797, 2094. HRMS (ESI) C₂₁H₃₅N₇: Calculated for [M+H] C₂₁H₃₆N₇, 386.3032; found: 386.3012.

8-azido-N-(8-azidoheptyl)-N-benzyloctan-1-amine

Synthesized by the general procedure used for 7-azido-N-(7-azidoheptyl)-N-benzylheptan-1-amine (38). The product 39 was isolated as a golden oil (310 mg, 39%), the structure of which was verified by 1H NMR. 1H NMR (500 MHz, CDCl₃) δ 7.37 – 7.22 (m, 5H), 3.56 (s, 2H), 3.28 (t, J = 6.8 Hz, 4H), 2.42 (t, J = 7.5 Hz, 4H), 1.62 (p, J = 7.1 Hz, 4H), 1.48 (s, 4H), 1.34 (d, J = 42.1 Hz, 14H). 13C NMR (126 MHz, CDCl₃) δ 128.79, 128.04, 126.58, 124.56, 58.67, 53.84, 51.50, 29.39, 29.15, 28.83, 27.32, 27.03,
26.70. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2932, 2857, 2797, 2094. HRMS (ESI) C$_{23}$H$_{39}$N$_7$: Calculated for [M+H] C$_{23}$H$_{40}$N$_7$, 414.3278; found: 414.3301.

N$^1$-7(-aminoheptyl)heptane-1,7-diamine

7-azido-N-(7-azidoheptyl)-N-benzylheptan-1-amine ($\text{38}$) (435mg, 1.12 mmol, 1.0 equiv.) was added to a round bottom flask and dissolved in methanol. The reaction was degassed by bubbling argon gas through the mixture for ten minutes. To this palladium on carbon (356 mg of 10 weight %, 0.34 mmol, 0.3 equiv.) was added and the reaction sealed with a septum. Using a balloon and needle, hydrogen gas was bubbled through the reaction for fifteen minutes. Then placed under a balloon of hydrogen at one atmosphere for 6 hours. Upon completion, argon gas was bubbled through the reaction mixture for fifteen minutes, sonicated in methanol, and filtered through a pad of Celite$^\text{TM}$. Concentration of this reaction mixture gave a white solid ($\text{40}$) (235 mg, 86%) the structure of which was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 2.61 (h, $J = 7.9, 6.8$ Hz, 4H), 2.53 (q, $J = 7.4, 6.1$ Hz, 4H), 1.78 – 1.73 (m, 9H), 1.45 – 1.34 (m, 11H), 1.33 – 1.17 (m, 5H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 50.19, 42.32, 33.86, 30.21, 29.53, 27.50, 26.95. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2923, 2851. HRMS (ESI) C$_{14}$H$_{33}$N$_3$: Calculated for [M+H] C$_{14}$H$_{34}$N$_3$, 244.2753; found: 244.2755.
N<sup>1</sup>-(8-aminooctyl)octane-1,8-diamine

The same general synthesis as N<sup>1</sup>-7(-aminohexyl)heptane-1,7-diamine (40) was used. The product was isolated as an off-white paste (41) (160 mg, 79%), the structure of which was verified by <sup>1</sup>H NMR. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 2.69 – 2.61 (m, 4H), 2.55 (td, J = 7.5, 2.8 Hz, 5H), 1.65 (bs, 8H), 1.48 – 1.37 (m, 11H), 1.28 (d, J = 5.7 Hz, 19H).<sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 50.28, 42.30, 33.85, 30.19, 29.65, 29.54, 27.47, 26.94. FTIR (thin film) λ (cm<sup>-1</sup>): 2923, 2851. HRMS (ESI) C<sub>16</sub>H<sub>37</sub>N<sub>3</sub>: Calculated for [M+H]<sup>+</sup> C<sub>16</sub>H<sub>38</sub>N<sub>3</sub>, 272.3066; found: 272.3063.

Benzyl (11-hydroxyundecyl)carbamate

To a reaction vessel 11-aminoundecanol<sup>137</sup> 43 (466mg, 2.49 mmol, 1.0 equiv.) was added, placed under a positive pressure of argon gas, and dissolved in tetrahydrofuran (8mL). Triethylamine (800 ul, 5.73 mmol, 2.3 equiv.) was added to the reaction mixture via a syringe, and the reaction was cooled to 0 °C. While stirring, benzyl chloroformate (400 ul, 2.74 mmol, 1.1 equiv.) was added slowly via a syringe. The reaction was allowed to stir 6 hours at 55 °C till completion. The reaction was filtered
through Celite™ and concentrated to a yellow-white solid 800 mg. The solid was purified on by column chromatography (SiO₂, 30 mm x 150 mm, 3% MeOH: CH₂Cl₂). Collect 463 mg of product (44) 58% yield, the structure of which was confirmed by ¹H NMR. MP = 66-69 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.39 – 7.27 (m, 5H), 5.08 (s, 2H), 4.88 – 4.78 (m, 1H), 3.62 (t, J = 6.7 Hz, 2H), 3.17 (q, J = 6.8 Hz, 2H), 1.64 (s, 1H), 1.55 (p, J = 6.8 Hz, 2H), 1.47 (q, J = 7.1 Hz, 2H), 1.36 – 1.20 (m, 14H). ¹³C NMR (126 MHz, CDCl₃) δ 156.54, 136.77, 128.61, 128.22, 128.18, 66.68, 63.09, 41.22, 32.90, 30.05, 29.66, 29.60, 29.57, 29.52, 29.35, 26.83, 25.85. HRMS (ESI) C₁₉H₃₁NO₃: Calculated for [M+H] C₁₉H₃₁NO₃, 188.2104; found: 188.2005. FTIR (thin film) λ (cm⁻¹): 3346, 2922, 2852, 1685, 1531, 1267, 1245.

benzyl (11-oxoundecyl)carbamate

To a flame dried round bottom flask, CH₂Cl₂ (83 ml) was added followed by distilled oxalyl chloride (960 ul, 11.2 mmol, 1.2 equiv.). The reaction vessel was cooled to -78 °C and stirred under a positive pressure of argon gas. DMSO (1.59 ml, 22.4 mmol, 2.4 equiv.) was added via a syringe, and allowed to stir 45 min until the reaction ceases bubbling. In a solution of CH₂Cl₂ (10 ml) Benzyl (11-hydroxyundecyl)carbamate (44) (3.00g, 9.33 mmol, 1.0 equiv.) was added to the reaction mixture, and allowed to stir for 30 minutes. Triethylamine (6.5 ml, 46.7 mmol) was added and the reaction was stirred 60 min warming to room temperature until the oxidation was complete. Upon completion,
the reaction was washed 3 x 20 mL with a saturated NH₄Cl solution, washed with brine, and the organic layer was dried over sodium sulfate. Concentration of the organic layer yielded a paste, which was dissolved in toluene and evaporated till a white solid (45) (2.8g, 94 %) forms. ¹H NMR (500 MHz, CDCl₃) δ 9.71 (s, 1H), 7.29 (dd, J = 20.5, 4.4 Hz, 5H), 5.05 (s, 2H), 4.96 (t, J = 6.1 Hz, 1H), 3.14 (q, J = 6.7 Hz, 2H), 2.37 (td, J = 7.3, 1.8 Hz, 2H), 1.59 (q, J = 7.2 Hz, 2H), 1.45 (p, J = 7.2 Hz, 2H), 1.36 – 1.17 (m, 13H). ¹³C NMR (126 MHz, CDCl₃) δ 202.97, 156.43, 136.73, 128.47, 128.05, 128.01, 66.46, 43.87, 41.08, 29.93, 29.43, 29.31, 29.30, 29.22, 29.11, 26.71, 22.04. FTIR (thin film) λ (cm⁻¹): 3325, 2922, 2851, 1688, 1634, 1556, 1469.

dibenzyldibenzyloxazanediylbis(undecane-11,1-diyl)dicarbamate

To a round bottom flask, compound (45) (2.8g, 8.8 mmol, 2.2 equiv.) was added and placed under a positive pressure of argon gas. CH₂Cl₂ was added (80 ml), then benzyl amine (435 ul, 3.98 mmol, 1.0 equiv.) was added via a syringe. Once the reaction becomes homogenous, sodium triacetoxyborohydride was added (4.24 g, 20 mmol, 5.0 equiv.). Upon completion, the reaction was diluted by half and excess sodium triacetoxyborohydride was quenched by reaction with an equal volume of 2N aqueous sodium hydroxide for 1 hour. The organic layer was separated, washed with brine, dried.
over sodium sulfate, and concentrated under reduced pressure. The crude material was purified by column chromatography (SiO₂, 45 mm x 150 mm, 3:1 Hexanes:THF). Yield a white solid (46) (2.33g, 82%). ¹H NMR (500 MHz, CDCl₃) δ 7.39 – 7.27 (m, 15H), 7.24 – 7.20 (m, 1H), 5.10 (s, 4H), 4.85 (s, 1H), 3.54 (s, 2H), 3.17 (t, J = 7.0 Hz, 4H), 2.39 (t, 4H), 1.52 – 1.41 (m, 8H), 1.34 – 1.19 (m, 32H). ¹³C NMR (126 MHz, CDCl₃) δ 156.46, 140.41, 136.80, 128.91, 128.59, 128.20, 128.15, 128.12, 126.65, 66.62, 58.72, 53.92, 41.22, 41.09, 30.05, 29.73, 29.68, 29.66, 29.65, 29.40, 27.56, 27.12, 26.86. FTIR (thin film) λ (cm⁻¹): 2923, 2852, 1685, 1531. HRMS (ESI) C₄₅H₆₇N₃O₃: Calculated for [M+Na] C₄₅H₆₇N₃O₄Na, 736.5032; found: 736.5029.

**N₁-(11-aminoundecyl)undecane-1,11-diamine**

To a round bottom flask, compound 46 (800mg, 1.12 mmol, 1.0 equiv.) was added, and dissolved in MeOH (12.5 ml). The reaction was then degassed by bubbling argon gas through the solution for 10 minutes. Palladium on carbon (10% by weight, 367 mg, 0.34 mmol, 0.3 equiv.) was added to the reaction mixture, and while stirring, hydrogen gas was bubbled through the reaction mixture for 15 minutes. The reaction was then placed under a balloon of hydrogen, and stirred till the starting material was
consumed. Upon completion the reaction was degassed by bubbling argon gas through the reaction for 15 minutes. Then, the reaction was sonicated and filtered through Celite™ using methanol. The methanol was concentrated yielding compound 47 (360 mg, 91%), as a white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 2.67 (t, $J = 7.0$ Hz, 4H), 2.58 (t, 3H), 1.45 – 1.39 (m, 1H), 1.35 – 1.22 (m, 34H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 50.20 42.44, 34.01, 30.16, 29.78, 29.74, 29.72, 29.67(2C), 27.58, 27.07 HRMS (ESI) C$_{22}$H$_{49}$N$_3$: Calculated for [M+H] C$_{22}$H$_{50}$N$_3$, 188.2104; found: 188.2005. FTIR (thin film) λ (cm$^{-1}$): 2925, 2850, 2812.

**General procedure for Buchwald-Hartwig amination**

To a vial, triamine (1.0 Eq), Arylhalide (2.2 eq)$^{55,56,156}$, Pd(OAc)$_2$ (0.05 eq), racemic BINAP (0.10 eq), and K$_3$PO$_4$ (3.0 eq) were added. The reaction vessel was sealed, evacuated, and placed under an argon atmosphere. To the vial, a solution of 10:1 1,4-dioxane:water (degassed by freeze-pump-thaw method) was added (0.4M final reaction concentration), and the reaction was heated to 120°C. Upon completion, the reaction was allowed to cool to 23°C and filtered through Celite™ using either CHCl$_3$ (DC compounds) or CH$_2$Cl$_2$ (DQ compounds) and concentrated under reduced pressure. The compounds were then dissolved in a minimum amount of either CHCl$_3$ (DC compounds), or CH$_2$Cl$_2$ (DQ compounds). The HCl salt was formed by added 3.0 eq of
HCl in diethyl ether. The precipitate was filtered and collected via vacuum filtration. The organic layer is washed 3 x with H$_2$O. The precipitate was dissolved in H$_2$O unless specified. The H$_2$O layer was then washed once with CH$_2$Cl$_2$. Following the organic wash, the pH of the aqueous layer was adjusted to 12 by the addition of aqueous ammonium hydroxide. The aqueous layer was then extracted with 25% 2-propanol in CHCl$_3$ and dried over anhydrous sodium sulfate. The organic reaction was concentrated to a solid or film yielding the dimeric inhibitor. Further purification used when specified.

**DC340**

DC340 (17) was synthesized and isolated by the general procedure above. The product as a white solid (785 mg, 91% yield), the structure of which was verified by $^1$H NMR to match literature precedent.$^{46}$ R$_f$ = 0.10 (EtOAc:MeOH:TEA; 80:15:5); $^1$H NMR (500 MHz, CD$_3$OD) δ 8.34 (t, $J$ = 5.4 Hz, 2H), 8.07 (dd, $J$ = 12.3, 9.0 Hz, 2H), 7.90 (d, $J$ = 0.8 Hz, 1H), 7.77 (d, $J$ = 2.3 Hz, 1H), 7.38 (ddd, $J$ = 8.7, 6.1, 2.2 Hz, 2H), 6.52 (t, $J$ = 6.3 Hz, 2H), 3.40 (dt, $J$ = 17.8, 7.0 Hz, 4H), 2.76 (t, $J$ = 7.1 Hz, 2H), 2.69 (t, $J$ = 7.5 Hz, 2H), 1.94 (p, $J$ = 7.0 Hz, 2H), 1.79 (p, $J$ = 7.2 Hz, 2H), 1.69 (q, $J$ = 7.6 Hz, 2H).
To a vial was added 3-Chloro-2,8-bis(trifluoromethyl)quinoline<sup>58</sup> (558.00 mg, 1.86 mmol, 2.40 equiv.), BINAP (72.00 mg, 0.12 mmol, 0.15 equiv.), Pd(OAc)<sub>2</sub> (14.00 mg, 0.06 mmol, 0.075 equiv.), finely ground K<sub>3</sub>PO<sub>4</sub> (659.00 mg, 3.10 mmol, 4.00 equiv.) and spermidine (120.0 uL, 0.78 mmol, 1.00 equiv.). The reagents were placed under a blanket of Argon; then a mixture of degassed dioxane and water (3 mL, 10:1) was added. The reaction vial was sealed and heated to 120 °C for 12 hours. The reaction was then cooled to room temperature and filtered on a pad of Celite<sup>TM</sup>; washing with chloroform (3 × 10 mL). Combined organic layers were acidified to pH 1 using 1M aqueous HCl solution (4.7 mL, 4.66 mmol, 6.00 equiv.) and diluted with water (20 mL). The resulting biphasic mixture was separated, and the water layer was washed with chloroform (2 × 15 mL). The pH of the aqueous layer was adjusted to 11 using ammonium hydroxide. The now alkaline mixture was washed with chloroform (3 × 20 mL). These 3 chloroform extracts were combined, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated which afforded the product as an off-white foam (33) (243.00 mg, 50% yield). The purity of this material was calculated to be ≥ 95% which negated the need for further purification. R<sub>f</sub> = 0.25 (CCl<sub>3</sub>:MeOH:TEA; 85:10:5); Mp = 84 – 87 °C. <sup>1</sup>H NMR (500 MHz, DMSO) 1.55 – 1.60 (2H, m, CH<sub>2</sub>), 1.70 – 1.75 (2H, m, CH<sub>2</sub>), 1.79 – 1.85 (2H, m, CH<sub>2</sub>), 2.58 (2H, t, J =
7.0 Hz, CH₂), 2.65 (2H, t, J = 6.0 Hz, CH₂), 3.39 – 3.43 (4H, m, CH₂), 6.86 (2H, d, J =
7.5 Hz, ArH), 7.59 – 7.66 (2H, m, ArH), 8.03 (1H, s(br), NH), 8.08 (1H, d, J = 7.0 Hz,
ArH), 8.12 (1H, d, J = 7.0 Hz, ArH), 8.26 (1H, s(br), NH), 8.53 (1H, d, J = 8.5 Hz, ArH),
8.61 (1H, d, J = 8.5 Hz, ArH) ppm. ¹³C (125 MHz, DMSO) 25.9, 27.3, 28.0, 41.6, 42.8,
47.5, 49.4, 94.4, 119.7, 119.7, 120.9, 123.1, 124.8 (d), 126.6, 126.8, 127.2, 127.3, 129.3
(br), 144.17, 144.23, 152.7, 152.8 ppm. FTIR (thin film) λ (cm⁻¹): 3406, 3020, 2976,
1523, 1215, 771. HRMS (ESI) C₂₉H₂₆F₁₂N₅: Calculated for [M+H] C₂₉H₂₇F₁₂N₅,
672.1997; found: 672.2033.

**DP340**

To a vial was added 8-Chloro-6-methoxyquinoline⁵⁶ (26) (193.0 mg, 1.0 mmol,
2.20 equiv.), BINAP (45.00 mg, 0.072 mmol, 0.16 equiv.), Pd₂(dba)₃ (33.00 mg, 0.036
mmol, 0.08 equiv.), NaOtBu (130.0 mg, 1.37 mmol, 3.0 equiv) and spermidine (71.0 uL,
0.46 mmol, 1.00 equiv.). The reagents were placed under a blanket of Argon; then 1 mL
of dioxane was added. The reaction vial 26 and filtered on a pad of Celite™; washing
with chloroform (3 × 10 mL). The filtrate was concentrated and dissolved with methanol
and dichloromethane and adsorbed onto 400 mg of SiO₂. These materials were separated
via flash column chromatography (EtOAc:MeOH:TEA; 90:9:1) to afford the product as a
red brown paste (32) (173 mg, 83%), the structure of which was confirmed by ¹H NMR.
$^1$H NMR (500 MHz, CDCl$_3$) δ 8.54 – 8.50 (m, 2H), 7.91 (ddd, $J = 8.1$, 4.0, 1.7 Hz, 2H), 
7.29 (d, $J = 1.3$ Hz, 2H), 6.37 – 6.27 (m, 5H), 6.14 (s, 1H), 3.88 (d, $J = 1.3$ Hz, 6H), 3.35 
(t, $J = 6.9$ Hz, 2H), 3.28 (q, $J = 6.2$ Hz, 2H), 2.82 (td, $J = 6.9$, 1.4 Hz, 2H), 2.75 – 2.67 
(m, 2H), 2.00 – 1.93 (m, 2H), 1.84 – 1.76 (m, 2H), 1.74 – 1.65 (m, 2H). $^{13}$C (125 MHz, 
CDCl$_3$) 26.2 (2C’s), 29.6, 40.8, 42.7, 46.3, 47.9, 55.1 (2C’s), 92.2, 92.6, 96.8, 97.1, 
121.75, 121.81, 129.60, 129.61, 134.6, 135.17, 135.19, 144.3, 144.5, 145.3, 145.5, 159.2, 
159.3 ppm. FTIR (thin film) λ (cm$^{-1}$): 2936, 1617, 1522, 1458, 1422, 1388, 1214, 1154, 
1050, 821, 791. HRMS (ESI) C$_{27}$H$_{34}$N$_{5}$O$_{2}$: Calculated for [M+H] C$_{27}$H$_{35}$N$_{5}$O$_{2}$, 460.2713; 
found: 460.2707.

![Chemical Structure](image_url)

**DQ 221 – Dr. Noel McLaughlin**

DQ 221 (52) was prepared by the above general procedure. Required flash 
column chromatography (EtOAc:MeOH:TEA; 90:9:1) to afford the product as a red solid 
(240.00 mg, 33%). $R_f = 0.15$ (EtOAc:MeOH:TEA; 92:7:1); Mp = 163 – 164 °C; $^1$H NMR 
(500 MHz, CDCl$_3$) 2.45 (3H, s, CH$_3$), 2.88 (4H, t, $J = 6.0$ Hz, CH$_2$), 3.73 (6H, s, CH$_3$), 
3.85 (4H, t, $J = 6.0$ Hz, CH$_2$), 5.65 (2H, s(br), NH), 7.16 (2H, d, $J = 9.0$ Hz, ArH), 7.21 
(2H, d, $J = 2.5$ Hz, ArH), 7.32 (2H, d, $J = 8.5$ Hz, ArH), 7.95 (2H, d, $J = 9.0$ Hz, ArH), 
8.01 – 8.07 (4H, m, ArH) ppm. $^{13}$C NMR (125 MHz, CDCl$_3$) 42.0, 47.4, 55.4, 57.9, 99.5,
115.5, 117.8, 124.3, 124.4, 127.5, 130.8, 135.1, 145.9, 147.8, 149.9, 156.1 ppm. FTIR (thin film) λ (cm⁻¹): 3688, 3619, 3019, 1219, 929. HRMS (ESI) C₃₃H₃₂Cl₂N₅O₂: Calculated for [M+H] C₃₃H₃₂Cl₂N₅O₂, 600.1933; found:600.1935.2707.

DQ 330

DQ 330 (53) was prepared by the above general procedure. The workup was altered from the general procedure as follows. After formation of the HCl salt, and following the standard workup showed little product moved into the aqueous layer, therefore the collected precipitate (380mg) was recrystallized from methanol yielding (229 mg). The HCl salt solubilized in a solution of a 1:1 mixture of dichloromethane to basic methanol. The residue was dissolved in dichloromethane, washed with water (2x20 mL) then washed with brine and dried over Na₂SO₄. Concentration of the organic phase yielded an orange solid (172 mg, 56%). Mp = 122 – 124 °C ¹H NMR (500 MHz, CDCl₃) δ 8.01 – 7.96 (m, 2H), 7.92 (d, J = 9.3 Hz, 4H), 7.33 (dd, J = 9.4, 2.7 Hz, 2H), 7.21 (d, J = 2.7 Hz, 2H), 7.10 (dd, J = 9.2, 2.2 Hz, 2H), 6.01 (s, 2H), 3.83 – 3.75 (m, 10H), 3.49 (s, 1H), 2.82 (t, J = 6.3 Hz, 4H), 1.89 (p, J = 6.4 Hz, 4H). ¹³C NMR (126 MHz, CDCl₃) δ 155.56, 149.93, 134.72, 124.37, 123.86, 123.72, 117.50, 115.26, 100.41, 55.52, 49.90, 30.87,
25.41. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2925, 2853, 1631, 1562, 1466, 1238. HRMS (ESI) $\text{C}_{34}\text{H}_{34}\text{Cl}_{2}\text{N}_{5}\text{O}_{2}$: Calculated for [M+H] $\text{C}_{34}\text{H}_{35}\text{Cl}_{2}\text{N}_{5}\text{O}_{2}$, 614.2090; found: 614.2088.

DQ 340 – Dr. Noel McLaughlin

DQ 340 (31) was prepared by the above general procedure. The isolated materials were separated via flash column chromatography (EtOAc:MeOH:TEA; 84:16:1) to afford the product as a red solid (160.00 mg, 56%). $R_f = 0.10$ (EtOAc:MeOH:TEA; 84:15:1). Mp = 115 – 118 °C. $^1$H NMR (500 MHz, CDCl$_3$) 1.68 – 1.74 (2H, m, CH$_2$), 1.80 – 1.86 (2H, m, CH$_2$), 1.88 – 1.92 (2H, m, CH$_2$), 2.73 (2H, t, $J = 7.0$ Hz, CH$_2$), 2.89 (2H, t, $J = 7.0$ Hz, CH$_2$), 3.74 (2H, t, $J = 7.0$ Hz, ArH), 3.87 (3H, s, CH$_3$), 3.89 – 3.92 (5H, m, CH$_3$ and CH$_2$), 7.11 – 7.24 (3H, m, ArH), 7.31 – 7.39 (3H, m, ArH), 7.95 – 8.04 (6H, m, ArH) ppm. $^{13}$C NMR (125 MHz, CDCl$_3$) 27.2, 29.5, 30.1, 49.0, 49.7, 50.5, 50.8, 55.6 (2C), 99.5, 101.3, 117.9, 118.4, 118.5, 120.6, 123.5, 123.6, 124.1, 124.4, 124.5, 124.8, 134.9, 135.2, 148.2, 149.7, 150.8, 155.4, 156.0 ppm. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3435, 3019, 1635, 1216, 929. HRMS (ESI) C$_{35}$H$_{36}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{35}$H$_{37}$Cl$_2$N$_5$O$_2$, 628.2246; found:628.2247.
DQ 440 (54) was prepared by the above general procedure yielding the product as an orange foam (0.35 g, 39% yield). R_f = 0.25 (EtOAc:MeOH:TEA; 80:15:5). Mp = 58 – 62 °C; 1H NMR (500 MHz, CDCl_3) 1.55 – 1.61 (4H, m, CH_2), 1.72 – 1.78 (4H, m, CH_2), 2.60 (4H, t, J = 7.0 Hz, CH_2), 3.68 – 3.71 (4H, m, CH_2), 3.92 (6H, s, CH_3), 7.23 (2H, d, J = 2.5 Hz, ArH), 7.26 (2H, dd, J = 2.0, 9.0 Hz, ArH), 7.40 (2H, dd, J = 2.5, 9.5 Hz, ArH), 7.98 – 8.00 (4H, m, ArH), 8.05 (2H, d, J = 1.5 Hz, ArH) ppm. 13C NMR (125 MHz, CDCl_3) 27.5, 29.5, 49.3, 50.6, 55.6, 67.1, 99.6, 115.9, 117.9, 124.1, 124.2, 124.4, 128.3, 131.6, 134.7, 146.9, 148.4, 149.8, 155.9 ppm. FTIR (thin film) λ (cm⁻¹): 3943, 3688, 3054, 2987, 2685, 1631, 1559, 1528, 1421, 1264, 1032, 896. HRMS (ESI) C_{36}H_{38}Cl_2N_5O_2: Calculated for [M+H] C_{36}H_{39}Cl_2N_5O_2, 642.2403; found: 642.2402.

DQ 550 – Dr. Noel McLaughlin
DQ 550 (55) was prepared by the above general procedure yielding the product as an orange solid (0.73 g, 83% yield). \( R_f = 0.15 \) (EtOAc:MeOH:TEA; 84:15:1). Mp = 52 – 54 °C. \(^1\)H NMR (500 MHz, CDCl\(_3\)) 1.47 (8H, s(br), CH\(_2\)), 1.73 – 1.75 (4H, m, CH\(_2\)), 2.51 – 2.53 (4H, m, CH\(_2\)), 3.65 – 3.66 (4H, m, CH\(_2\)), 3.93 (6H, s, CH\(_3\)), 7.19 (2H, s(br), ArH), 7.25 (2H, s(br), ArH), 7.38 – 7.40 (2H, m, ArH), 7.96 – 7.98 (4H, m, ArH), 8.04 (2H, s(br), ArH) ppm. \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) 24.7, 29.7, 31.6, 49.7, 50.5, 55.5, 99.3, 115.8, 117.9, 124.0, 124.4 (2C), 128.1, 131.4, 134.8, 146.6, 148.3, 149.7, 156.0 ppm. FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 3943, 3692, 3054, 2987, 2934, 2858, 2685, 1631, 1560, 1519, 1421, 1265, 896. HRMS (ESI) \( C_{38}H_{42}Cl_2N_5O_2 \): Calculated for \([M+H] C_{38}H_{43}Cl_2N_5O_2\), 670.2716; found: 670.2715.

DQ 660

DQ 660 (56) was prepared by the above general procedure. This compound, the precipitated HCl salt was recrystallized in MeOH, and then solubilized in a solution of a 1:1 mixture of CH\(_2\)Cl\(_2\) and MeOH. Ammonium hydroxide was added until the solid completely dissolved. The residue was concentrated under reduced pressure to remove MeOH. The residue was dissolved in CH\(_2\)Cl\(_2\), washed with water (2 x 20 mL) then washed with brine and dried over Na\(_2\)SO\(_4\). Evaporation yielded an orange solid (4.28g,
68%). \( R_f = 0.30 \) (EtOAc:MeOH:TEA; 80:15:5). Mp = 52 – 54 °C; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 1.31 – 1.36 (4H, m, CH\(_2\)), 1.40 – 1.47 (8H, m, CH\(_2\)), 1.71 – 1.77 (4H, m, CH\(_2\)), 2.51 (4H, t, \( J = 7.0 \) Hz, CH\(_2\)), 3.69 (4H, t, \( J = 7.0 \) Hz, CH\(_2\)), 3.95 (6H, s, CH\(_3\)), 7.20 (2H, d, \( J = 2.5 \) Hz, ArH), 7.30 (2H, dd, \( J = 2.0, 9.5 \) Hz, ArH), 7.42 (2H, dd, \( J = 2.5, 9.5 \) Hz, ArH), 8.00 (2H, dd, \( J = 3.0, 9.5 \) Hz, ArH), 8.06 (2H, d, \( J = 2.0 \) Hz, ArH) ppm; \(^1\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 26.8, 27.1, 31.8, 49.9, 50.7, 55.6, 99.1, 115.9, 118.0, 124.0, 124.5, 124.6, 128.4, 131.6, 134.8, 146.3, 149.0, 149.7, 156.0 ppm. FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 3943, 3692, 3054, 2987, 1560, 1421, 1265, 896. HRMS (ESI) C\(_{40}\)H\(_{46}\)Cl\(_2\)N\(_5\)O\(_2\): Calculated for [M+H] C\(_{40}\)H\(_{47}\)Cl\(_2\)N\(_5\)O\(_2\), 698.3029; found: 698.3029.

**DQ 770**

DQ 770 (57) was prepared by the above general procedure. Solid HCl salt was recrystallized from EtOH yielding 331 mg. This solid was converted to its free base form by dissolution in a one to one mixture of aqueous ammonium hydroxide and 25% 2-propanol/75% CHCl\(_3\) yielding an orange foam (145 mg, 38%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 8.07 (d, \( J = 2.1 \) Hz, 2H, ArH), 8.00 (dd, 4H, ArH), 7.42 (dd, \( J = 9.4, 2.7 \) Hz, 2H, ArH), 7.30 (dd, \( J = 9.2, 2.1 \) Hz, 2H, ArH), 7.20 (d, \( J = 2.7 \) Hz, 2H, ArH), 4.67 (t, \( J = 5.9 \) Hz, 2H, NH), 3.96 (s, 6H), 3.68 (q, 4H, CH\(_2\)), 2.54 (t, \( J = 8.1, 7.4 \) Hz, 4H, CH\(_2\)), 1.74
(p, J = 7.3 Hz, 4H, CH₂), 1.48 – 1.37 (m, 8H, CH₂), 1.36 – 1.25 (m, 10H, CH₂). ¹³C NMR (126 MHz, CDCl₃) δ 156.04, 149.87, 148.43, 146.80, 134.85, 131.53, 128.27, 124.56, 124.53, 124.18, 117.96, 115.87, 99.30, 77.39, 55.64, 50.78, 50.14, 31.84, 30.11, 29.36, 27.37, 26.95. FTIR (thin film) λ (cm⁻¹): 2927, 2853, 2358, 2330, 1558, 1507. HRMS (ESI) C₄₂H₄₉Cl₂N₅O₂: Calculated for [M+H] C₄₂H₅₀Cl₂N₅O₂, 725.3263; found: 725.3339.

DQ 880

DQ 880 (58) was prepared by the above general procedure. The product required recrystallization from methanol, and flash column chromatography (Ethyl Acetate with 5% Triethylamine). Yielded an orange paste (125 mg, 33%). ¹H NMR (500 MHz, CDCl₃) δ 8.07 (d, J = 2.1 Hz, 2H, ArH), 8.04 – 7.97 (m, 4H, ArH), 7.42 (dd, J = 9.4, 2.7 Hz, 2H, ArH), 7.34 – 7.24 (m, 7H, ArH), 7.21 (d, J = 2.7 Hz, 2H, ArH), 4.68 (bs, 2H, N-H), 3.97 (d, J = 2.7 Hz, 6H, CH₃), 3.69 (t, 4H, CH₂), 2.55 (t, J = 7.2 Hz, 4H, CH₂), 1.78 – 1.71 (m, 4H, CH₂), 1.44 (t, J = 7.0 Hz, 8H, CH₂), 1.36 – 1.23 (m, 12H, CH₂). ¹³C NMR (126 MHz, CDCl₃) δ 155.98, 149.72, 148.44, 146.88, 134.70, 131.64, 128.38, 124.51, 124.41, 123.98, 117.96, 115.91, 99.16, 55.52, 50.77, 50.15, 31.81, 30.19, 29.43, 29.26, 27.28, 26.86. FTIR (thin film) λ (cm⁻¹): 3292, 2927, 2854, 2359, 2341, 1632, 1559, 1434, 1336,
DQ 11110

DQ 11110 (59) was prepared by the above general procedure except for purification. The reaction, post Celite™ filtration is adsorbed onto silica, and purified by flash chromatography (20 mm x 150 mm, 4% MeOH, 25% ethyl acetate, 1% triethylamine, and 70% CH₂Cl₂) to yield an orange paste (95 mg, 30%). ¹H NMR (500 MHz, Chloroform-d) δ 8.07 – 7.91 (m, 6H), 7.41 (dd, J = 9.4, 2.7 Hz, 2H), 7.29 (dd, J = 9.3, 2.2 Hz, 2H), 7.21 (d, J = 2.8 Hz, 2H), 3.96 (s, 6H), 3.70 (t, J = 7.2 Hz, 4H), 2.61 (t, J = 7.4 Hz, 4H), 1.78 – 1.70 (m, 4H), 1.50 (d, J = 7.7 Hz, 4H), 1.46 – 1.37 (m, 4H), 1.25 (d, J = 16.7 Hz, 28H). ¹³C NMR (126 MHz, CDCl₃) δ 156.15, 149.97, 148.50, 146.88, 134.94, 131.65, 128.39, 124.66, 124.60, 124.19, 118.06, 115.99, 99.38, 55.70, 29.47, 29.47, 27.54, 27.08.

FTIR (thin film) λ (cm⁻¹): 3302, 2925, 2852, 2360, 2314, 1631, 1561, 1518, 1465, 1434, 1235. HRMS (ESI) C₅₀H₆₆Cl₂N₅O₂: Calculated for [M+H] C₅₀H₆₆Cl₂N₅O₂, 838.4515; found: 838.4574.
General reductive alkylation procedure

Method 1: Reductive alkylation with sodium triacetoxyborohydride

Dimeric inhibitor was added to a flask and dissolved in CH₂Cl₂, to give a final reaction concentration of 0.3M. Aqueous formaldehyde (37% w/v) (2.0 equiv.) was added to the reaction and allowed to stir for 15 minutes. Once the reaction becomes homogenous, sodium triacetoxyborohydride was added (4 equiv.). Upon disappearance of the starting DQ compound as observed by TLC, approximately 16 hours, the reaction was diluted by half with CH₂Cl₂ and the excess sodium triacetoxyborohydride was quenched by reaction with a volume of 2N aqueous sodium hydroxide, equal to the CH₂Cl₂, for 1 hour at 23°C. To the dichloromethane solution was added 1 M aq HCl solution. The mixture was stirred vigorously at room temperature for 1 hour, resulting in the formation of a water-soluble salt of the desired product. Both layers were separated. The aqueous layer was further washed with CHCl₃ (2 × 15 mL) and combined chloroform washings were discarded at this point. Using NH₄·OH, the pH of the aqueous layer was adjusted to 11, resulting in the liberation of the free base of the product. The product was extracted into a solvent mixture of CHCl₃ and i-PrOH (4:1). These combined extracts were washed with brine and dried over Na₂SO₄. The combined organic layers were concentrated under reduced pressure to afford a pure product unless specified.

Method 2: Eschweiler-Clarke Methylation
The dimeric inhibitor was added to a flask under an atmosphere of argon and dissolved in 88% formic acid (to give a reaction concentration of 0.5M). Aqueous formaldehyde was added to the reaction (3 equiv.), and then heated to reflux at 105 °C till consumption of the starting material is observed by TLC. Upon completion, between one and three hours, the reaction was poured over water, resulting in a twofold dilution. The pH of the aqueous layer was adjusted to pH 12 by addition of aqueous ammonium hydroxide. When a precipitate formed, it was dissolved in a solution of 50:50 MeOH:CH₂Cl₂. To this solution aqueous ammonium hydroxide was added. The solution was then concentrated under reduced pressure to near dryness and dissolved in both CH₂Cl₂ and water. The organic layer was separated, and the aqueous layer was then extracted three times CH₂Cl₂. The combined organic layer was then dried over sodium sulfate and concentrated under reduced pressure to yield the methylated inhibitor. The compound required no further purification unless specified.

DQ 331

DQ 331 (60) was synthesized by general method 2. The product was isolated as an orange film (21mg, 86%). Mp = 62 – 65 °C. ¹H NMR (500 MHz, CDCl₃) δ 7.99 (d, J = 2.1 Hz, 2H, ArH), 7.89 (dd, J = 19.6, 9.3 Hz, 4H, ArH), 7.32 (dd, J = 9.4, 2.7 Hz, 2H,
ArH), 7.15 – 7.09 (m, 4H, ArH), 3.78 (s, 6H, CH3), 3.76 (t, J = 6.4 Hz, 4H, CH2), 2.56 (t, J = 6.3 Hz, 4H, CH2), 2.35 (s, 3H, CH3), 1.88 (p, J = 6.4 Hz, 4H, CH2). 13C NMR (126 MHz, CDCl3) δ 155.71, 149.84, 148.64, 146.92, 134.78, 131.59, 128.38, 124.21, 124.13, 123.93, 117.51, 115.37, 100.20, 57.06, 55.61, 50.10, 42.91, 28.34. FTIR (thin film) λ (cm⁻¹): 3254, 295, 2926, 2359, 2341, 1633, 1236. HRMS (ESI) C35H35Cl2N5O2: Calculated for [M+H] C35H36Cl2N5O2, 628.2246; found: 628.2244.

![Image of a chemical structure](image)

**DQ 341 – Dr. Noel McLaughlin**

DQ 341 (61) was prepared by the general method 2. The product as a dark-red solid (34.0 mg, 94% yield). Mp = 61 – 66 °C. ¹H NMR (500 MHz, CD2Cl2) 1.61 – 1.67 (4H, m, CH2), 1.84 (2H, pent, J = 5.5 Hz, CH2), 2.32 (3H, s, CH3), 2.44 (2H, t, J = 7.0 Hz, CH2), 2.57 (2H, t, J = 7.0 Hz, CH2), 3.67 (2H, t, J = 6.5 Hz, CH2), 3.83 (3H, s, CH3), 3.86 (3H, s, CH3), 3.89 (2H, t, J = 4.5 Hz, CH2), 7.07 – 7.14 (4H, m, ArH, NH), 7.24 (1H, d, J = 2.5 Hz, ArH), 7.30 – 7.34 (3H, m, ArH), 7.86 (2H, dd, J = 3.5, 9.5 Hz, ArH), 7.92 (3H, d, J = 9.5 Hz, ArH), 8.02 (1H, d, J = 9.5 Hz, ArH) ppm. 13C NMR (125 MHz, CD2Cl2) 25.1, 27.7, 30.4, 43.0, 51.0, 51.7, 56.0, 56.1, 57.8, 58.7, 99.7, 101.3, 115.0, 116.4, 117.2, 118.4, 123.5, 124.0, 124.7 (2C), 124.8, 125.6, 128.4, 128.7, 131.7, 132.0, 134.9 (2C), 148.8, 149.2 (2C), 150.0, 150.9, 155.8, 156.5. FTIR (thin film) λ (cm⁻¹): 3434, 1653, 1213, 929.
HRMS (ESI) C$_{36}$H$_{37}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{36}$H$_{38}$Cl$_2$N$_5$O$_2$, 642.2403; found: 642.2398.

DQ 441 – Dr. Noel McLaughlin

DQ 441 (62) was synthesized by general method 1. Filtration followed by solvent evaporation afforded the product as a red solid (0.13 g, 83% yield). $R_f$ = 0.55 (EtOAc:MeOH:TEA; 80:15:5); Mp = 60 – 62 °C. $^1$H NMR (500 MHz, CDCl$_3$) 1.52 – 1.58 (4H, m, CH$_2$), 1.67 – 1.83 (4H, m, CH$_2$), 2.15 (3H, s, CH$_3$), 2.30 (4H, t, $J = 7.0$ Hz, CH$_2$), 3.68 (4H, t, $J = 6.5$ Hz, CH$_2$), 3.91 (6H, s, CH$_3$), 5.06 (2H, s(br), NH), 7.22 (2H, d, $J = 2.5$ Hz, ArH), 7.24 (1H, d, $J = 2.0$ Hz, ArH), 7.26 (1H, s(br), ArH), 7.39 (2H, dd, $J = 2.5$, 9.5 Hz, ArH), 7.97 – 7.99 (4H, m, ArH), 8.04 (2H, d, $J = 2.0$ Hz, ArH) ppm. $^{13}$C NMR (125 MHz, CDCl$_3$) 24.6, 29.6, 42.2, 50.5, 55.6, 57.0, 99.6, 115.8, 117.9, 124.1, 124.2, 124.4, 128.3, 131.6, 134.7, 146.8, 148.4, 149.9, 155.9 ppm. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3054, 2987, 1559, 1422, 1265, 896. HRMS (ESI) C$_{37}$H$_{40}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{36}$H$_{41}$Cl$_2$N$_5$O$_2$, 656.2559; found: 656.2565.
DQ 551 – Dr. Noel McLaughlin

DQ 551 (63) was synthesized by general method 1. The product was isolated as a red foam (0.30 g, 50% yield). Mp = 48 – 52 °C. $^1$H NMR (500 MHz, CD$_2$Cl$_3$) 1.45 (8H, s(br), CH$_2$), 1.78 (4H, s(br), CH$_2$), 2.12 (3H, s, CH$_3$), 2.26 (4H, s(br), CH$_2$), 3.73 (4H, t, $J$ = 6.5 Hz, CH$_2$), 3.98 (6H, s, CH$_3$), 7.31 – 7.34 (4H, m, ArH), 7.41 – 7.45 (2H, m, ArH), 7.96 – 7.98 (2H, m, ArH), 8.03 (2H, s, ArH), 8.10 (2H, d, $J$ = 8.0 Hz, ArH) ppm. $^{13}$C NMR (125 MHz, CD$_2$Cl$_3$) 24.7, 26.9, 31.5, 41.8, 50.6, 55.5, 57.4, 99.3, 115.7, 117.8, 124.0, 124.3, 124.5, 128.0, 131.4, 134.4, 146.7, 148.4, 149.8, 155.9 ppm. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3944, 3692, 3054, 2987, 1631, 1560, 1422, 1262, 896. HRMS (ESI) C$_{39}$H$_{43}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{39}$H$_{44}$Cl$_2$N$_5$O$_2$, 684.2872; found: 684.2897.

DQ 661
DQ 661 (64) was synthesized by general method 2. The product was isolated as a brown orange solid/foam (2.98g, 91%). IR λ (neat/cm⁻¹) 3293, 2934, 2854, 1687, 1657, 1235, 925, 829. 

\[ ^1H \text{ NMR (500 MHz, CDCl}_3) \delta 8.08 (d, J = 2.1 \text{ Hz}, 2H, \text{ArH}), 8.02 (t, J = 8.9 \text{ Hz}, 4H, \text{ArH}), 7.43 \text{ (dd}, J = 9.4, 2.7 \text{ Hz}, 2H, \text{ArH}), 7.32 \text{ (dd}, J = 9.3, 2.1 \text{ Hz}, 1H, \text{ArH}), 7.23 \text{ (d}, J = 2.7 \text{ Hz}, 2H, \text{ArH}), 4.75 \text{ (s}, 2H, \text{NH}), 3.97 \text{ (d}, J = 2.9 \text{ Hz}, 6H, \text{CH}_3), 3.70 \text{ (s}, 4H, \text{CH}_2), 2.25 \text{ (t}, J = 7.6 \text{ Hz}, 4H, \text{CH}_2), 2.17 \text{ (s}, 3H, \text{CH}_3), 1.75 \text{ (p}, J = 7.5 \text{ Hz}, 4H, \text{CH}_2), 1.43 \text{ (d}, J = 7.6 \text{ Hz}, 8H, \text{CH}_2), 1.32 \text{ (m}, 4H, \text{CH}_2). \]

\[ ^13C \text{ NMR (126 MHz, CDCl}_3) \delta 155.98, 150.00, 148.06, 146.29, 134.97, 131.02, 127.80, 124.55, 24.36, 124.30, 117.74, 115.56, 99.48, 57.73, 55.63, 50.57, 42.30, 31.74, 27.32, 27.24, 26.93. \]

\[ \text{FTIR (thin film) } \lambda (\text{cm}^{-1}) : 3293, 2934, 2854, 1687, 1657, 1235, 925, 829. \]

HRMS (ESI) \text{C}_4\text{H}_4\text{Cl}_2\text{N}_5\text{O}_2: Calculated for [M+H] \text{C}_4\text{H}_4\text{Cl}_2\text{N}_5\text{O}_2, 712.3185; \text{found: 712.3164.} \]

DQ 771

DQ 771 (65) was synthesized by general method 2. The product was isolated as a black/orange solid film. (31mg, 74%). \n
\[ ^1H \text{ NMR (500 MHz, CDCl}_3) \delta 8.07 (d, J = 2.1 \text{ Hz}, 2H), 8.00 (dd, J = 9.3, 5.4 \text{ Hz}, 4H), 7.42 (dd, J = 9.4, 2.7 \text{ Hz}, 2H), 7.31 (dd, J = 9.2, 2.1 \text{ Hz}, 2H), 7.21 (d, J = 2.7 \text{ Hz}, 2H), 3.96 (s, 6H), 3.69 (t, J = 7.2 \text{ Hz}, 4H), 2.27 (t, J = 7.6 \text{ Hz}, 3H), 2.17 (s, 2H), 1.74 (p, J = 7.3 \text{ Hz}, 4H), 1.47 − 1.22 (m, 16H). \]

\[ ^13C \text{ NMR (126} \]
MHz, CDCl$_3$) $\delta$ 156.19, 149.93, 148.54, 146.94, 134.94, 131.75, 128.49, 124.73, 124.60, 124.15, 118.13, 116.07, 99.39, 57.95, 55.72, 50.92, 42.42, 31.92, 29.45, 27.60, 27.35, 27.07. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3302, 2930, 2854, 2793, 2359, 2341, 1632, 1559, 1236. HRMS (ESI) C$_{43}$H$_{51}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{41}$H$_{52}$Cl$_2$N$_5$O$_2$, 739.3420; found: 739.3522.

DQ 881

DQ 881 (66) was synthesized by general method 2. The product was isolated as a red orange film (15 mg, 72%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.06 (s, 2H, ArH), 8.03 – 7.97 (m, 4H, ArH), 7.42 (dd, $J$ = 9.4, 2.9 Hz, 2H, ArH), 7.30 (dd, $J$ = 9.2, 3.4, 2.1 Hz, 2H, ArH), 7.21 (d, $J$ = 2.8 Hz, 2H, ArH), 4.72 (s, 2H, NH), 3.96 (d, $J$ = 4.6 Hz, 6H, CH$_3$), 3.68 (t, $J$ = 7.1 Hz, 4H, CH$_2$), 2.27 (dd, $J$ = 8.9, 6.2 Hz, 4H, CH$_2$), 2.18 (d, $J$ = 3.5 Hz, 3H, CH$_3$), 1.73 (p, $J$ = 7.5 Hz, 4H, CH$_2$), 1.49 – 1.19 (m, 24H, CH$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 156.17, 149.93, 148.57, 146.99, 134.91, 131.77, 128.51, 124.71, 124.59, 124.15, 118.13, 116.08, 99.36, 58.06, 55.71, 50.95, 42.48, 31.98, 29.64, 29.47, 27.63, 27.47, 27.05. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2930, 2854, 2359, 1632, 1559, 1236. HRMS (ESI) C$_{45}$H$_{55}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{45}$H$_{56}$Cl$_2$N$_5$O$_2$, 767.3733; found: 767.3719.
DQ 11111

DQ 11111 (67) was synthesized by general method 2. The product was isolated as a red orange paste (53mg, 75%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.04 (d, $J = 2.1$ Hz, 2H, ArH), 7.98 (dd, $J = 9.4$, 2.3 Hz, 4H, ArH), 7.40 (dd, $J = 9.4$, 2.7 Hz, 2H, ArH), 7.30 – 7.26 (m, 4H, ArH), 7.18 (d, $J = 2.7$ Hz, 2H, ArH), 3.94 (s, 6H, CH$_3$), 3.66 (t, $J = 7.2$ Hz, 4H, CH$_2$), 2.31 – 2.24 (m, 4H, CH$_2$), 2.19 (s, 3H, CH$_3$), 1.72 (p, $J = 7.3$ Hz, 4H, CH$_2$), 1.46 – 1.36 (m, 8H, CH$_2$), 1.34 – 1.15 (m, 30H, CH$_2$). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 156.09, 149.90, 148.54, 146.95, 134.85, 131.67, 128.41, 124.59, 124.56, 124.16, 118.03, 115.97, 99.31, 58.13, 55.64, 50.90, 42.52, 31.95, 29.76, 29.70, 29.64, 29.61, 29.47, 27.77, 27.52, 27.06. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2925, 2852, 2793, 1631, 1465, 1236. HRMS (ESI) C$_{51}$H$_{68}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{45}$H$_{69}$Cl$_2$N$_5$O$_2$, 852.4672; found: 852.4634.
Synthesis of longer linked dimeric chloroquine inhibitors

To a flame-dried round bottom flask, bishexamethylene triamine (TCI America) (51) (3.799 g, 17.64 mmol, 1.0 eq.), 4-bromo-7-chloroquinoline (13) (9.43, 38.8 mmol, 2.2 eq.), Pd(OAc)$_2$ (199 mg, 0.71 mmol, 0.05 eq.), BINAP (1098 mg, 1.41 mmol, 0.1 eq.), K$_3$PO$_4$ (11.27 g, 52.9 mmol, 3.0 eq.) was added. The reagents were placed under an argon atmosphere and dissolved in 1,4-dioxane (44 mL) to give a 0.4M reaction concentration. The reaction was then heated in an oil bath preset to 105 °C under a reflux condenser. The reaction was monitored by $^1$H NMR (CDCl$_3$) where the consumption of primary amine-containing linker marks complete consumption of the limiting reagent (peak at ~2.7 ppm). The reaction was then cooled to 23 °C and filtered through Celite™ using 300 mL of a 4:1 mixture of CHCl$_3$:MeOH. The solution was concentrated under reduced pressure to afford an orange-yellow solid (13.37 g), which was recrystallized from CH$_2$Cl$_2$ (heated to reflux and then cooled to -20 °C) to afford a white solid (8.516 g, 90%), the structure (68) of which was verified by $^1$H NMR. TLC ($R_f$ = 0.25, 90:10:1, CH$_2$Cl$_2$, MeOH, NH$_4$OH). Melting point (mp) = 100 – 102 °C; $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 1.38 – 1.55 (12H, m), 1.73 – 1.78 (4H, m), 2.55 (4H, t, $J$ = 7.5 Hz), 3.36 (4H,
t, $J = 7.0$ Hz), 6.50 (2H, d, $J = 5.5$ Hz), 7.38 (2H, dd, $J = 2.0$, 9.0 Hz), 7.76 (2H, d, $J = 2.0$ Hz), 8.09 (2H, d, $J = 9.0$ Hz), 8.34 (2H, d, $J = 5.5$ Hz). $^{13}$C NMR (125 MHz, CD$_3$OD): δ 26.7 (2C), 27.9, 28.8, 42.5, 49.1, 98.2, 117.4, 122.9, 124.5, 126.2, 134.8, 148.3, 151.0 (2C). FTIR (thin film) $\tilde{\nu}$ (cm$^{-1}$): 3054, 1420, 1265, 896. HRMS (ESI) C$_{30}$H$_{37}$Cl$_2$N$_5$: Calculated for [M+H] C$_{30}$H$_{38}$Cl$_2$N$_5$, 538.2507; found: 538.2509.

**DC661**

Compound 68 (7.02 g, 13.0 mmol, 1.0 eq.) was added to a round bottom flask, under a reflux condenser and placed under an atmosphere of argon. Formic acid (HCOOH, 19.3 mL) was added to the reaction vessel to give a 0.3M reaction concentration. While stirring, aqueous formaldehyde (37 % w/w) (0.95 mL, 12.75 mmol, 2.2 eq.) was added to the reaction vessel via a syringe. The reaction was then heated to reflux in an oil bath preset to 105 °C. The reaction was stirred at reflux until the starting material (68) was observed by TLC (90:10:1, CH$_2$Cl$_2$, MeOH, NH$_4$OH, $R_f = 0.25$) to be consumed. The formic acid was diluted with water (300 mL) resulting in a black aqueous solution. The pH of the solution was brought to 12 using concentrated NH$_4$OH (50 mL), and then extracted with CHCl$_3$ (3 x 100 mL). Upon extraction, a gray precipitate forms which was identified by $^1$H NMR to be mostly product. The solid (1.1g) was collected via vacuum filtration of the remaining aqueous layer. The combined organic layers were
dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a gray-brown foam (5.4g). The foam was dissolved in CH₂Cl₂, and stirred with decolorizing carbon (2.0 g) for 45 min. The carbon was then removed by filtration through a plug of Celite™, using CH₂Cl₂ (100 mL). The gray solid was dissolved in a 4:1 mixture of CHCl₃:MeOH (100 mL) and stirred with decolorizing carbon (1.0 g) for 45 min. The carbon was then removed by filtration through a plug of Celite™. The filtered carbon was washed using CH₂Cl₂ (100 mL). The combined filtrates were then concentrated under reduced pressure to afford a beige-white crude solid. Purification was affected by recrystallization from a three-solvent system, where boiling CH₂Cl₂ yields a slightly cloudy solution, which was not clarified by the addition of more boiling CH₂Cl₂. To this solution methanol was added dropwise stirring until the boiling solution clarifies. The solution was then cooled to 20 °C, diethyl ether was then added until a droplet caused the solution to turn cloudy. The solution was cooled to -20°C for 24 hours to yield white circular crystals which were collected via vacuum filtration (4.508g, 63%), the structure (69) of which was verified by ¹H NMR. TLC (R_f = 0.35 (EtOAc:MeOH:TEA; 80:15:5)). MP (CH₂Cl₂/Et₂O): 58 – 60 °C. ¹H NMR (500 MHz, CD₃OD): δ 1.35 – 1.47 (12H, m, CH₂), 1.69 – 1.73 (4H, m, CH₂), 2.17 (3H, s, CH₃), 2.29 – 2.31 (4H, m, CH₂), 3.30 (4H, s(br), CH₂), 6.45 (2H, s(br), ArH), 7.34 – 7.37 (2H, m, ArH), 7.75 (2H, s, ArH), 8.06 – 8.08 (2H, m, ArH), 8.32 (2H, s(br), ArH). ¹³C NMR (125 MHz, CD₃OD): δ 26.2, 26.6, 27.0, 27.9, 41.0, 42.5, 57.1, 98.2, 117.4, 122.9, 124.5, 126.2, 134.8, 148.3, 151.0, 151.3. FTIR (thin film) λ (cm⁻¹): 3683, 3455, 3019, 2936, 2859, 1582, 852. HRMS (ESI) C₃₁H₃₉Cl₂N₅: Calculated for [M+H] C₃₁H₄₀N₅Cl₂, 552.2655; found: 552.2654.
DC661 (Water Soluble Salt)

DC661 (69) (126 mg, 0.23 mmol, 1.0 eq) was added to a round bottom flask. MeOH (3 mL) was added to dissolve DC661, and the solution was stirred at 23 °C. A solution of HCl in methanol (3 mL, 0.25 M) was added to the reaction vessel, and the resulting solution was stirred for 1 hour at 23 °C. The solvent and excess HCl was removed by concentration under reduced pressure to afford a yellow foam (150 mg, 99%). The foam was found to be a hygroscopic solid, which must be stored under a dry inert atmosphere. Attempts to determine a melting point result in phase transition of the foam to a paste.\(^1\)\(^{1}\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 8.45 (d, \(J = 9.1\) Hz, 2H), 8.35 (d, \(J = 7.1\) Hz, 2H), 7.83 (d, \(J = 2.1\) Hz, 2H), 7.60 (dd, \(J = 9.1, 2.1\) Hz, 2H), 6.86 (d, \(J = 7.2\) Hz, 2H), 3.58 (t, \(J = 7.3\) Hz, 4H), 3.25 – 3.02 (m, 4H), 2.83 (s, 3H), 1.87 – 1.70 (m, 8H), 1.57 – 1.40 (m, 8H).\(^{1}\)\(^{1}\)C NMR (126 MHz, CD\(_3\)OD) \(\delta\) 157.47, 143.64, 140.84, 139.91, 128.53, 126.26, 120.18, 116.83, 99.81, 57.23, 44.69, 40.44, 28.80, 27.35, 27.16, 25.08. FTIR (thin film) \(\lambda\) (cm\(^{-1}\)): 3220, 3020, 2938, 2729, 1612, 1591, 1453, 1212. HRMS (ESI) C\(_{31}\)H\(_{42}\)Cl\(_5\)N\(_5\): Calculated for [M+H-3HCl] C\(_{31}\)H\(_{40}\)N\(_5\)Cl\(_2\), 552.2661; found: 552.2656. Purity of the final compound was determined by UPLC-MS analysis. DC661-3HCl was found to be a single peak, with a mass corresponding to the reported literature precedent, [M+2H-3HCl]/2 = 277.8.
To a screw cap vial, 11,11 triamine (47) (178 mg, 0.5 mmol, 1.0 eq.) was added, followed by 4-bromo-7chloroquinoline\textsuperscript{156} (13) (267 mg, 1.1 mmol, 2.2 eq.), Pd(OAc)\textsubscript{2} (6 mg, 0.03 mmol, 0.05 eq.), BINAP (32 mg, 0.05 mmol, 0.1 eq.) and K\textsubscript{3}PO\textsubscript{4} (320 mg, 1.5 mmol, 3.0 eq.). The reaction is placed under an atmosphere of argon and 1,4-dioxane (1.25 mL) was added. The vial was sealed and heated to 120 °C until the reaction was complete. The reaction was deemed complete when all the primary amine starting material was consumed as observed by \textsuperscript{1}H NMR (~2.7 ppm). The reaction was cooled to room temperature and filtered through a pad of Celite\textsuperscript{TM} with a mixture of 1:1 CHCl\textsubscript{3}:MeOH (ca. 50 mL). The filtrated was concentrated under reduced pressure to afford a crude paste (250 mg) which was purified by column chromatography (SiO\textsubscript{2}; dry load on 300 mg SiO\textsubscript{2}, 25 mm x 15 mm; 5:2:93, MeOH:TEA:EtOAc) to afford an off-white solid (69) (52 mg, 16%). The structure of the product was verified by \textsuperscript{1}H NMR. TLC (R\textsubscript{f} = 0.45, 10:90:1, MeOH:CH\textsubscript{2}Cl\textsubscript{2}:NH\textsubscript{4}OH). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ 8.52 (d, J = 5.3 Hz, 2H), 7.94 (d, J = 2.2 Hz, 2H), 7.67 (d, J = 9.0 Hz, 2H), 7.34 (dd, J = 8.9, 2.2 Hz, 2H), 6.40 (d, J = 5.4 Hz, 2H), 5.07 (d, J = 5.4 Hz, 2H), 3.29 (td, J = 7.2, 5.1 Hz, 4H), 2.61 (t, J = 7.4 Hz, 4H), 1.74 (p, J = 7.3 Hz, 4H), 1.55 – 1.19 (m, 36H). \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}) δ 152.19, 149.93, 149.30, 134.98, 128.95, 125.38, 121.07, 117.31, 99.23, 50.10, 43.45, 29.91, 29.65, 29.63, 29.62, 29.47, 29.04, 27.50, 27.28. FTIR (thin film) λ (cm\textsuperscript{-1}):
3277, 2924, 2851, 1611, 1578, 1536, 1456, 1373, 1135. HRMS (ESI) C_{40}H_{57}Cl_{2}N_{5}:

Calculated for [M+H] C_{40}H_{58}Cl_{2}N_{5}, 678.4069; found: 678.4076.
DC11110 (71) (25 mg, 0.037 mmol, 1.0 equiv.) is added to a round bottom flask and placed under an atmosphere of argon. The flask contents were dissolved in CH$_2$Cl$_2$ (750 μL). Aqueous formaldehyde is added and the reaction is stirred at 23 °C. Sodium triacetoxyborohydride (78 mg, 0.37, 10.0 equiv.) was added to the flask in one portion, and the reaction was stirred until the starting inhibitor was consumed as observed via TLC ($R_f = 0.45$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). The reaction was quenched by first dilution with CH$_2$Cl$_2$ (5 mL) and 2N NaOH (5 mL). The reaction was stirred for 60 minutes to quench any excess reducing agent. The layers of the resulting biphasic mixture were separated, and the aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated to a film on a vial (72) (19 mg, 74%). The structure of the product was verified by $^1$H NMR. TLC ($R_f = 0.70$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.52 (d, $J = 5.3$ Hz, 2H), 7.95 (d, $J = 2.2$ Hz, 2H), 7.65 (d, $J = 8.9$ Hz, 2H), 7.35 (dd, $J = 8.9$, 2.2 Hz, 2H), 6.40 (d, $J = 5.4$ Hz, 2H), 5.00 (s, 2H), 3.29 (td, $J = 7.2$, 5.2 Hz, 4H), 2.34 – 2.27 (m, 4H), 2.21 (s, 3H), 1.79 – 1.70 (m, 4H), 1.51 – 1.40 (m, 8H), 1.40 – 1.22 (m, 23H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 152.21, 149.82, 149.28, 134.92, 129.00, 125.36, 120.93, 117.23, 99.20, 58.05, 53.57, 43.42, 42.45, 29.74, 29.69, 29.64, 29.48, 29.03,
FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 3273, 2924, 2851, 1578, 1540, 1456, 1364, 1126. HRMS (ESI) \( \text{C}_{41}\text{H}_{59}\text{Cl}_{2}\text{N}_{5} \): Calculated for [M+H] \( \text{C}_{41}\text{H}_{60}\text{Cl}_{2}\text{N}_{5} \), 692.4226; found: 692.4242.

The bisazide (39) (920 mg, 2.2 mmol, 1.0 eq.) was added to a round bottom flask and dissolved in THF (4 mL). Water (500 uL, mmol, eq.) was added via a syringe, and the reaction was heated in an oil bath at 50 °C for 16 hours. The reaction was observed to be complete by LC-MS, where no bis- or mono-azide was found. The reaction was then stirred with 2N HCl (5 mL) to form the ammonium salt of the product. The layers of the biphasic mixture were separated. The resulting organic layer was extracted with 0.5 N HCl (3 x 10 mL). The combined aqueous layers were extracted with CH\(_2\)Cl\(_2\) (2 x 30 mL) to remove all traces of triphenylphosphine oxide in the aqueous layer. The aqueous layer was saturated with solid NaCl, and the pH brought to 12 by the addition of NH\(_4\)OH (ca. 10 mL). The aqueous layer was then extracted with CH\(_2\)Cl\(_2\) (3 x 30 mL). The resulting organic layers were combined, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to afford an off white waxy solid (73) (679 mg, 85%). The structure of the resulting product was verified by \(^1\)H NMR. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \)

7.26 – 7.19 (m, 4H), 7.15 (d, \( J = 6.8 \) Hz, 1H), 3.46 (s, 2H), 3.35 (d, \( J = 2.7 \) Hz, 4H), 2.60 (t, \( J = 7.1 \) Hz, 4H), 2.35 – 2.25 (m, 4H), 2.10 (s, 6H), 1.37 (d, \( J = 7.8 \) Hz, 8H), 1.28 – 1.13 (m, 16H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 140.27, 128.93, 128.12, 126.67, 58.73,
53.90, 50.17, 42.15, 33.54, 29.62, 29.55, 27.49, 27.06, 26.93. IR (thin film) $\lambda$ (cm$^{-1}$):

2927, 2851, 2360, 1604, 1468. HRMS (ESI) $\text{C}_{23}\text{H}_{43}\text{N}_3$: Calculated for $[\text{M+H}]$ $\text{C}_{23}\text{H}_{44}\text{N}_3$, 362.3535; found: 362.3516.
To a screw cap vial, diamine (73) (99mg, 0.3 mmol, 1.0 eq.) was added, followed by 4-bromo-7chloroquinoline (13) (160mg, 0.66 mmol, 2.2 eq.), Pd(OAc)$_2$ (97mg, 0.03 mmol, 0.05 eq.), BINAP (38mg, 0.06mmol, 0.1 eq.) and K$_3$PO$_4$ (192 mg, 0.9 mmol, 3.0 eq.). The reaction is placed under an atmosphere of argon and 1,4-dioxane (0.75 mL) was added. The vial was sealed and heated to 105 °C for 8 hours until the reaction was complete. The reaction was deemed complete when all the primary amine starting material was consumed as observed by $^1$H NMR. The reaction was cooled to room temperature and filtered through a pad of Celite™ with CH$_2$Cl$_2$ (3 mL). The filtrate was concentrated under reduced pressure to afford a crude paste (270 mg). The crude product was purified by column chromatography (SiO$_2$; 25 mm x 15 mm; 2:98:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH) to afford an off-white solid (74) (107 mg, 65%). The structure of the product was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.52 (d, $J$ = 5.4 Hz, 2H), 7.96 (d, $J$ = 2.2 Hz, 2H), 7.70 (d, $J$ = 8.9 Hz, 2H), 7.40 – 7.27 (m, 6H), 7.25 – 7.21 (m, 2H), 6.40 (d, $J$ = 5.5 Hz, 2H), 5.13 (s, 2H), 3.59 (s, 2H), 3.30 (td, $J$ = 7.2, 5.2 Hz, 4H), 2.50 – 2.38 (m, 4H), 1.72 (q, $J$ = 7.4 Hz, 11H), 1.55 – 1.22 (m, 22H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 152.24, 149.87, 149.36, 140.45, 134.98, 129.06, 128.95, 128.21, 126.78, 125.41, 120.99, 117.30, 99.24, 58.83, 54.00, 43.43, 29.58, 29.48, 29.03, 27.49, 27.25, 27.23. IR (thin film) λ (cm$^{-1}$):2925, 1577, 1541, 1456, 1366. HRMS (ESI) C$_{41}$H$_{51}$Cl$_2$N$_3$: Calculated for [M+H] C$_{41}$H$_{52}$Cl$_2$N$_3$, 684.3600; found: 684.3591.
The bisazide (39) (208 mg, 0.5 mmol, 1.0 eq.) was to a screwcap vial, followed by NaHCO₃ (21 mg, 0.6 mmol, 1.0 eq.) and toluene (700 uL). Methyl chloroformate was added to the reaction, and the vial was sealed under an atmosphere of argon. The reaction was heated to 85 °C in an oil bath for 36 hours, until the reaction was complete. The reaction was observed to be complete by the loss of UV active starting material, as observed by TLC (Rₓ = 0.3; 8:1, Hex:EtOAc). The reaction was then concentrated under reduced pressure to afford a crude paste which was purified by column chromatography (SiO₂; 25 mm x 110 mm; 6:1, Hex:EtOAc). The product was isolated as an oil (75) (187 mg, 97%), the structure of which was verified by ¹H NMR. The ¹³C NMR contains a mixture of two rotamers which have split the signal of two methylenes. ¹H NMR (500 MHz, CDCl₃) δ 3.68 (s, 3H), 3.26 (t, J = 6.8 Hz, 4H), 3.19 (s, 4H), 1.59 (h, J = 6.7 Hz, 4H), 1.56 – 1.47 (m, 4H), 1.44 – 1.22 (m, 16H). ¹³C NMR (126 MHz, CDCl₃) δ 157.00, 52.44, 51.61, 47.60, 47.02, 29.38, 29.23, 28.97, 28.73, 28.28, 26.86, 26.80. FTIR (thin film) λ (cm⁻¹): 2929, 2857, 2090, 1699, 1477, 1438, 1408, 1350, 1253, 1191, 1134, 1090, 1035. HRMS (ESI) C₁₈H₃₅N₇O₂: Calculated for [M+H] C₁₈H₃₆N₇O₂, 382.2930; found: 382.2190.
Bisazide (75) (210 mg, 0.55 mmol, 1.0 eq.) was added to a round bottom flask and dissolved in THF (1 mL). Triphenylphosphine (286 mg, 1.1 mmol, 2.0 eq.) was added to the solution in two portions, and gas evolution was observed after each addition. Water (400 μL) was added to the reaction via a syringe and the reaction was stirred at 23 °C for 2.5 hours, until the starting bis-azide was observed to be consumed by LC-MS. The reaction was concentrated under reduced pressure to remove THF. The crude paste was then dissolved in CH₂Cl₂ (10 mL) and extracted with 0.2 N HCl (2 x 5 mL). The combined organic layers were then washed with CH₂Cl₂ (2 x 10 mL) to ensure the removal of triphenylphosphine oxide. The aqueous layer was saturated with NaCl and the pH was raised to 12 by the addition of concentrated NH₄OH (ca. 3 mL). The basic aqueous solution was then extracted with CHCl₃ (3 x 5 mL) and the combined organic layers were dried over anhydrous sodium sulfate. The dried organic fraction was concentrated under reduced pressure to afford the product as a paste (76) (154 mg, 86%), the structure of which was verified by ¹H NMR. The ¹³C NMR contains a mixture of two rotamers which have split the signal of two methylenes. ¹H NMR (500 MHz, CDCl₃) δ 3.65 (s, 3H), 3.22 – 3.09 (m, 4H), 2.66 (t, J = 7.0 Hz, 4H), 1.54 – 1.19 (m, 30H). ¹³C NMR (126 MHz, CDCl₃) δ 157.02, 52.43, 47.64, 47.06, 42.38, 33.93, 29.58, 29.53, 28.75, 28.30, 26.99, 26.94. IR (thin film) λ (cm⁻¹): 3362, 2925, 2854, 1704, 1575, 1478,
1408, 1309, 1305, 1196, 1092. HRMS (ESI) C$_{18}$H$_{39}$N$_{3}$O$_{2}$: Calculated for [M+H]
C$_{18}$H$_{40}$N$_{3}$O$_{2}$, 330.3121; found: 330.3112.
To a screw cap vial, diamine (76) (99 mg, 0.3 mmol, 1.0 eq.) was added, followed by 4-bromo-7-chloroquinoline 13 (160 mg, 0.66 mmol, 2.2 eq.), Pd(OAc)$_2$ (97 mg, 0.03 mmol, 0.05 eq.), BINAP (38 mg, 0.06 mmol, 0.1 eq.) and K$_3$PO$_4$ (192 mg, 0.9 mmol, 3.0 eq.). The reaction is placed under an atmosphere of argon and 1,4-dioxane (0.75 mL) was added. The vial was sealed and heated to 105 °C for 8 hours until the reaction was complete. The reaction was deemed complete when all the primary amine starting material was consumed as observed by $^1$H NMR. The reaction was cooled to room temperature and filtered through a pad of Celite™ with CH$_2$Cl$_2$ (3 mL). The filtrate was concentrated under reduced pressure to afford a crude paste (270 mg). The crude product was purified by column chromatography (SiO$_2$; 25 mm x 15 mm; 2:98:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH) to afford an off-white foam (77) (107 mg, 65%). The structure of the product was verified by $^1$H NMR. The $^{13}$C NMR was observed to contain a mixture of rotamers which suppressed the signal of two methylenes. $^1$H NMR (CDCl$_3$) $\delta$ 8.52 (d, $J = 5.3$ Hz, 2H), 7.97 (d, $J = 2.2$ Hz, 2H), 7.68 (d, $J = 8.9$ Hz, 2H), 7.35 (dd, $J = 8.9$, 2.2 Hz, 2H), 6.41 (d, $J = 5.3$ Hz, 2H), 5.07 (s, 2H), 3.67 (s, 3H), 3.32 (q, $J = 6.5$ Hz, 4H), 3.20 (t, $J = 7.5$ Hz, 4H), 1.76 (p, $J = 7.3$ Hz, 4H), 1.60 – 1.22 (m, 20H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.07, 151.96, 150.05, 149.08, 135.12, 128.80, 125.47, 121.15, 117.28, 99.20, 52.52, 43.42, 29.39, 28.98, 27.20, 26.85. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2915,
1693, 1581, 1479, 1452, 1122. HRMS (ESI) C$_{36}$H$_{47}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{36}$H$_{48}$Cl$_2$N$_5$O$_2$, 652.3185; found: 652.3173.
The carbamate (77) (108 mg, 0.165 mmol, 1.0 eq.) was added to a round bottom flask and dissolved in HBr/AcOH (33% HBr) (v/v) (500 uL). The reaction was heated at reflux until the starting material was consumed as observed by TLC (starting material R_f = 0.25; 2:98:1, MeOH:CH₂Cl₂:NH₄OH). The reaction was complete at 45 minutes and allowed to cool to room temperature. The reaction was then poured onto 0.1N NH₄OH (20 mL) to deprotonate the product. The aqueous phase was then extracted with a mixture of CHCl₃ and MeOH (3:1, 3 x 10 mL). The combined organic layers were washed once with brine (30 mL) and dried over anhydrous sodium sulfate. The resulting organic layer was decanted and concentrated under reduced pressure to afford a crude paste. The crude material was then purified by column chromatography (SiO₂; 20 mm x 200 mm; 7:92:1, MeOH: CH₂Cl₂:NH₄OH) yielding a tan-white solid (47 mg, 48%). The structure of the isolated product (78) was verified ¹H NMR. ¹H NMR (500 MHz, CDCl₃) δ 8.51 (d, J = 5.3 Hz, 2H), 7.94 (d, J = 2.2 Hz, 2H), 7.67 (d, J = 8.9 Hz, 2H), 7.33 (dd, J = 8.9, 2.2 Hz, 2H), 6.39 (d, J = 5.4 Hz, 2H), 5.08 (t, J = 5.2 Hz, 2H), 3.27 (td, J = 7.2, 5.1 Hz, 4H), 2.59 (t, J = 7.3 Hz, 4H), 1.73 (p, J = 7.3 Hz, 4H), 1.54 – 1.27 (m, 21H). ¹³C NMR (126 MHz, CDCl₃) δ 152.22, 149.89, 149.34, 134.94, 129.00, 125.36, 121.06, 117.31, 99.23, 50.20, 43.41, 30.14, 29.56, 29.40, 29.01, 27.44, 27.21. FTIR (thin film) λ (cm⁻¹): 2926, 2853,
1610, 1578, 1541, 1451, 1367, 1331, 1281, 1135, 1079. HRMS (ESI) C$_{34}$H$_{45}$Cl$_2$N$_5$:

Calculated for [M+H] C$_{34}$H$_{46}$Cl$_2$N$_5$, 594.3130; found: 594.3149.
DC880 (78) (24 mg, 0.04 mmol, 1.0 eq) was added to a screwcap vial followed by HCOOH (270 uL) and CH₂O (10 uL, 37% aqueous, 0.12 mmol, 3.0 eq.). The reaction was sealed and heated to reflux in an oil bath, preset to 105 °C, until the starting material is observed to be consumed by TLC (DC880 Rf = 0.5; 7:92:1, MeOH:CH₂Cl₂:NH₄OH). The reaction was cooled to room temperature and diluted with CHCl₃ (1 mL) and 2 N NaOH (500 uL). The reaction was stirred for 60 minutes at room temperature to quench excess sodium triacetoxyborohydride. The resulting biphasic mixture was separated, and the aqueous layer was extracted with CHCl₃ (3 x 1 mL). The combined organic layers were dried over anhydrous sodium sulfate, decanted, and concentrated under reduced pressure to afford a crude paste. The resulting crude product was purified by filtration through a plug of silica, first with CH₂Cl₂ (5 mL), followed by an elution gradient of (2 mL each: 7:93:1, MeOH:CH₂Cl₂:NH₄OH; 10:90:1, MeOH:CH₂Cl₂:NH₄OH; 15:85:1, MeOH:CH₂Cl₂:NH₄OH). The product was isolated as a translucent film on a vial (79) (36 mg, 76%). The structure of the product was verified by ¹H NMR. ¹H NMR (500 MHz, CDCl₃) δ 8.52 (d, J = 5.4 Hz, 2H), 7.95 (d, J = 2.2 Hz, 2H), 7.65 (d, J = 8.9 Hz, 2H), 7.34 (dd, J = 8.9, 2.3 Hz, 2H), 6.40 (d, J = 5.4 Hz, 2H), 5.01 (t, J = 5.3 Hz, 2H), 3.28 (td, J = 7.3, 5.1 Hz, 4H), 2.30 (dd, J = 8.8, 6.4 Hz, 4H), 2.20 (s, 3H), 1.73 (p, J = 7.4 Hz, 4H), 1.51 – 1.20 (m, 22H). ¹³C NMR (126 MHz, CDCl₃) δ 152.26, 149.86, 149.37, 134.97,
In a flame-dried round bottom flask under an argon atmosphere, a solution of freshly distilled oxalyl chloride (2.0 mL, 23.0 mmol, 1.2 eq.) in CH$_2$Cl$_2$ (95 mL), was cooled to -78°C. DMSO (3.25 mL, 45.8 mmol, 2.4 eq.) was added to the flask, causing the evolution of gas. The reaction mixture was stirred for 30 min at -78°C, then a solution of 7-azidoheptanol$^{158}$ (80) (3.00 g, 19.1 mmol, 1.0 eq.) in EtOAc was added slowly. The resulting solution was stirred at -78°C for 1 hour, at which time freshly distilled triethylamine (13.3 mL, 96.0 mmol, 5.0 eq.) was added to the reaction. The resulting mixture was slowly warmed to 23 °C and stirred at 23 °C for 16 hours. The progress of the reaction was determined via TLC by consumption of the starting alcohol (TLC R$_f$ = 0.5, EtOAc) and the appearance of a new TLC spot for the aldehyde product (R$_f$ = 0.8,
EtOAc). The reaction mixture was then washed with saturated aqueous NH₄Cl (3 x 20 mL), brine (1 x 20 mL), and the resulting organic solution dried over anhydrous Na₂SO₄. The filtrate was concentrated under reduced pressure yielding a crude oil, the structure of the product was verified by ¹H NMR. Due to stability concerns, the crude aldehyde was used without further purification in the next reaction.

A solution of the resulting 7-azidoheptanal (2.3 g, 14.2 mmol, 1.2 eq.) in CH₂Cl₂ (35 mL) was added to a round bottom flask. The solution was stirred under an atmosphere of argon and 2,4-dimethoxybenzylamine 81 (1.1 mL, 7.0 mmol, 1.0 eq.) was added to the reaction via a syringe. The reaction was stirred for 10 minutes to allow imine intermediates to form. Sodium triacetoxyborohydride was added in one portion and the reaction was stirred at 23 °C until the starting aldehyde was consumed as observed by TLC (Rf = 0.8, EtOAc). The reaction was then quenched by dilution with CH₂Cl₂ (30 mL), followed by the addition of 2N NaOH (30 mL). The reaction was stirred for 60 min. The layers of the biphasic mixture were separated, and the aqueous layer was extracted with CH₂Cl₂ (3 x 20 mL). The combined organic layers were dried over sodium sulfate, concentrated under reduced pressure, and purified by column chromatography (SiO₂, 45 mm x 150 mm, 1:1 Hexanes:EtOAc). Purification yields a golden oil (82) (1.25 g, 39%), the structure of which was verified by ¹H NMR. TLC (Rf = 0.3, 1:1 Hex:EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.26 (t, J = 4.1 Hz, 1H), 6.51 – 6.41 (m, 2H), 3.79 (d, J = 8.3 Hz, 6H), 3.50 (s, 2H), 3.24 (t, J = 7.0 Hz, 4H), 2.45 – 2.36 (m, 4H), 1.58 (p, J = 7.0 Hz, 4H), 1.47 (p, J = 7.2 Hz, 4H), 1.41 – 1.23 (m, 12H). ¹³C NMR (126 MHz, CDCl₃) δ 159.71, 158.80, 130.88, 103.99, 98.42 (2C), 55.49, 55.47, 54.09, 51.62, 51.59, 29.26,
28.96, 27.52, 27.12, 26.89. FTIR (thin film) λ (cm⁻¹): 2931, 2855, 2094, 1612, 1588, 1506, 1464, 1289, 1259, 1207, 1156, 1039.

HRMS (ESI) C₂₃H₃₉N₇O₂: Calculated for [M+H] C₂₃H₄₀N₇O₂, 446.3243; found: 446.3228.
Bisazide (82) (1.00g, 2.25 mmol, 1.0 equiv.) was added to a round bottom flask and dissolved in THF (4.5 mL). Next, triphenylphosphine (1.18 g, 4.5 mmol, 2.0 equiv.) was added to the round bottom flask in one portion, and gas evolution from the solution was observed. The solution was heated under an atmosphere of argon to 50 °C, and water (1.2 ml, 68 mmol, 30 equiv.) was added to the reaction vessel via a syringe. The reaction was heated for 16 hours until complete, where no bis- or mono-azide was observed via LC-MS of the crude reaction mixture. The reaction was concentrated under reduced pressure and the resulting oil was dissolved in EtOAc (25 mL). Water was added to the solution causing a biphasic mixture. Dilute acetic acid (3% aq., 10 mL) was added to lower the pH of the aqueous layer. The layers were separated, and the resulting organic layer was extracted with dilute acetic acid (3% aq., 2x 25 mL). The resulting combined aqueous layers were extracted with EtOAc (2 x 10 mL) to remove any remaining triphenylphosphine oxide. The aqueous layer was saturated with sodium sulfate, and the pH of the resulting solution was raised to 12 by the addition of ammonium hydroxide (ca. 3 mL). The aqueous layer was extracted with EtOAc (2x 10 mL) and the combined organic layers were dried over anhydrous sodium sulfate. The organic solution was decanted and concentrated under reduced pressure to yield a golden yellow oil (83) (776 mg, 88%), the structure of which was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$)
δ 7.25 (s, 1H), 6.47 – 6.39 (m, 2H), 3.78 (d, J = 8.7 Hz, 6H), 3.49 (s, 2H), 2.66 (t, J = 7.0 Hz, 3H), 2.38 (t, J = 7.5 Hz, 4H), 1.91 – 1.71 (m, 4H), 1.42 (dq, J = 14.0, 7.3 Hz, 4H), 1.27 (s, 14H). 13C NMR (126 MHz, CDCl3) δ 159.67, 158.79, 130.88, 120.71, 104.00, 98.40, 55.49, 55.47, 54.17, 51.56, 42.33, 33.83, 29.63, 27.70, 27.16, 27.07. FTIR (thin film) λ (cm⁻¹): 3357, 2928, 2854, 1612, 1587, 1505, 1465. HRMS (ESI) C23H43N3O2: Calculated for [M+H] C23H44N3O2, 394.3434; found: 394.3448.
To a flame-dried round bottom flask, triamine (83) (393 mg, 1.0 mmol, 1.0 eq.), 4-bromo-7-chloroquinoline\(^{156}\) (13) (535 mg, 2.2 mmol, 2.2 eq.), Pd(OAc)\(_2\) (11 mg, 0.05 mmol, 0.05 eq.), BINAP (62 mg, 0.1 mmol, 0.1 eq.), K\(_2\)PO\(_4\) (640 mg, 52.92 mmol, 3.0 eq.) were added. The reagents were placed under an argon atmosphere and dissolved in 1,4-dioxane (44 mL) to give a 0.4 M reaction concentration. The reaction was then heated to 105 °C under in a sealed vessel. The reaction was monitored by \(^1\)H NMR (CDCl\(_3\)) where the consumption of primary amine-containing linker marks complete consumption of the limiting reagent (peak at ~2.7 ppm). The reaction was then cooled to 23 °C and filtered through Celite\(^{TM}\) using EtOAc (10 mL). The solution was concentrated under reduced pressure to afford a paste which was dissolved in CH\(_2\)Cl\(_2\) (20 mL). Water (10 mL) and 1N HCl (3 mL) were added to the CH\(_2\)Cl\(_2\) and stirred for 60 minutes to form the tris-HCl salt of the product. The layers of the biphasic mixture were separated, and the resulting aqueous layer was washed with CH\(_2\)Cl\(_2\) (3 x 10 mL) to ensure the removal excess ligand and bromo-quinoline starting materials. The pH of the aqueous solution was adjusted to 12 with 2N NaOH (6 mL). The resulting aqueous layer was extracted with CHCl\(_3\) (3 x 25 mL), and the combined aqueous layers were dried over anhydrous
sodium sulfate. The organic solution was decanted and concentrated under reduced pressure to afford a paste which was purified by column chromatography (SiO₂, 35 mm x 120 mm, 5:94:1, MeOH: CH₂Cl₂:NH₄OH, gradient to 7:93, MeOH: CH₂Cl₂). The product was isolated as a golden solid-foam (84) (512 mg, 72%), the structure of which was verified by ¹H NMR. TLC (Rₐ = 0.45, 7:93, MeOH: CH₂Cl₂). ¹H NMR (500 MHz, CDCl₃) δ 8.50 (dd, J = 5.4, 2.2 Hz, 2H), 7.93 (t, J = 2.3 Hz, 2H), 7.69 (dd, J = 8.9, 2.2 Hz, 2H), 7.32 (dt, J = 8.9, 2.3 Hz, 2H), 7.28 (dd, J = 8.3, 2.1 Hz, 1H), 6.48 – 6.34 (m, 4H), 5.16 (s, 2H), 3.76 (d, J = 2.3 Hz, 6H), 3.55 (s, 2H), 3.26 (dt, J = 7.3, 4.9 Hz, 4H), 2.50 – 2.36 (m, 4H), 1.75 – 1.65 (m, 4H), 1.55 – 1.21 (m, 18H). ¹³C NMR (126 MHz, CDCl₃) δ 159.97, 158.86, 152.15, 149.95, 149.29, 134.95, 131.14, 128.89, 125.34, 121.20, 117.32, 104.17, 99.17, 98.43, 55.53, 55.49, 53.96, 51.56, 43.36, 29.31, 28.91, 27.44, 27.21, 26.87. FTIR (thin film) λ (cm⁻¹): 2926, 2851, 2611, 1578, 1537, 1504, 1455,1370,1329, 1288,1207,1133.1037. HRMS (ESI) C₄₁H₅₁Cl₂N₅O₂: Calculated for [M+H] C₄₁H₅₂Cl₂N₅O₂, 716.3498; found: 716.3497.
Compound 84 (42 mg, 0.06 mmol 1.0 equiv.) was added to a microwave vial and dissolved in TFA (2 mL), water (350 μL), and triisopropylsilane (350 μL). The reaction was sealed and placed under an atmosphere of argon. The sealed reaction was heated to 130 °C under microwave irradiation for 3 hours. The reaction was considered complete when starting inhibitor was consumed as observed by LC-MS. The reaction was cooled to room temperature and the solvent was removed under reduced pressure. The crude paste was dissolved in CHCl₃ (10 mL) and washed with NH₄OH (2 x3 mL) to create the free base of the crude product. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield a crude paste. The crude material was then purified by column chromatography (SiO₂, 15 x 130 mm, 10:90:1, MeOH: CH₂Cl₂:NH₄OH) to afford the product as a film on a vial (85) (24 mg, 72%). The structure of the resulting product was verified by ¹H NMR. ¹H NMR (500 MHz, CDCl₃) \( \delta \) 8.51 (d, \( J = 5.4 \) Hz, 2H), 7.93 (d, \( J = 2.1 \) Hz, 2H), 7.68 (d, \( J = 8.9 \) Hz, 2H), 7.33 (dd, \( J = 8.9, 2.2 \) Hz, 2H), 6.38 (d, \( J = 5.3 \) Hz, 2H), 5.13 (t, \( J = 5.4 \) Hz, 2H), 3.27 (td, \( J = 7.2, 5.2 \) Hz, 4H), 2.61 (t, \( J = 7.3 \) Hz, 4H), 1.72 (p, \( J = 7.3 \) Hz, 4H), 1.57 – 1.28 (m, 16H). ¹³C NMR (126 MHz, CDCl₃) \( \delta \) 152.20, 149.90, 149.32, 134.95, 128.96, 125.36, 121.14, 117.31, 99.21, 50.02, 43.35, 29.87, 29.33, 28.92, 27.36, 27.16. FTIR (thin film) \( \lambda \) (cm⁻¹):
3269, 2928, 2854, 1610, 1579, 1540, 1452. HRMS (ESI) C$_{32}$H$_{41}$Cl$_2$N$_5$: Calculated for [M+H] C$_{32}$H$_{42}$Cl$_2$N$_5$, 566.2817; found: 566.2837.

DC770 (85) (46 mg, 0.08 mmol, 1.0 eq.) was added to a screwcap vial and dissolved in CHCl$_3$ (350 uL). Aqueous formaldehyde was added (10 uL, 37% aq, 0.24 mmol, 1.5 eq.) to the vial, and the reaction was vortexed for 30 seconds. Solid sodium triacetoxyborohydride (52 mg, 0.24 mmol. 3.0 eq.) was added to the vial, and the reaction was stirred for 48 hours. The reaction was observed to be complete via LC-MS, where only a single peak corresponding to the [M+H] for the product (580) was observed. The reaction was quenched by first dilution with CH$_2$Cl$_2$ (500 uL), 2N NaOH (500 uL) and stirred for 60 minutes. The layers of the resulting biphasic mixture were separated, and the resulting aqueous layer was extracted with CHCl$_3$ (3 x 1 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated to a film (40 mg), which was purified by column chromatography (SiO$_2$, 15 mm x 100 mm, gradient elution[100% CH$_2$Cl$_2$;7:93:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH; 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH; 15:85:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH]) to yield a film (86) (36 mg, 76%). The structure of the purified product was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.50 (d, $J = 5.3$ Hz, 2H), 7.93 (d, $J = 2.3$ Hz, 2H), 7.66 (d, $J = 8.9$ Hz, 2H), 7.35 – 7.28 (m, 2H), 6.37 (d, $J = 5.4$ Hz, 2H), 5.14 (d, $J = 5.2$ Hz, 2H), 3.26 (td, $J = 7.2$, 5.0 Hz, 4H), 2.30 (dd, $J = 8.7$, 6.5 Hz, 4H), 2.19 (d, $J = 3.7$ Hz, 3H), 1.72 (p, $J = 7.2$ Hz, 4H), 1.51 – 1.26 (m, 16H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.16, 149.89, 149.30, 134.91, 128.92, 125.32, 121.11,
117.30, 99.18, 57.96, 43.35, 42.45, 29.40, 28.93, 27.57, 27.37, 27.21. IR (thin film) λ
(cm⁻¹): 3277, 2928, 2854, 1608, 1579, 1541, 1454. HRMS (ESI) C₃₃H₄₂Cl₂N₅: Calculated
for [M+H] C₃₃H₄₃Cl₂N₅, 580.2974; found: 580.2976.
Compound\textsuperscript{75} 87 (3.7 g, 28.0 mmol, 1.0 eq.) was added to a round bottom flask along with a stir bar and place under an atmosphere of argon. THF (70 mL) was added to the round bottom flask via a syringe. The solution was cooled to 0 °C in an ice bath and triphenylphosphine (11.8 g, 45 mmol, 1.6 eq.) was added in one portion. Carbon tetrabromide (14.97 g, 45 mmol, 1.6 eq) was added to the reaction in three portions, adding the next portion when the first portion has dissolved. All additions performed while maintaining the 0 °C bath. The reaction was first clear but becomes increasingly brown and opaque through the duration of the reaction. The reaction was stirred for 1 hour at 0 °C. The reaction was then warmed to 23 °C and stirred for 4 hours. The reaction was considered complete when the starting azido alcohol was consumed by TLC (starting material $R_f = 0.25$, 1:3 EtOAc:Hex, KMnO\textsubscript{4}). The reaction was then concentrated under reduced pressure to afford a paste. The paste was triturated with Et\textsubscript{2}O (300 mL), and the resulting solid was washed with Et\textsubscript{2}O (2 x 100 mL). The combined ethereal fractions were dried over MgSO\textsubscript{4} and concentrated to a crude oil. The resulting crude product was purified by column chromatography (SiO\textsubscript{2}, 65 mm x 150 mm, 1:5, EtOAc:Hex) to afford an oil (3.5 g, 64%). The structure of the product (88) was verified by $^1$H NMR. TLC ($R_f = 0.6$, 3:1, Hex: EtOAc). $^1$H NMR (500 MHz, CDCl\textsubscript{3}) $\delta$ 3.41 (t, $J = 5.0$ Hz, 2H), 3.48 (t, $J =$
6.2 Hz, 2H), 3.70 (dd, J = 5.5, 4.6 Hz, 2H), 3.83 (t, J = 6.2 Hz, 2H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 71.35, 70.13, 50.89, 30.14. FTIR (thin film) \(\lambda\) (cm\(^{-1}\)): 2867, 2107. HRMS (ESI) C\(_4\)H\(_8\)BrN\(_3\)O: Calculated for [M-N\(_2\)+H] C\(_8\)H\(_9\)BrNO, 165.9868; found: 165.9888.

Compound\(^7\)\(^6\) 89 (6.16 g, 28.0 mmol, 1.0 eq.) was added to a round bottom flask along with a stir bar and placed under an atmosphere of argon. THF (70 mL) was added to the round bottom flask via a syringe. The solution was cooled to 0 °C in an ice bath and triphenylphosphine (11.8 g, 45 mmol, 1.6 eq.) was added in one portion. Carbon tetrabromide (14.92 g, 45 mmol, 1.6 eq) was added to the reaction in three portions, adding the next portion when the first portion has dissolved. All additions performed while maintaining the 0 °C bath. The reaction was first clear but becomes increasingly brown and opaque through the duration of the reaction. The reaction was stirred for 1 hour at 0 °C. The reaction was then warmed to 23 °C and stirred for 4 hours. The reaction was considered complete when the starting azido alcohol was consumed by TLC (starting material R\(_f\) = 0.25, 1:3 EtOAc:Hex, KMnO\(_4\)). The reaction was then concentrated under reduced pressure to afford a paste. The paste was triturated with Et\(_2\)O (200 mL), and the resulting solid was washed with Et\(_2\)O (100 mL). The combined ethereal fractions were dried over MgSO\(_4\) and concentrated to a crude oil. The resulting crude product was purified by column chromatography (SiO\(_2\), 65 mm x 150 mm, 1:3, EtOAc:Hex) to afford an oil (4.49 g, 57%). The structure of the product (90) was verified by \(^1\)H NMR. TLC (R\(_f\)
\( \delta = 0.25, 1:3, \text{EtOAc:Hex}. \) \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 3.81 (td, \( J = 6.3, 1.4 \text{ Hz}, 2\text{H} \)), 3.70 – 3.65 (m, 10H), 3.47 (td, \( J = 6.3, 1.4 \text{ Hz}, 2\text{H} \)), 3.39 (t, \( J = 4.6 \text{ Hz}, 2\text{H} \)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 71.12, 70.63, 70.60 (2C), 70.46, 69.97, 50.61, 30.21. FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 2868, 2108, 1449, 1352, 1281. HRMS (ESI) \( \text{C}_8\text{H}_{16}\text{BrN}_3\text{O}_3 \): Calculated for \([\text{M+Na}] \text{C}_8\text{H}_{16}\text{BrN}_3\text{O}_3\text{Na}, 304.0273; \) found: 304.0279.

To a round bottom flask the azido-bromide \( \textbf{88} \) was added (3.581 mmol, 18.5 mmol, 3.0 eq), the reaction was placed under an atmosphere of argon, EtOH (14 mL) was added to dissolve to form a solution, and anhydrous potassium carbonate was added (2.55g, 18.5 mmol, 3.0 eq). The reaction was placed under a reflux condenser, benzylamine was added (672 uL, 6.2 mmol, 1.0 eq), and the reaction was heated to reflux in an oil bath (75 °C). The reaction was heated until the benzylamine has been completely bisalkylated, as observed by \(^1\)H NMR. The reaction was concentrated under reduced pressure to afford a paste. The reaction was partitioned between EtOAc (20 mL) and water (20 mL). The layers were separated, and the water was extracted with EtOAc (25 mL). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure to afford a crude paste. The paste was purified by flash chromatography (SiO\(_2\), 55 mm x 150 mm, Hex EtOAc, 4:1) to afford a translucent oil \( \textbf{91} \) (1.33 g, 65%), the structure of which was confirmed by \(^1\)H NMR. TLC (\( R_f = 0.7 \),
2:1, Hex:EtOAc, UV254 nm). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.36 – 7.28 (m, 4H), 7.23 (td, $J = 7.3, 6.9, 1.5$ Hz, 1H), 3.73 (s, 2H), 3.58 (dt, $J = 7.2, 5.3$ Hz, 8H), 3.35 (t, $J = 5.0$ Hz, 4H), 2.79 (t, $J = 6.0$ Hz, 4H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 139.87, 128.92, 128.35, 127.05, 70.22, 69.88, 60.02, 54.08, 50.98. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2864, 2107. HRMS (ESI) C$_{15}$H$_{23}$N$_7$O$_2$: Calculated for [M+H] C$_{15}$H$_{24}$N$_7$O$_2$, 334.1991; found: 334.1991.

To a round bottom flask the azido-bromide 90 was added (1.53 g, 5.4 mmol, 3.0 equiv.) and the reaction was placed under an atmosphere of argon. EtOH (4 mL) and anhydrous potassium carbonate were added (749 mg, 5.4 mmol, 3.0 equiv.) to the reaction vessel. The reaction was placed under a reflux condenser, benzylamine was added (200 uL, 1.8 mmol, 1.0 eq), and the reaction was heated to reflux in an oil bath preset to 75 °C. The reaction was heated for 20 hours, until the benzylamine has been completely bisalkylated as observed by $^1$H NMR. The reaction was concentrated under reduced pressure to afford a paste and dissolved in CH$_2$Cl$_2$ (10 mL). Water (5 mL) and saturated aqueous sodium bicarbonate (5 mL) were added to the crude solution. The layers of the biphasic mixture were separated, and the resulting aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a crude paste. The paste was purified by flash chromatography (SiO$_2$, 35 mm x 150 mm, Gradient
1:1 to 0:1, Hex:EtoAc) to afford a translucent oil (92) (753 mg, 82%) the structure of which was verified by $^1$H NMR. TLC ($R_f = 0.6$, 99:1, EtOAc:MeOH). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.30 (dt, $J = 15.0$, 7.7 Hz, 4H), 7.22 (t, $J = 7.0$ Hz, 1H), 3.75 – 3.51 (m, 30H), 3.36 (t, $J = 5.1$ Hz, 4H), 2.75 (t, $J = 6.3$ Hz, 4H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 139.88, 128.84, 128.17, 126.85, 70.75, 70.72, 70.43, 70.07, 69.98, 59.79, 53.86, 50.73. FTIR (thin film) λ (cm$^{-1}$): 2868, 2106. HRMS (ESI) C$_{23}$H$_{39}$N$_7$O$_6$: Calculated for [M+H] C$_{23}$H$_{39}$N$_7$O$_6$, 510.3040; found: 510.3042.

The tertiary amine (91) (900 mg, 2.7 mmol, 1.0 equiv.) was added to a round bottom flask followed by sodium bicarbonate (125mg, 1.5 mmol, 0.6 eq), and was placed under an argon atmosphere with a stir bar. The reaction was dissolved in toluene (3.8 mL) and methylchloroformate (250 uL, 3.2 mmol, 1.2) was added to the reaction dropwise while the reaction was stirring. The reaction was then heated to 85 °C in an oil bath until the starting tertiary amine was consumed as observed by TLC ($R_f = 0.7$, 2:1, Hex:EtoAc, UV 254). The reaction was cooled to 23 °C and concentrated under reduced pressure to afford a crude paste. The paste was purified by column chromatography (SiO$_2$, 2:1, Hex:EtoAc) to afford a translucent oil (93) (770 mg, 97%). The structure of the crude oil was verified by $^1$H NMR. TLC ($R_f = 0.2$, 2:1, Hex:EtoAc). The $^{13}$C NMR was observed to contain a mixture of rotamers. $^1$H NMR (500 MHz, CDCl$_3$) δ 3.70 (s,
3H), 3.67 – 3.56 (m, 8H), 3.56 – 3.50 (m, 4H), 3.36 (t, \( J = 5.0 \text{ Hz}, 4H \)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 156.85, 69.98, 69.89, 69.74, 52.67, 52.64, 50.88, 48.41, 47.96. FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 2867, 2109, 1702. HRMS (ESI) C\(_{10}\)H\(_{19}\)N\(_7\)O\(_4\): Calculated for [M+H] C\(_{10}\)H\(_{20}\)N\(_7\)O\(_4\), 302.1577; found: 302.1590.

The tertiary amine 92 (426 mg, 0.84 mmol, 1.0 equiv.) was added to a round bottom flask followed by sodium bicarbonate (40 mg, 0.46 mmol, 0.6 equiv.) and was placed under an argon atmosphere with a stir bar. The reaction was dissolved in toluene (1.2 mL) and methylchloroformate (80 uL, 1 mmol, 1.2 equiv.) was added to the reaction dropwise while the reaction was stirring. The reaction was then heated in an oil bath preset to 85 °C until the starting tertiary amine was consumed as observed by TLC (R\(_f\) = 0.2, EtOAc, UV 254). The reaction was then concentrated under reduced pressure to afford a crude paste which was purified by flash chromatography (SiO\(_2\), 25 mm x 150 mm, EtOAc). The product was isolated as a clear oil (94) (243 mg, 61%), the structure of which was verified by \(^1\)H NMR. The \(^{13}\)C NMR was observed to contain a mixture of rotamers. TLC (R\(_f\) = 0.4, EtOAc, KMnO\(_4\)). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 3.73 – 3.42 (m, 32H), 3.37 (q, \( J = 4.8 \text{ Hz}, 4H \)). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \( \delta \) 156.86, 70.82, 70.79, 70.60, 70.50, 70.15, 69.77, 69.60, 52.59, 50.80, 48.22, 47.76. FTIR (thin film) \( \lambda \) (cm\(^{-1}\)): 2868, 2106, 1701. HRMS (ESI) C\(_{18}\)H\(_{35}\)N\(_7\)O\(_8\): Calculated for [M+Na] C\(_{18}\)H\(_{35}\)N\(_7\)O\(_8\)Na, 500.2445; found: 500.2455.
The bisazide (93) (540 mg, 1.9 mmol, 1.0 equiv.) was added to a round bottomed flask along with a stir bar. The reaction vessel was placed under an atmosphere of argon, and the contents of the flask were dissolved in THF (2.8 mL). Triphenylphosphine (992 mg, 3.8 mmol, 2.0 equiv.) was added to the reaction vessel, and the reaction was stirred at 50 °C. Water (1 mL, 55 mmol, 29.0 equiv.) was added via a syringe to the reaction vessel and the reaction was heated until all azide containing compounds were reduced as observed via LCMS. The reaction was then concentrated to a paste and dissolved in CH₂Cl₂ (10 mL). The organic solution was washed with 1N HCl (4 mL). The organic fraction was extracted (2 x 5 mL) with 1N HCl and the aqueous layers were combined. The product, now a HCl salt in the aqueous layer, was then back extracted with methylene chloride. The water layer was now frozen and lyophilized to obtain the pure product as a white HCl salt (95) (518 mg, 85%). The structure of the salt was verified by ¹H NMR. The ¹³C NMR was observed to contain a mixture of rotamers. MP (Water) 165-175 °C. TLC (Rf = 0.0, 10:90:1, MeOH:NH₄OH:CH₂Cl₂). ¹H NMR (500 MHz, CD₃OD) δ 3.73 – 3.63 (m, 12H), 3.59 – 3.51 (m, 4H), 3.16 – 3.10 (m, 4H). ¹³C NMR (126 MHz, CD₃OD) δ 158.91, 70.57, 70.02, 67.73, 53.40, 48.74, 48.02, 40.57. FTIR (thin film) λ (cm⁻¹): 2971, 1672, 1485, 1246, 1107. HRMS (ESI) C₁₀H₂₃N₃O₄(HCl)₂: Calculated for [M+H-2HCl] C₁₀H₂₄N₃O₄, 250.1767; found: 250.1750.
The bisazide 94 (200 mg, 0.42 mmol, 1.0 equiv.) was added to a round bottomed flask along with a stir bar. The reaction vessel was placed under an atmosphere of argon, and the contents of the flask were dissolved in THF (0.75 mL). Triphenylphosphine (219 mg, 0.84 mmol 2.0 equiv.) was added to the reaction vessel, and the reaction was stirred at 50 °C. Water (250 μL) was added via a syringe to the reaction vessel and the reaction was heated until the all azide containing compounds were reduced as observed via 1H NMR. The reaction was then concentrated to a paste and dissolved in EtOAc (2 mL). The EtOAc was washed with 1N HCl (6 x 2 mL) and the aqueous layers were combined. The product, now a HCl salt in the aqueous layer was frozen and lyophilized to obtain a solid clear paste as the HCl salt (96) (147mg, 70%). The structure of which was determined by 1H NMR and HRMS. The 13C NMR was observed to contain a mixture of rotamers. TLC (Rf = 0.0, 10:90:1, MeOH:NH4OH:CH2Cl2). 1H NMR (500 MHz, DMSO-d$_6$) δ 3.64 – 3.45 (m, 14H), 3.33 (d, J = 3.8 Hz, 24H), 3.16 (s, 1H), 2.94 (s, 1H). 13C NMR (126 MHz, CDCl3) δ 155.40, 69.19, 69.13, 66.08, 51.78, 37.93. FTIR (thin film) λ (cm$^{-1}$): 3390, 1670. HRMS (ESI) C$_{18}$H$_{39}$N$_3$O$_4$(HCl)$_2$: Calculated for [M+H-2HCl] C$_{18}$H$_{40}$N$_3$O$_4$, 426.2815; found: 426.2807.

To the reaction vessel 4-bromo-7-chloroquinoline (13) (290 mg, 1.2 mmol, 2.2 equiv.) was added followed by the diamine (95) (175 mg, 1.0 mmol, 1.0 equiv.),
Pd(OAc)$_2$ (6 mg, 0.03 mmol, 0.05 equiv.), BINAP (74 mg, 0.12 mmol, 0.1 equiv.), K$_3$PO$_4$ (578 mg, 3.0 mmol, 5.0 equiv.). The reaction vessel was then sealed and placed under an atmosphere of argon. 1,4-dioxane was degassed and added to the reaction via a syringe (1.4 mL). The reaction was then heated at 105 °C for 20 hours. The reaction was cooled to room temperature and was considered to be complete when 4-bromo-7-chloroquinoline was consumed as observed via TLC ($R_f = 0.65$, 3 Hexanes :1 EtOAc). The reaction was filtered through Celite™ using EtOAc and concentrated to a solid (530mg). The reaction was then purified by flash chromatography (SiO$_2$, 25 mm x 130 mm, gradient: MeOH:NH$_4$OH:CH$_2$Cl$_2$; 1:98:1 (50 mL), 2:97:1 (50 mL), 3:96:1 (50 mL), 4:95:1 (50 mL), 8:91:1 (50 mL), 10:89:1 (100 mL)). The product was isolated as a yellow white solid (97) (266mg, 86%), the structure of which was confirmed by $^1$H NMR. The $^{13}$C NMR was observed to contain a mixture of rotamers. MP 56 – 60 °C. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.50 (d, $J = 5.4$ Hz, 2H), 7.94 (d, $J = 2.2$ Hz, 2H), 7.32 (s, 2H), 6.37 (d, $J = 5.9$ Hz, 2H), 5.73 (d, $J = 143.3$ Hz, 2H), 3.82 – 3.35 (m, 19H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 157.32, 151.80, 148.98, 135.18, 128.63, 128.42, 125.60, 125.32, 122.10, 121.41, 117.44, 99.30, 70.01, 69.44, 68.89, 68.48, 53.07, 53.05, 47.84, 43.18, 42.95. FTIR (thin film) λ (cm$^{-1}$): 2869, 1691, 1611, 1579, 1539. HRMS (ESI) C$_{28}$H$_{31}$Cl$_2$N$_5$O$_4$: Calculated for [M+H] C$_{28}$H$_{32}$Cl$_2$N$_5$O$_4$, 572.1831; found: 572.1823.
To a reaction vessel 4-bromo-7-chloroquinoline (13) (119 mg, 1.2 mmol, 2.2 equiv.) was added followed by the diamine (96) (111 mg, 1.0 mmol, 1.0 equiv.), Pd(OAc)$_2$ (3 mg, 0.03 mmol, 0.05 equiv.), BINAP (17 mg, 0.12 mmol, 0.1 equiv.), K$_3$PO$_4$ (235 mg, 1.10 mmol, 5.0 equiv.). The reaction vessel was then sealed and placed under an atmosphere of argon. 1,4-dioxane was degassed and added to the reaction via a syringe (560 mL). The reaction was then heated at 105 °C for 20 hours. The reaction was cooled to room temperature and considered to be complete when 4-bromo-7-chloroquinoline was consumed as observed via TLC ($R_f = 0.65$, 3 Hexanes :1 EtOAc).

The reaction was filtered through Celite™ using 10% MeOH in CHCl$_3$ (v/v) and concentrated to a solid (168 mg). The reaction was then purified by flash chromatography (SiO$_2$, 25 mm x 130 mm, gradient: MeOH:NH$_4$OH:CH$_2$Cl$_2$; 3:96:1 (50 mL), 5:94:1 (50 mL)) The product was isolated as a yellow white paste (98) (167 mg, 72%) the structure of which was confirmed by $^1$H NMR. The $^{13}$C NMR was observed to contain a mixture of rotamers. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.50 (d, $J = 5.3$ Hz, 2H), 7.93 (d, $J = 2.2$ Hz, 2H), 7.80 (d, $J = 8.9$ Hz, 2H), 7.32 (dd, $J = 8.9, 2.2$ Hz, 2H), 6.37 (d, $J = 5.4$ Hz, 2H), 5.81 (d, $J = 15.2$ Hz, 2H), 3.79 (t, $J = 5.2$ Hz, 4H), 3.70 – 3.34 (m, 33H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 156.88, 152.09, 150.10, 149.26, 135.01, 128.74, 125.36, 121.74, 117.58,
99.30, 70.75, 70.69, 70.62, 70.46, 69.79, 69.47, 68.78, 52.71, 48.18, 47.68, 42.89. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3380, 2869, 1698, 1610, 1580, 1538. HRMS (ESI) C$_{36}$H$_{47}$Cl$_2$N$_5$O$_8$: Calculated for [M+H] C$_{36}$H$_{48}$Cl$_2$N$_5$O$_8$, 748.2880; found: 748.2894.

The carbamate 97 (37 mg, 0.07 mmol, 1.0 equiv.) was added to a screw-cap vial followed by solid KOH (150 mg, 3 mmol, 45 equiv.). The reaction contents were placed under an atmosphere of argon and dissolved in a 2:1 mixture of methanol and water (600 uL). The reaction was then heated for 48 hours at 100 °C. The reaction was monitored by consumption of the starting material via TLC ($R_f$ = 0.5, 5:95:1, MeOH:NH$_4$OH:CH$_2$Cl$_2$). The completed reaction was then concentrated under reduced pressure to remove the methanol. The resulting pasted was partitioned between CHCl$_3$ (1 mL) and water (1 mL). The layers were separated, and the water layer was extracted with CHCl$_3$ (3 x 1 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated to a crude paste. Purification of the paste by column chromatography (15 mm x 150 mm, gradient 3:96:1 to 6:93:1, MeOH:NH$_4$OH:CH$_2$Cl$_2$) yielding a white yellow film (99) (27 mg, 79%). TLC ($R_f$ = 0.2, 5:95:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.48 (d, $J = 5.3$ Hz, 2H), 7.91 (d, $J = 2.1$ Hz, 2H), 7.70 (d, $J = 9.0$ Hz, 2H), 7.31 – 7.24 (m, 2H), 6.33 (d, $J = 5.3$ Hz, 2H), 5.63 (t, $J = 5.1$ Hz, 2H), 3.72 (dd, $J = 5.7$, 4.6 Hz, 4H), 3.62 – 3.56 (m, 4H), 3.38 (q, $J = 5.1$ Hz, 4H), 2.84 (t, $J = 5.1$ Hz, 4H), 1.23 (t, $J = 7.0$ Hz, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.19, 149.92, 149.32, 135.00, 128.93, 125.39, 121.43, 117.50, 99.39, 77.44, 77.19, 76.93, 70.61, 68.82, 49.40, 42.85. FTIR (thin film) $\lambda$ (cm$^{-1}$): 3269, 2869, 1611, 1580, 1541, 1451, 1429, 1368, 1332, 1280, 1251, 1142, 1118.
HRMS (ESI) C_{26}H_{29}Cl_{2}N_{5}O_{2}: Calculated for [M+H] C_{26}H_{30}Cl_{2}N_{5}O_{2}, 514.1777; found: 514.1760.

The carbamate (98) (50 mg, 0.7 mmol, 1.0 equiv.) was added to a screw-cap vial and placed under an atmosphere of argon. The reaction was dissolved in CHCl₃ (200 uL). TMS-I (60 uL, 0.21 mmol, 3.2 equiv.) was added and the reaction was heated to 65 °C for 3 hours. The reaction was observed to be complete by the consumption of starting carbamate (TLC R_f = 0.7, 1:10 MeOH:CH₂Cl₂). The reaction was concentrated under reduced pressure to afford a crude paste. The crude product was purified by column chromatography (SiO₂, 15 mm x 150 mm, 10:90:1, MeOH:CH₂Cl₂:NH₄OH) to yield a film on a vial (100) (34 mg, 71%). The structure of the product was verified by ¹H NMR. TLC (R_f = 0.2, 10:90:1, MeOH:CH₂Cl₂:NH₄OH). ¹H NMR (500 MHz, CDCl₃) δ 8.46 (d, J = 5.6 Hz, 2H), 8.00 – 7.92 (m, 4H), 7.36 (dd, J = 8.9, 2.2 Hz, 2H), 6.42 (d, J = 5.7 Hz, 2H), 6.32 (s, 2H), 3.86 – 3.80 (m, 4H), 3.70 – 3.49 (m, 27H), 2.83 (t, J = 5.2 Hz, 4H). ¹³C NMR (126 MHz, CDCl₃) δ 150.86, 150.45, 147.56, 135.53, 127.14, 125.51, 122.49, 117.15, 99.05, 70.50, 70.35, 70.22, 70.14, 69.24, 68.75, 68.65, 42.88. FTIR (thin film) λ (cm⁻¹): 3292, 2871, 1610, 1538, 1451, 1426, 1367, 1332, 1281, 1243, 1098. HRMS (ESI) C_{34}H_{45}Cl_{2}N_{5}O_{6}: Calculated for [M+H] C_{34}H_{46}Cl_{2}N_{5}O_{6}, 690.2825; found: 690.2844.
The dimeric inhibitor 99 (11 mg, 0.02 mmol, 1.0 equiv.) was added to a screw-cap vial and placed under an atmosphere of argon. A stock solution was made of CH2O (3.5 uL/110 uL) in CH2Cl2. The inhibitor was dissolved in CH2Cl2 (110 uL) which contained CH2O (3.5 uL, 0.04 mmol, 2.0 equiv.). The reaction was stirred for 2 minutes before adding solid sodium triacetoxyborohydride (18 mg, 0.9 mmol, 4.0). The reaction was stirred for three hours until the starting material was consumed as observed by TLC (Rf = 0.3, 5:94:1, MeOH:CH2Cl2:NH4OH) and LCMS. The excess sodium triacetoxyborohydride was quenched by the addition of 2N NaOH (500 uL). CH2Cl2 (500 uL) was added to the reaction mixture, and the biphasic mixture was separated. The aqueous layer was extracted with CH2Cl2, the combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a crude paste. The crude paste was then purified by column chromatography (SiO2 (pipet), 5:94:1, MeOH:CH2Cl2:NH4OH) to afford a film on a vial 101 (9 mg, 80%). TLC (Rf = 0.3, 5:94:1, MeOH:CH2Cl2:NH4OH). 1H NMR (500 MHz, CDCl3) δ 8.50 (d, J = 5.3 Hz, 2H), 7.94 (d, J = 2.3 Hz, 2H), 7.71 (d, J = 8.9 Hz, 2H), 7.34 – 7.24 (m, 4H), 6.32 (d, J = 5.3 Hz, 2H), 5.63 (d, J = 5.4 Hz, 3H), 3.71 (t, J = 5.1 Hz, 5H), 3.61 (t, J = 5.4 Hz, 5H), 3.36 (q, J = 5.1 Hz, 6H), 2.69 (t, J = 5.4 Hz, 5H), 2.34 (s, 2H). 13C NMR (126 MHz, CDCl3) δ 152.23, 149.95, 149.36, 135.00, 128.94, 125.30, 121.55, 117.56, 99.36, 68.88, 68.43, 57.23, 43.55, 42.78. FTIR (thin film) λ (cm⁻¹): 3265, 2870, 1611, 1578, 1538,
The dimeric inhibitor 100 (33 mg, 0.048 mmol, 1.0 equiv.) was added to a round bottom flask, placed under an atmosphere of argon, and dissolved in CH$_2$Cl$_2$. Aqueous formaldehyde (10 uL) was added via a microsyringe, and the reaction was stirred for ten minutes. Sodium triacetoxyborohydride (31 mg, 0.14 mmol, 3.0 equiv.) was added to the reaction in one portion, and the reaction was stirred for 20 hours. In LC-MS of the crude reaction only the mass of the methylated product (704 [M+H]) was observed. Excess sodium triacetoxyborohydride was quenched by dilution with CH$_2$Cl$_2$ (2 mL) and the addition of 2N NaOH (2 mL). The reaction was vortexed for 60 seconds and the resulting emulsion was allowed to settle. The resulting biphasic mixture was separated, and the remaining aqueous fraction was extracted with CH$_2$Cl$_2$ (3 x 2 mL). The combined organic layers were dried over sodium sulfate, concentrated under reduced pressure to a paste, and purified by flash chromatography (SiO$_2$, 15 mm x 125 mm, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). The product was isolated as a film on a vial (102) (23 mg, 68%), the structure of which was verified by $^1$H NMR. TLC ($R_f$ = 0.2, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.50 (d, $J$ = 5.3 Hz, 2H), 7.93 (d, $J$ = 2.0 Hz, 2H), 7.86 (d, $J$ = 9.2 Hz, 2H), 7.33 (dd, $J$ = 9.0, 2.2 Hz, 2H), 6.36 (d, $J$ = 5.4 Hz, 2H), 6.01 (s, 2H), 3.80 (t, $J$ = 5.2 Hz, 4H), 3.69 – 3.58 (m, 12H), 3.56 – 3.43 (m, 12H), 2.58 – 2.49 (m, 7H), 2.24 (s, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 152.14, 150.22, 1482, 1451, 1428, 1367, 1332, 1280, 1252, 1141, 1120, 1080. HRMS (ESI) C$_{27}$H$_{31}$Cl$_2$N$_5$O$_2$: Calculated for [M+H] C$_{27}$H$_{32}$Cl$_2$N$_5$O$_2$, 528.1933; found: 528.1917.
Diamine\(^{77}\) \(\text{103}\) (197 mg, 0.4023 mmol, 1.0 equiv) aryl halide \(\text{13}\) (215 mg, 0.8851 mmol, 2.2 equiv), Pd(OAc)\(_2\) (4.54 mg, 0.02 mmol, 0.05 equiv), BINAP (25 mg, 0.04 mmol, 0.1 equiv), and K\(_3\)PO\(_4\) (257 mg, 1.207 mmol, 3.0 equiv) were added to a vial. The vial was sealed and placed under an atmosphere of argon. Through the septum, 1,4-dioxane (1 mL), was added to the reaction vessel. The reaction was heated to 105 °C until the consumption of the linker was observed via proton NMR. Protons of the methylene next to the primary amine were easily visible (~2.7 ppm CDCl\(_3\)). When the reaction was complete, it was cooled to 23 °C and the reaction was filtered through a pad of celite using CHCl\(_3\) to remove inorganic salts. The crude reaction mixture was then concentrated under reduced pressure and purified via flash chromatography (SiO\(_2\), 25 mm X 150 mm, eluted via a gradient 2%-5% MeOH with 1% TEA in EtOAc). Isolate the product 92% pure by HPLC as a brown solid (115 mg, 32%) \(\text{104}\). The product is a mixture of rotamers at 300K. Heating the sample to 340 K causes the mixture of rotamers to coalesce. The
structure of the compound was then verified by 1H NMR and HRMS. The $^{13}$C NMR contained a mixture of rotamers. MP 62 – 64 °C. TLC ($R_f = 0.45, 7:93:1$, MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.40 (d, $J = 5.4$ Hz, 2H), 8.25 – 8.16 (m, 3H), 7.78 (s, 2H), 7.39 (d, $J = 9.0$ Hz, 2H), 6.48 (s, 2H), 3.34 (d, $J = 6.5$ Hz, 4H), 2.86 (s, 5H), 2.68 – 2.59 (m, 27H).$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.81, 155.10, 151.74, 148.77, 134.97, 128.25, 125.28, 121.91, 117.12, 97.98, 81.36, 80.54, 53.39, 46.29, 45.73, 45.48, 45.06, 43.98, 43.75, 28.37. FTIR (thin film) $\lambda$ (cm$^{-1}$): 1673, 1611, 1579, 1541, 1477, 1465, 1453, 1392, 1366, 1333, 1308, 1279, 1241, 1216, 1156, 1138. HRMS (ESI) C$_{41}$H$_{55}$Cl$_2$N$_7$O$_6$: Calculated for [M+H] C$_{41}$H$_{56}$Cl$_2$N$_7$O$_6$, 812.3669; found: 812.3665.

![Chemical diagram]

The starting carbamate 104 (77 mg, 0.095 mmol, 1.0 equiv.) was added to a vial and placed under an atmosphere of argon. The carbamate was dissolved in CH$_2$Cl$_2$ (400 uL) and TFA was added (400 uL). The reaction was monitored via LC-MS, where after 3 hours of stirring the reaction was complete. The reaction was concentrated under reduced pressure to afford a paste. The paste was partitioned between CHCl$_3$ (5 mL) and 50% concentrated NH$_4$OH in water (2 mL). The layers were separated, and the water layer was saturated with NaCl. The saturated aqueous solution was then extracted with CHCl$_3$ (2 x 3 mL). The combined organic layers were then dried over Na$_2$SO$_4$ and concentrated to afford a paste (105) (23 mg, 47%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.52 – 8.46 (m, 2H),
7.92 (t, J = 2.3 Hz, 2H), 7.68 (dd, J = 9.1, 2.1 Hz, 2H), 7.29 (dd, J = 8.9, 2.3 Hz, 2H),
6.33 (dd, J = 5.7, 2.0 Hz, 2H), 5.84 (s, 2H), 3.30 – 3.24 (m, 4H), 3.00 – 2.94 (m, 4H),
2.74 (q, J = 2.3 Hz, 8H), 1.62 (bs, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 152.24, 150.00,
149.29, 134.91, 128.87, 125.27, 121.45, 117.52, 99.36, 49.55, 49.04, 47.63, 42.24.
FTIR (thin film) λ (cm⁻¹): 3280, 2938, 2840, 1611, 1579, 1533, 1450, 1428, 1367, 1330,
1280, 1246, 1215, 1137, 1080. HRMS (ESI) C₂₆H₃₁Cl₂N₇: Calculated for [M+H]
C₂₆H₃₂Cl₂N₇, 512.2096; found: 512.2105.

To a screw cap vial the DC inhibitor 105 (50 mg, 0.1 mmol, 1.0 equiv.) was
added and placed under an atmosphere of argon. The vial contents were dissolved in
HCOOH (500 uL) and aqueous formaldehyde (74 uL, 1.0 mmol, 10 equiv.) was added.
The reaction was heated at 105 °C for four hours, until LCMS showed tris-methylated
product and no mass for the bis- or mono-methylated species was observed. The reaction
contents were concentrated under reduced pressure to afford a paste. The resulting paste
was then dissolved in water (3 mL), CHCl₃ (4 mL), and concentrated NH₄OH (1 mL).
The resulting biphasic mixture was separated, and the resulting aqueous layer was
extracted with CHCl₃ (3 x 3 mL). The combined organic layers were then dried over
anhydrous sodium sulfate and concentrated under reduced pressure to yield a crude paste.
The crude material was purified by flash chromatography (SiO₂, 15 mm x 150 mm,
5:94:1 to 10:90:1, MeOH:NH₄OH:CH₂Cl₂) to yield the product as film on a vial (106)
(33 mg, 60%). The structure of the product was confirmed by $^1$H NMR. $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.53 – 8.37 (m, 4H), 7.90 (d, $J = 2.5$ Hz, 2H), 7.73 – 7.62 (m, 2H), 7.05 – 6.98 (m, 2H), 4.07 (s, 4H), 3.64 – 3.53 (m, 4H), 3.44 (s, 4H), 3.05 – 2.95 (m, 9H), 2.40 (d, $J = 2.5$ Hz, 3H).$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.82, 144.40, 141.30, 140.01, 129.02, 126.16, 120.41, 117.17, 100.13, 55.11, 54.34, 53.06, 41.31, 40.99, 39.56. FTIR (thin film) $\lambda$ (cm$^{-1}$): 1674, 1614, 1457, 1200, 1131, 720. HRMS (ESI) C$_{29}$H$_{37}$Cl$_2$N$_7$: Calculated for [M+H] C$_{29}$H$_{38}$Cl$_2$N$_7$, 554.2566; found: 554.2589.

Piperaquine Dimeric Inhibitors

To a screw-cap vial the secondary amine$^{78}$ 107 (248 mg, 1.0 mmol, 2.0 equiv.) and potassium carbonate (166 mg, 1.2 mmol, 2.4 equiv.) were added as solids and placed under an atmosphere of argon. DMF (1 mL) was added to the reaction mixture followed by 1,2-dibromoethane 108 (45 uL, 0.5 mmol, 1.0 equiv.). The reaction was heated until 107 was consumed by TLC ($R_f = 0.3$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH, UV254). The reaction was cooled to room temperature and diluted with water (1 mL). The crude mixtures were all purified specific to each reaction. The reaction was poured onto water (20 mL) and a white-brown precipitate is observed. Vacuum filtration and washing with water (20 mL) allowed collection of the product as a brown solid (109) (214 mg, 82%),
the structure of which was verified by 1H NMR. MP (DMF/Water) 193-195 °C. TLC (Rf = 0.25, 7:92:1, MeOH:CH₂Cl₂:NH₄OH, UV254). ¹H NMR (500 MHz, CDCl₃) δ 8.69 (d, J = 5.0 Hz, 2H), 8.07 – 7.98 (m, 2H), 7.93 (d, J = 8.8 Hz, 2H), 7.44 – 7.37 (m, 2H), 6.81 (d, J = 5.1 Hz, 2H), 3.30 – 3.19 (m, 8H), 2.88 – 2.64 (m, 10H). ¹³C NMR (126 MHz, CDCl₃) δ 157.03, 152.06, 150.27, 134.99, 129.01, 126.23, 125.31, 122.04, 109.09, 56.07, 53.69, 52.29. FTIR (thin film) λ (cm⁻¹): 2947, 2827, 1606, 1574, 1497, 1459, 1424, 1381, 1333, 1299, 1253, 1234, 1193, 1134, 1072. HRMS (ESI) C₂₈H₃₁Cl₂N₆: Calculated for [M+H] C₂₈H₃₁Cl₂N₆, 521.1987; found: 521.2007.

![Chemical Structure](image)

To a screw-cap vial the secondary amine⁷⁸ ¹⁰⁷ (100 mg, 0.4 mmol, 2.0 equiv.) and potassium carbonate (66 mg, 0.48 mmol, 2.4 equiv.) were added as solids and placed under an atmosphere of argon. DMF (1 mL) was added to the reaction mixture followed by 1,7-dibromoheptane (34 uL, 0.2 mmol, 1.0 equiv.). The reaction was heated until ¹⁰⁷ was consumed by TLC (Rf = 0.3, 10:90:1, MeOH:CH₂Cl₂:NH₄OH, UV254). The reaction was cooled to room temperature and diluted with water (10 mL). The resulting aqueous mixture was extracted with EtOAc (3 x 10 mL) and the resulting organic fractions were collected. The combined organic layers were washed with water (2 x 10 mL), brine (1 x 10 mL) and the resulting organic layers were dried over anhydrous sodium sulfate. The
dried organic layer was concentrated under reduced pressure to a crude paste. The paste was purified by column chromatography (SiO₂, 20 x 150 mm, 5:95:1, MeOH:CH₂Cl₂:NH₄OH) resulting in a brown paste (110) (35 mg, 29%). The structure of the product was verified by ¹H NMR. TLC (R_f = 0.5, 10:90:1, MeOH:CH₂Cl₂:NH₄OH).

¹H NMR (500 MHz, CDCl₃) δ 8.68 (d, J = 5.0 Hz, 2H), 8.02 (d, J = 2.1 Hz, 2H), 7.92 (d, J = 8.9 Hz, 2H), 7.39 (dd, J = 9.0, 2.1 Hz, 2H), 6.81 (d, J = 5.0 Hz, 2H), 3.29 – 3.19 (m, 8H), 2.78 – 2.66 (m, 8H), 2.46 (dd, J = 9.1, 6.4 Hz, 4H), 2.21 (s, 4H), 1.56 (t, J = 7.5 Hz, 4H), 1.42 – 1.30 (m, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 157.14, 152.02, 150.21, 135.00, 128.93, 126.21, 125.35, 122.04, 109.07, 58.85, 53.27, 52.29, 29.66, 27.66, 26.98. FTIR (thin film) λ (cm⁻¹): 3373, 3066, 297, 2885, 2822, 1606, 1575, 1497, 1458, 1424, 1379, 1357, 1334, 1298, 1252, 1233, 1192, 1159, 1142, 1072, 1020. HRMS (ESI) C₃₃H₄₀Cl₂N₆: Calculated for [M+H] C₃₃H₄₁Cl₂N₆, 591.2770; found: 591.2770.

To a screw-cap vial the amine 107 (242 mg, 1.0 mmol, 2.0 equiv.) and potassium carbonate (161 mg, 1.2 mmol, 2.4 equiv.) were added as solids and placed under an atmosphere of argon. DMF (1 mL) was added to the reaction mixture followed by 1,8-dibromoocotane 35 (82 uL, 0.44 mmol, 1.0 equiv.). The reaction was heated until 107 was...
consumed by TLC ($R_f = 0.3$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH, UV254). The reaction was cooled to room temperature and diluted with water (1 mL). The crude mixtures were all purified specific to each reaction. The reaction was poured onto water (20 mL) and a white-brown precipitate is observed. The water and DMF was decanted and discarded.

The resulting precipitate (247 mg) was purified by flash chromatography (SiO$_2$, 25 x 150 mm, 4:96:1 then 6:94:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH) resulting in a brown paste (111) (176 mg, 65%). TLC ($R_f = 0.5$, 10:90:1, MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.68 (dd, $J = 5.2$, 2.0 Hz, 2H), 8.01 (d, $J = 2.2$ Hz, 2H), 7.91 (dd, $J = 9.0$, 2.0 Hz, 2H), 7.38 (dt, $J = 9.0$, 2.2 Hz, 2H), 6.80 (dd, $J = 5.0$, 2.0 Hz, 2H), 3.23 (t, $J = 4.6$ Hz, 8H), 2.79 – 2.64 (m, 8H), 2.45 (td, $J = 7.6$, 2.0 Hz, 4H), 1.60 – 1.48 (m, 4H), 1.34 (t, $J = 5.1$ Hz, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 157.11, 152.04, 150.26, 134.95, 128.97, 126.16, 125.36, 122.05, 109.05, 58.87, 53.27, 52.31, 29.67, 27.66, 27.02. FTIR (thin film) $\lambda$ (cm$^{-1}$ $^1$): 2930, 2851, 2820, 1606, 1574, 1497, 1458, 1424, 1380, 1357, 1335, 1297, 1252, 1233, 1191, 1161, 1139, 1071, 1020. HRMS (ESI) C$_{34}$H$_{42}$Cl$_2$N$_6$: Calculated for [M+H]C$_{33}$H$_{43}$Cl$_2$N$_6$, 605.2926; found: 605.2946.
Dimeric ferroquine inhibitors

Ferroquine (6) (20 mg, 0.046 mmol, 1.0 eq) was added to a screw cap vial under an atmosphere of argon. In a separate vial a mixture of 3:1 CHCl₃:MeOH is made (1.2 mL), and methyl iodide (50 uL) is added to the solution. The methyl iodide, CHCl₃, and method solution (400 uL, 0.27 mmol of MeI, 5.8 equiv.) is added to the screw cap vial, and the vessel is sealed. The reaction mixture is heated at 40 °C for four hours until the starting material is consumed as observed by LC-MS. The solvent is removed from the reaction vessel under reduced pressure, and the reaction is placed under high vacuum for one hour to ensure all excess methyl iodide is removed. The amine (46 mg, 0.19 mmol, 4.0 equiv.) is added to the reaction vessel in a mixture of CHCl₃:MeOH (300 uL). The reaction is sealed and stirred at 56 °C for four days. The reaction is then concentrated under reduced pressure to afford an orange-yellow paste. The paste is then purified by flash chromatography (SiO₂, 15 x 150 mm, 3:94:1, MeOH:CH₂Cl₂:NH₄OH) yielding an orange yellow film which contained a coeluted impurity. The film was recrystallized from methanol boiling methanol to yield the product as orange crystals (113) (11 mg, 37%). The structure of the product was verified by ¹H NMR. TLC (R_f = 0.4, 8:92:1,
MeOH:CH₂Cl₂:NH₄OH). ¹H NMR (500 MHz, CDCl₃) δ 8.78 (d, J = 4.9 Hz, 1H), 8.58 (d, J = 5.4 Hz, 1H), 8.06 (d, J = 2.1 Hz, 1H), 7.97 (d, J = 2.1 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.80 (d, J = 8.9 Hz, 1H), 7.42 (dd, J = 8.9, 2.2 Hz, 1H), 7.19 (dd, J = 8.9, 2.2 Hz, 1H), 6.71 (d, J = 5.0 Hz, 1H), 6.56 (d, J = 5.3 Hz, 1H), 6.22 (s, 1H), 4.44 – 4.32 (m, 2H), 4.32 – 4.15 (m, 8H), 3.86 (d, J = 12.7 Hz, 1H), 3.25 – 3.06 (m, 5H), 2.83 (s, 2H), 2.64 (s, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 156.85, 152.27, 150.37, 135.21, 129.23, 126.54, 125.56, 125.13, 122.90, 122.08, 117.33, 109.19, 99.41, 83.72, 82.83, 72.01, 70.77, 69.59(5C), 69.43, 66.95, 57.44, 53.29, 51.94, 51.14, 42.57. FTIR (thin film) λ (cm⁻¹): 2929, 2847, 1606, 1578. HRMS (ESI) C₃₄H₃₁Cl₂FeN₅: Calculated for [M+H] C₃₄H₃₂Cl₂FeN₅, 636.1384; found: 636.1394.
Ferroquine (6) (20 mg, mmol, 1.0 eq) was added to a screw cap vial under an atmosphere of argon. In a separate vial a mixture of 3:1 CHCl₃:MeOH is made (1.2 mL), and methyl iodide (50 uL) is added to the solution. The methyl iodide, CHCl₃, and method solution (400 uL, 0.27 mmol of MeI, 5.8 equiv.) is added to the screw cap vial, and the vessel is sealed. The reaction mixture is heated at 40 °C for four hours until the starting material is consumed as observed by LC-MS. The solvent is removed from the reaction vessel under reduced pressure, and the reaction is placed under high vacuum for one hour to ensure all excess methyl iodide is removed. The secondary amine, 114 (49 mg, 0.19 mmol, 4.0 equiv.) is added to the reaction vessel in a mixture of CHCl₃:MeOH (400 uL). The reaction is sealed and stirred at 40 °C for four days. The reaction is then concentrated under reduced pressure to afford an orange-yellow paste. The reaction is then concentrated under reduced pressure to afford an orange-yellow paste. The paste is then purified by flash chromatography (SiO₂, 15 x 150 mm, 3:94:1, MeOH:CH₂Cl₂:NH₄OH) yielding an orange yellow film which contained a coeluted impurity (13 mg). The paste is then recrystallized from methanol boiling methanol to yield orange crystals (6 mg). The remaining supernatant was purified by RPHPLC (C₁₈,
19 mm x 250 mm, 10-45% MeCN in water (0.1% TFA) over 40 minutes). The combined products resulted in an orange film on a vial (150) (9 mg, 30%), the structure of which was verified by 1H NMR. TLC (R_f = 0.4, 8:92:1, MeOH:CH_2Cl_2:NH_4OH). 1H NMR (500 MHz, CDCl_3) δ 8.59 (d, J = 5.3 Hz, 1H), 8.52 (d, J = 5.3 Hz, 1H), 7.98 (dd, J = 7.5, 2.2 Hz, 2H), 7.82 (d, J = 8.9 Hz, 1H), 7.65 (d, J = 9.0 Hz, 1H), 7.45 (dd, J = 8.9, 2.2 Hz, 1H), 7.32 (dd, J = 8.9, 2.2 Hz, 1H), 6.56 (d, J = 5.4 Hz, 1H), 6.41 (d, J = 5.4 Hz, 1H), 6.36 (d, J = 7.7 Hz, 1H), 4.72 (d, J = 7.0 Hz, 1H), 4.40 – 4.31 (m, 2H), 4.23 (dd, J = 2.5, 1.4 Hz, 1H), 4.17 (s, 7H), 3.80 (d, J = 12.9 Hz, 1H), 3.53 (s, 1H), 3.07 (d, J = 12.8 Hz, 1H), 2.99 (d, J = 11.6 Hz, 1H), 2.86 (s, 1H), 2.34 (s, 1H), 2.13 (d, J = 13.3 Hz, 1H), 2.00 (d, J = 13.8 Hz, 2H), 1.45 (q, J = 8.5, 5.3 Hz, 2H), 1.25 (s, 1H). 13C NMR (126 MHz, CDCl_3) δ 152.33, 151.92, 150.20, 149.36, 148.50, 135.32, 135.09, 129.08, 128.90, 125.94, 124.56, 123.07, 120.84, 117.85, 117.28, 99.69, 99.58, 84.08, 83.33, 71.90, 70.64, 69.50, 66.73, 57.32, 53.60, 50.93, 49.37, 42.46, 31.74, 31.51, 29.89. FTIR (thin film) λ (cm⁻¹): 2925, 1609, 1578, 1530, 1448. HRMS (ESI) C_{35}H_{33}Cl_2FeN_5: Calculated for [M+H]+ C_{35}H_{34}Cl_2FeN_5, 650.1541; found: 650.1519.
Chapter 3: Synthesis of photoaffinity analogs

(4-hydroxyphenyl)(4-(prop-2-yn-1-yloxy)phenyl)methanone (compound 142) (1.02g, 4.05 mmol) was added to a round bottom flask, under an argon atmosphere, followed by K$_2$CO$_3$ (Fisher Scientific) (1.10g, 8.1 mmol, 2.0 eq.). 2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (compound 143) (1.55g, 4.46 mmol, 1.1 eq.) was dissolved in absolute ethanol (8 mL) and added to the reaction vessel. The reaction was heated to reflux under a condenser for 16 hours. The reaction was halted upon consumption of the tosylate starting material as determined by TLC ($R_f = 0.2$, EtOAc). The reaction was concentrated under reduced pressure to remove ethanol. The crude paste was dissolved in 30 mL of a 1:1 mixture of water and EtOAc. The layers were separated, and the aqueous layer was washed with EtOAc (2 x 25 mL). The combined organic layers were washed once with brine and dried over anhydrous Na$_2$SO$_4$. Evaporation under reduced pressure yielded a yellow clear paste (2.06g). The crude material was purified by column chromatography (45mm x 150mm, SiO$_2$, EtOAc) which yielded a translucent paste (141) (1.314g, 76%), the structure of which was verified by $^1$H NMR to match the reported literature.$^3$ TLC ($R_f = 0.2$, EtOAc) $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 2.56 (1H, s, CH), 3.76-3.61 (m, 12H, CH$_2$), 3.90 (2H, t, $J=4.84$, CH$_2$), 4.22 (2H, t, $J=4.81$, CH$_2$), 4.78 (2H, d, $J=2.4$ Hz, CH$_2$), 6.98 (2H, d, $J=8.7$ Hz, CH$_2$), 7.46 (8H, d, $J=8.7$ Hz, CH$_2$).
ArH), 7.04 (2H, d, J= 8.7 Hz, ArH), 7.80-7.77 (4H, m, ArH). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 194.47, 162.27, 160.77, 132.35, 132.22, 131.68, 130.85, 114.49, 114.24, 78.05, 76.27, 71.00, 70.81, 70.73, 70.48, 69.70, 67.75, 61.87, 56.00. FTIR $\lambda$ (neat/cm$^{-1}$): 2873, 1644, 1600, 1507. HRMS (ESI) Calculated for [M+H] C$_{24}$H$_{29}$O$_7$, 429.1913; found: 429.1933.
In a flame-dried round bottom flask under an argon atmosphere, a solution of freshly distilled oxalyl chloride (0.31 mL, 3.6 mmol, 1.2 eq.) in dichloromethane (20 mL), was cooled to -78°C. Anhydrous DMSO (Acros Organics) (0.51 mL, 7.2 mmol, 2.4 eq.) was added to the flask, causing the evolution of gas. The reaction mixture was stirred for 30 min at -78°C, then a solution of the alcohol 141 (1.28g, 3.0 mmol, 1.0 eq.) in CH₂Cl₂ was added slowly. Prior to addition, the alcohol was dried azeotropically with benzene and then placed under high vacuum for 16 hours to remove water. The resulting solution was stirred at -78°C for 1 hour, at which time freshly distilled triethylamine (Acros Organics) (2.0 mL, 15.0 mmol, 5.0 eq.) was added to the reaction. The resulting mixture was slowly warmed to 23 °C and stirred for 16 hours. The progress of the reaction was determined via TLC by consumption of the starting alcohol (TLC Rf = 0.18, EtOAc) and the appearance of a new TLC spot for the aldehyde product (Rf = 0.6, EtOAc). Upon completion, the reaction mixture was then washed with saturated aqueous NH₄Cl (3 x 20 mL), brine (1 x 20 mL), and the resulting organic solution dried over anhydrous Na₂SO₄. The filtrate was concentrated under reduced pressure yielding a crude paste, the presences of the product was verified by ¹H NMR to match the literature precedent.³ Due to stability concerns, the crude aldehyde 144 was used without further
purification in the next reaction. TLC (Rf = 0.6, EtOAc). 1H NMR (500 MHz, CDCl3): δ 2.56 (1H, s, CH), 3.76-3.61, (10H, m, CH2), 3.87 (2H, t, J= 3.86, CH2), 4.13 (2H, s, CH2), 4.18 (2H, t, J= 4.35, CH2), 4.75 (2H, d, J= 2.0 Hz, CH2), 6.98 (2H, d, J=8.0 Hz, ArH), 7.04 (2H, d, J= 9.0 Hz, ArH), 7.8-7.5 (4H, m, ArH), 9.69 (1H, s, CHO).

**DC661-Alkyne**

To a round bottom flask, solid DC660 inhibitor (66) was added (307 mg, 0.57 mmol, 1.0 eq.). The solid was then placed under an atmosphere of argon. The crude aldehyde (123) (505mg, 1.2 mmol, 2.1 eq.) was then dissolved in CH2Cl2 (7mL). The aldehyde solution was then added to the reaction vessel via a syringe to give a reaction concentration of 0.1 M. The reaction was stirred at 23 °C for 10 minutes. Next, sodium triacetoxyborohydride was added to the reaction as a solid (483mg, 2.4 mmol, 4.0 eq.). The reaction was stirred for 22 hours at 23 °C, when the starting DC660 (66) was consumed as observed via TLC (Rf = 0.10 (EtOAc:MeOH:TEA; 80:15:5). Upon completion of the reaction, CH2Cl2 was added (10 mL) to the reaction, and an equal volume of 2N sodium hydroxide was added (10 mL). The biphasic solution was stirred for 60 min to quench the remaining sodium triacetoxyborohydride. After stirring, the
layers were separated, and the aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 10 mL), and the resulting organic layers were combined. The combined organic layers were washed once with an equal volume of brine (50 mL) and dried over anhydrous Na$_2$SO$_4$. The organic phase was concentrated under reduced pressure to afford a crude paste (580 mg), which was purified by flash chromatography (SiO$_2$, gradient starting 3:1:96 MeOH:triethylamine:EtOAc, moving to 3:1:50:44 MeOH:triethylamine:EtOAc:CHCl$_3$) to afford the pure product (118) as a yellow paste (200mg, 51%), the structure of which was verified by $^1$H NMR. TLC ($R_f = 0.25$, 5:1:50:44, MeOH:triethylamine:EtOAc:CHCl$_3$, UV 254 nm) $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 1.27 – 1.50 (m, 12H), 1.72 (p, $J = 7.2$ Hz, 4H), 2.44 (t, $J = 7.4$ Hz, 4H), 2.56 (t, $J = 2.3$ Hz, 1H), 2.65 (t, $J = 6.4$ Hz, 2H), 3.28 (td, $J = 7.1$, 5.3 Hz, 4H), 3.49 – 3.73 (m, 10H), 3.83 – 3.89 (m, 2H), 4.17 (t, $J = 4.8$ Hz, 2H), 4.76 (d, $J = 2.3$ Hz, 2H), 5.15 (s, 2H), 6.39 (d, $J = 5.4$ Hz, 2H), 6.91 – 6.98 (m, 2H), 7.03 (d, $J = 9.0$ Hz, 2H), 7.34 (dd, $J = 8.9$, 2.2 Hz, 2H), 7.69 – 7.80 (m, 7H), 7.95 (d, $J = 2.1$ Hz, 2H), 8.52 (d, $J = 5.4$ Hz, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 194.50, 162.27, 160.82, 151.90, 150.10, 149.08, 135.00, 132.34, 132.22, 131.59, 130.83, 128.62, 125.31, 121.46, 117.31, 114.51, 114.21, 99.08, 78.01, 76.29, 71.02, 70.82, 70.75, 70.54, 69.68, 67.76, 56.02, 54.70, 53.43, 43.29, 28.81, 27.21, 27.10, 26.98. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2921.63, 2848.35, 1756.83, 1719.23, 1627.63, 1594.84, 1558.2. HRMS (ESI) C$_{54}$H$_{63}$Cl$_2$N$_5$O$_6$: Calculated for [M+H] C$_{54}$H$_{64}$Cl$_2$N$_5$O$_6$, 948.4234; found: 948.4229.
DC221-Alkyne

To a round bottom flask, solid DC220 (8) was added (95 mg, 0.22 mmol, 1.0 eq.). The solid was then placed under an atmosphere of argon. The crude aldehyde (144) (189 mg, 0.44 mmol, 2.0 eq.) was then dissolved in CH₂Cl₂ (2.2 mL). The aldehyde solution was then added to the reaction vessel via a syringe, to give a total reaction concentration of 0.1 M. The reaction was stirred at 23 °C for 10 minutes. Next, sodium triacetoxyborohydride was added to the reaction as a solid (190 mg, 0.89 mmol, 4.0 eq.). The reaction was stirred for 22 hours at 23 °C, when the starting DC inhibitor was consumed as observed via TLC ($R_f = 0.2, 10:89:1$, MeOH:CH₂Cl₂:NH₄OH, UV 254 nm). Upon completion of the reaction, CH₂Cl₂ was added (10 mL) to the solution, and an equal volume of 2N sodium hydroxide was added (10 mL). The biphasic solution was stirred for 60 min to quench the remaining sodium triacetoxyborohydride. After stirring, the layers were separated, and the aqueous layer was extracted with CH₂Cl₂ (2 x 15 mL), and the resulting organic layers were combined. The combined organic layers were
washed once with an equal volume of brine (40 mL) and dried over anhydrous Na₂SO₄. The organic phase was concentrated under reduced pressure to afford a crude paste (185 mg), which was purified by flash chromatography (SiO₂, 25 mm x 150 mm, 7.5:1:92, MeOH: NH₄OH: CH₂Cl₂) to yield the product as a yellow paste (145) (55 mg, 38%), the structure of which was verified by ¹H NMR. TLC (Rₑ = 0.5, 7.5:1:92, MeOH: NH₄OH: CH₂Cl₂, UV 254 nm). ¹H NMR (500 MHz, CDCl₃): δ 2.57 (d, J = 2.4 Hz, 1H), 2.84 (t, 2H), 2.97 (dd, J = 6.6, 4.4 Hz, 4H), 3.23 – 3.28 (m, 4H), 3.44 – 3.53 (m, 4H), 3.55 – 3.59 (m, 2H), 3.64 – 3.73 (m, 6H), 4.04 (t, 2H), 4.77 (d, J = 2.4 Hz, 2H), 6.19 (s, 2H), 6.24 (d, J = 5.4 Hz, 2H), 6.75 (dd, J = 8.9, 2.2 Hz, 2H), 6.87 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 8.9 Hz, 2H), 7.55 (d, J = 8.9 Hz, 2H), 7.74 (dd, J = 17.5, 8.8 Hz, 3H), 7.87 (d, J = 2.2 Hz, 2H), 8.41 (d, J = 5.3 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 194.50, 162.15, 160.82, 151.63, 150.05, 148.65, 135.17, 132.34, 132.25, 131.56, 130.85, 128.28, 125.29, 121.70, 117.26, 114.52, 114.14, 99.10, 78.03, 76.30, 70.82, 70.63, 70.58, 70.23, 70.12, 69.61, 67.60, 56.03, 53.30, 53.04, 41.08. FTIR (thin film) λ (cm⁻¹): 3435.56, 2871.49, 1644.98, 1600.63, 1581.34. HRMS (ESI) C₄₆H₄₇Cl₂N₅O₆: Calculated for [M+H] C₄₆H₄₆Cl₂N₅O₆, 836.2982; found: 836.2982.
In a flame dried round bottom flask under an argon atmosphere, a suspension of DQ660 (56) (320mg, 0.458 mmol) in 2 mL of dichloromethane was reacted with a solution of aldehyde (144) (558mg, 1.31 mmol, 2.9 eq) in 3.5 mL of dichloromethane, bringing the reaction to a final concentration of 0.08 M. The two components were stirred for 15 minutes at 23°C and the resulting orange mixture became homogeneous. 

NaBH(OAc)₃ (388mg, 1.832 mmol, 4.0 eq) was added as a solid to the reaction mixture and the resulting mixture was stirred for 16 hours at 23°C, until the DQ 660 (56) was consumed as observed by TLC. The reaction mixture was then diluted with dichloromethane (50 mL) and stirred with an equal volume of 2N NaOH (50 mL) for 1 hour at 23°C. The layers were separated, and the aqueous layer was washed with 2 x 20 mL of dichloromethane. The combined organic layers were washed once with brine and dried over anhydrous Na₂SO₄. Evaporation under reduced pressure afforded an orange paste, which was purified by column chromatography (35mm x 150mm, SiO₂, 5% MeOH:94% EtOAc:1%TEA) to yield an orange paste (146) (200mg, 39%). ¹H NMR (500 MHz, CDCl₃/CD₃OD): δ 8.05 (2H, s, ArH), 8.00 (4H, t, J= 9.0  Hz, ArH), 7.77-7.74 (4H, m, ArH), 7.42 (2H, dd, J=9.2 Hz, J= 2.5 Hz, ArH), 7.30 (2H, dd, J=9.3  Hz, J=2.5
Hz, ArH), 7.23 (2H, d, J=2.6 Hz, ArH), 7.03 (2H, d, J=8.9 Hz, ArH), 6.94 (2H, d, J=8.3, ArH), 4.75 (2H, d, J= 2.4 Hz, CH₂), 4.17 (2H, t, J=5.0 Hz, CH₂), 3.95 (6H, s, OMe), 3.85 (2H, t, J=3.8 Hz, CH₂), 3.70-3.56 (14H, m, CH₂) 2.59 (2H, t, J=6.5 Hz, CH₂), 2.55 (1H, s, CH), 2.36 (4H, t, J= 7.0 Hz, CH₂), 1.72 (4H, m, CH₂), 1.39 (8H, m, CH₂), 1.27 (4H, m, CH₂). 

^13^C NMR (126 MHz, CDCl₃): δ 194.23, 162.03, 160.57, 155.88, 149.712, 148.187, 134.67, 132.108, 131.99, 131.39, 130.62, 128.11, 124.36, 124.32, 123.99, 117.80, 115.70, 114.26, 113.97, 99.21, 77.80, 76.02, 70.80, 70.58, 70.32, 69.71, 69.45, 67.52, 55.78, 55.45, 54.58, 53.21, 50.54, 31.65, 27.08, 26.98, 26.77. IR λ (neat/cm⁻¹): 2921, 2848, 1756, 1719, 1627, 1594, 1558, 1254. HRMS (ESI) C₆₄H₇₁N₅O₈Cl₂: Calculated for [M + H] C₆₄H₇₂N₅O₈Cl₂ 1108.4758; found: 1108.4749.
Desthiobiotin Azide

To a flame-dried round bottom flask, d-desthiobiotin (131) (Sigma Aldrich) (200 mg, 0.94 mmol, 1.0 eq.) was added, followed by TBTU (449mg, 1.4 mmol, 1.5 eq.). The reactants were placed under an atmosphere of argon and dissolved in dimethylformamide (DMF) (2 mL). The solution was stirred and (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-amine)\textsuperscript{105} (147) (305mg, 1.4 mmol,1.5 eq.) was added as a solution in DMF (1mL). Then after stirring for 5 min, DIPEA (490 uL, 3.7 mmol, 4.0 eq.) was added to the reaction via micro-syringe. The reaction was stirred for 24 hours until the starting desthiobiotin was consumed, as observed by TLC ($R_f = 0.1$, 10:1 CHCl\textsubscript{3}:MeOH, KMnO\textsubscript{4} stain). The reaction was quenched by pouring onto 30 mL of brine. The opaque solution was extracted with EtOAc (3 x 30mL). The combined organic extracts were then washed with water (5 x 10mL), resulting in a second aqueous fraction. TLC analysis ($R_f = 0.35$, 1:10, MeOH:CHCl\textsubscript{3}, KMnO\textsubscript{4} stain) revealed the product was found exclusively in the second aqueous fraction. The second aqueous fraction was then extracted with EtOAc (5 x 50mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield a crude
brown oil. The crude product was then purified by column chromatography [SiO₂, 20 mm x 180 mm, gradient elution of increasing MeOH in CHCl₃ (MeOH: 2% for 100 mL, 4% for 100 mL, and 6% for 200 mL)]. The product was isolated as a purple-translucent oil (140) (163mg, 42%). TLC (Rf = 0.35, 1:10, MeOH:CHCl₃, KMnO₄ stain). ¹H NMR (500 MHz, CDCl₃): δ 1.14 (d, J = 6.5 Hz, 3H), 1.56 – 1.23 (m, 7H), 1.66 (t, J = 7.3 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 3.50 – 3.36 (m, 4H), 3.74 – 3.54 (m, 13H), 3.91 – 3.82 (m, 1H), 6.16 (s, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 173.22, 164.19, 70.66, 70.51, 70.50, 70.13, 70.02, 69.98, 56.09, 51.40, 50.67, 39.14, 35.84, 29.51, 28.56, 25.82, 25.19, 15.72. FTIR λ (neat/cm⁻¹): 3287.07, 2 2933.2, 2106.85, 1699.94, 1545.67. HRMS (ESI) C₁₈H₃₅N₆O₅: Calculated for [M+H], C₁₈H₃₆N₆O₅, 415.2669; found: 415.2666.
DC661 Pulldown

Alkyne 139 (50mg, 0.053 mmol, 1.0 eq.) and desthiobiotin azide 140 (23mg, 0.080 mmol, 1.5 eq.), were added to a screwcap vial. The vial contents were dissolved in 500 uL of a 1:2:1 mixture of acetone:water:DMSO and placed under an argon atmosphere. CuSO₄ was added from a 1M aqueous solution (38uL, 0.05 mmol, 1.0 eq.), followed by the addition of solid sodium ascorbate (37mg, 0.2 mmol, 5.0 eq.). The reaction was complete after 30 min when the starting alkyne was consumed, as observed by TLC (Rₛ = 0.25, 5:1:50:44, MeOH:triethylamine:EtOAc:CHCl₃, UV 254 nm). The reaction was concentrated to a paste and partitioned between water (1mL) and CHCl₃ (1.5 mL), which caused a film to deposit on the wall of the vial. The aqueous layer was removed, and methanol was added to the organic layer to solubilize the film. The aqueous layer was extracted with a 1:3 mixture of 2-propanol:CHCl₃ (2 x 2.5 mL). The combined organic layers were then dry loaded onto silica for flash chromatography. Crude material
was chromatographed (SiO₂, 15 mm x 150 mm, 1:10 MeOH:CHCl₃ gradient to 10:1:100 MeOH:NH₄OH:CHCl₃) to afford the product as a yellow translucent paste (138) (35mg, 51%), the structure of which was verified by ¹H NMR. TLC (Rf = 0.3, 10:1:100 MeOH:NH₄OH:CHCl₃). ¹H NMR (500 MHz, CDCl₃): δ 1.09 (d, J = 6.5 Hz, 3H), 1.15 – 1.51 (m, 19H), 1.61 (p, J = 7.2 Hz, 2H), 1.71 (p, J = 7.3 Hz, 4H), 2.14 (t, J = 7.5 Hz, 2H), 2.42 (t, J = 7.4 Hz, 4H), 2.63 (t, J = 6.3 Hz, 2H), 3.27 (td, J = 7.2, 5.3 Hz, 4H), 3.37 – 3.74 (m, 28H), 3.75 – 3.82 (m, 1H), 3.87 (dt, J = 15.5, 4.9 Hz, 4H), 4.17 (dd, J = 5.6, 4.0 Hz, 2H), 4.51 (s, 1H), 4.55 (t, J = 5.0 Hz, 2H), 5.04 (s, 1H), 5.27 (s, 4H), 6.38 (d, J = 5.4 Hz, 2H), 6.90 – 6.97 (m, 2H), 7.04 (d, J = 8.8 Hz, 1H), 7.32 (dd, J = 8.9, 2.2 Hz, 2H), 7.69 – 7.78 (m, 6H), 7.85 (s, 1H), 7.93 (d, J = 2.2 Hz, 2H), 8.50 (d, J = 5.4 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃): δ 194.60, 173.37, 163.53, 162.28, 161.62, 151.94, 150.18, 149.08, 143.41, 135.02, 132.37, 131.29, 130.80, 128.58, 125.32, 124.46, 121.57, 117.35, 114.48, 114.26, 99.07, 70.97, 70.82, 70.72, 70.66, 70.59, 70.49, 70.47, 70.23, 70.15, 69.67, 69.55, 69.52, 67.74, 62.19, 56.13, 54.66, 53.57, 51.53, 50.53, 43.27, 39.29, 36.21, 29.59, 28.96, 28.76, 27.22, 27.07, 26.77, 26.10, 25.44, 15.91. FTIR (thin film) λ (cm⁻¹): 3352.64, 2929.34, 2854.13, 1696.09, 1645.95, 1596.77, 1578.45, 1539.88. HRMS (ESI) C₆₇H₈₇Cl₂N₁₁O₁₁: Calculated for [M+2H]/2 C₆₇H₈₉Cl₂N₁₁O₁₁, 646.8060; found: 646.8046. [α]D ²² 24.1 (c 1.0, CH₂Cl₂).
DC221 Pulldown

Alkyne 145 (16mg, 0.019 mmol, 1.0 eq.) was added to a screwcap vial, followed by desthiobiotin azide 140 (12mg, 0.029 mmol, 1.5 eq.). The contents of the vial were dissolved in MeOH (200 uL) to give a 0.1 M reaction concentration. In a separate vial, THPTA and CuSO₄ dissolved in water to form a dark blue solution (100 mM CuSO₄ and 200 mM THPTA). This aqueous solution was used to deliver THPTA and CuSO₄ (0.0036 mmol and 0.0018 mmol respectively) to the screwcap vial. While stirring, sodium ascorbate was added to the vial (20 mg, 0.10 mmol, 5.3 eq.). The reaction was monitored by RPHPLC (C₁₈, 4.6 mm x 250 mm, gradient elution: 10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 40 min). The reaction was complete when no starting alkyne remains. The reaction was concentrated to remove the methanol and dissolved in CHCl₃ (5 mL). The CHCl₃ washed with saturated sodium bicarbonate (3 x 5 mL). The organic layer was separated and dried over anhydrous Na₂SO₄. The organic layer was then concentrated under reduced pressure to afford a film. The film was then
solubilized in MeOH and DMSO (300 uL and 60 uL respectively) and purified by RPHPLC (C18, 19 mm x 250 mm, gradient elution: 10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 40 min). The pH of the fractions was brought to 12 by addition of NH4OH (1 mL), and then saturated with NaCl. The aqueous layer was then extracted with CHCl3 (3 x 5 mL). The CHCl3 was dried over sodium sulfate and concentrated under reduced pressure to afford a film on a vial (148) (8mg, 34%), the structure of which was verified by 1H NMR. TLC (Rf = 0.2, 10:1:100 MeOH:NH4OH:CHCl3). 1H NMR (500 MHz, CDCl3): δ 8.44 (d, J = 5.3 Hz, 2H), 7.89 – 7.85 (m, 3H), 7.73 (dd, J = 14.1, 8.9 Hz, 4H), 7.53 (d, J = 8.8 Hz, 2H), 7.05 (d, J = 8.9 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 6.76 (dd, J = 8.9, 2.2 Hz, 2H), 6.31 (s, 1H), 6.26 (d, J = 5.6 Hz, 2H), 6.06 (s, 2H), 4.95 (s, 1H), 4.57 (t, J = 5.1 Hz, 2H), 4.45 (s, 1H), 4.05 (t, J = 4.6 Hz, 2H), 3.90 (t, J = 5.1 Hz, 2H), 3.81 (p, J = 6.4 Hz, 1H), 3.73 – 3.39 (m, 27H), 3.27 (q, J = 5.5 Hz, 4H), 2.98 (t, J = 5.3 Hz, 4H), 2.84 (t, J = 4.7 Hz, 2H), 2.16 – 2.13 (m, 2H), 1.61 (q, J = 7.1 Hz, 2H), 1.50 – 1.28 (m, 8H), 1.10 (d, J = 6.5 Hz, 3H). 13C NMR (126 MHz, CDCl3) δ 194.47, 173.10, 163.27, 162.23, 161.63, 152.00, 149.92, 149.04, 143.48, 135.09, 132.37, 132.35, 131.34, 130.86, 128.65, 125.30, 124.40, 121.53, 117.36, 114.50, 114.21, 99.23, 70.85, 70.74, 70.68, 70.64, 70.60, 70.30, 70.28, 70.24, 70.10, 69.63 (2C), 69.57, 67.67, 62.27, 56.15, 53.15, 51.54, 50.57, 41.12, 39.30, 36.36, 29.66, 29.10, 26.25, 25.50, 15.97. FTIR (thin film) λ (cm⁻¹): 3350.71, 2933.2, 2865.7, 1697.05, 1684.52, 1650.77, 1599.66, 1578.45. HRMS (ESI) C64H81Cl2N11O11: Calculated for [M+2H]/2 C64H83Cl2N11O11, 625.7825; found: 625.7823. [α]D 22 °20.7 (c 1.0, CH2Cl2).
To a solution of DQ661-alkyne (146) (58mg, 0.052 mmol) and desthiobiotin-azide (140) (33mg, 0.08mmol, 1.5 eq) in 250 μL of MeOH in a resealable vial was added 200 μL of a solution that was 20 mM in CuSO₄ (0.65mg, 0.004mmol) and 44 mM in THPTA (3.9mg, 0.009mmol; Tris(3-hydroxypropyltriazolylmethyl)amine) which had been treated with 2 mg (10 μmoles) of sodium ascorbate to discharge the blue color before addition to the reaction mixture. The resulting mixture was stirred for 20 hours at 23°C. The reaction mixture was concentrated to a paste under reduced pressure and the residue partitioned between chloroform (5mL) and water (5mL). The water was extracted 6 x 5 mL with 3:1 chloroform: isopropanol, and the combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to give 94 mg of crude product, which was chromatographed (20 mm x 150 mm, SiO₂, 10% MeOH: 90%CH₂Cl₂). The resulting product required further purification (RP-HPLC). The chromatographed product 149 (36mg) was purified as the TFA salt by RP-HPLC (C₁₈, gradient elution using 35%-53% acetonitrile in water with 0.1% TFA over 15 min) yielded a yellow film (18 mg, 23%). ¹H NMR (500 MHz, CD₃OD): δ 8.51 (2H, d, J = 9.3
Hz, ArH), 8.18 (1H, s, ArH), 7.86 (4H, dd, J = 12.5 Hz, J = 2.5 Hz, ArH), 7.81 – 7.77
(6H, m, ArH), 7.72 (2H, dd, J = 9.0 Hz, J = 2.5 Hz, ArH), 7.57 (2H, dd, J = 9.3, J = 2.1
Hz, ArH), 7.06 (2H, d, J = 8.7 Hz, ArH), 6.94 (2H, d, J = 8.7 Hz, ArH), 4.61 (2H, t, J =
4.3 Hz, CH2), 4.26 (2H, t, J = 4.5 Hz, CH2), 3.99 (6H, s, OMe), 3.92-3.88 (4H, m, CH2),
3.85 – 3.78 (4H, m, CH2), 3.73 – 3.56 (18H, m, CH2), 3.49 (2H, t, J = 5.5 Hz, CH2), 3.18
– 3.13 (4H, m, CH2), 2.92 (4H, t, J= 8.0 Hz), 2.17 (2H, t, J = 8.0 Hz, CH2), 1.97 (4H, q, J
= 7.6 Hz), 1.72-1.58 (13H, m), 1.50 – 1.29 (14H, m), 1.35 (2H, q, J = 6.5 Hz), 1.10 (3H,
d, J = 6.5 Hz, CH3).13C NMR (126 MHz, CD3OD): δ 194.32, 174.82, 171.61, 164.75,
162.33, 161.82, 156.61, 142.74, 140.39, 131.87, 131.82, 130.26, 130.08, 127.25, 125.03,
123.57, 120.20 ,117.27, 114.03, 113.72, 70.33,70.09, 70.03, 69.98, 69.82, 69.28, 69.14,
68.89, 67.61, 65.51, 64.26, 61.09, 60.16, 60.14, 55.96, 55.28, 53.06, 52.54, 52.08, 51.28,
50.11, 48.90, 38.89, 35.48, 31.36, 29.31, 29.12, 28.80, 25.96, 25.79, 25.42, 23.08, 22.31,
19.50, 19.47, 14.25, 14.04, 13.06. IR λ (neat/cm-1): 3040, 1652, 1447, 1197, 1123. HRMS
(ESI) C82H104Cl2N11O13: Calculated for ([M+2H]/2) C82H106Cl2N11O13 761.8713; found:
761.8740. [α]d 22 –22.1 (c 1.0, CH2Cl2).
Tetrahydrofuran (THF) (70 mL) was added to a flame-dried round bottom flask, placed under an atmosphere of argon, and cooled to 0°C. Lithium aluminum hydride was added (7.09 g, 186 mmol, 3.5 eq.) to the reaction vessel, causing the evolution of gas. Ethyl 4-nitropentanoate\textsuperscript{107} \textbf{154} (9.334 g, 53.3 mmol, 1.0 eq.) was dissolved in THF (70 mL) and added to the reaction mixture using a syringe pump at a rate of 1 mL/min. The reaction was stirred for 30 min at 0 °C then allowed to warm to 23 °C. The reaction was then stirred at 23°C for 14 hours. The reaction was cooled to 0 °C and quenched by the addition of three solutions: First, 8 mL of water were added via a syringe pump at a rate of 0.5 mL/min. Second, 8 mL of 15% (w/v) NaOH (aq) were added at a rate of 0.5 mL/min. Third, 24 mL of water were added to the reaction at a rate of 1 mL/min. The reaction was then warmed to room temperature and filtered through a pad of Celite\textsuperscript{TM} using 100 mL of EtOAc. The combined organic layer was then dried over anhydrous sodium sulfate and concentrated to a translucent oil (\textbf{153}) (4.77 g, 87%), the structure of which was verified by \textsuperscript{1}H NMR. TLC (R\textsubscript{f} = 0.0, regardless of solvent choice, KMnO\textsubscript{4} stain). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}): δ 3.66 – 3.56 (m, 2H), 2.98 – 2.90 (m, 1H), 1.77 – 1.68 (m, 1H), 1.66 – 1.56 (m, 2H), 1.41 – 1.32 (m, 1H), 1.13 (d, J = 6.5 Hz, 3H). NO\textsubscript{N-H} showed up. \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}): δ 25.32, 30.45, 37.77, 47.17, 63.04. FTIR (thin
4-aminopentan-1-ol, 153, (1.00 g, 9.71 mmol, 1.0 eq.), 4-bromo-7-chloroquinoline\(^1\) 13 (1.80 g, 7.28 mmol, 0.75 eq.), Pd(OAc)\(_2\) (109 mg, 0.485 mmol, 0.05 eq.), BINAP (605 mg, 0.971 mmol, 0.1 eq.), and K\(_3\)PO\(_4\) (3.10 g, 14.6 mmol, 1.5 eq.) were added to a flame-dried round bottom flask. The flask contents under a reflux condenser were then placed under an argon atmosphere. 1,4-dioxane, which was degassed, was then added to the round bottom flask. The reaction was heated to reflux in a bath preset to 105 °C for 2 hours. After the first 20 minutes, the reaction takes on a deep red color. The reaction was complete when the 4-bromo-7-chloroquinoline 13 was consumed as observed by TLC (\(R_f = 0.65, 4:3:1\) Hex:CH\(_2\)Cl\(_2\):EtOAc, UV 254 nm). The reaction was then filtered through a pad of Celite\(^\text{TM}\) using a 100 mL of a 1:10 mixture, MeOH:CHCl\(_3\). Removal of solvent under reduced pressure affords a red paste which was then dry loaded on silica gel and purified by flash chromatography (SiO\(_2\), 5:94:1 MeOH:CH\(_2\)Cl\(_2\):NH\(_4\)OH (aq)) to afford a brown white solid. This solid required triturated with boiling CH\(_2\)Cl\(_2\) to yield a white solid which still contains an inseparable impurity (152) (~10% by \(^1\)H NMR) (695 mg, 295
36%). TLC \( R_f = 0.25 \), 5:94:1 MeOH:CH₂Cl₂:NH₄OH). \(^1\)H NMR (500 MHz, CD₃OD): \( \delta \) 8.33 (d, \( J = 5.6 \) Hz, 1H), 8.17 (dd, \( J = 9.0, 0.5 \) Hz, 1H), 7.76 (dd, \( J = 2.2, 0.5 \) Hz, 1H), 7.38 (dd, \( J = 9.0, 2.2 \) Hz, 1H), 6.54 (dd, \( J = 5.7, 0.7 \) Hz, 1H), 3.81 (h, \( J = 6.4 \) Hz, 1H), 3.59 (t, \( J = 6.4 \) Hz, 2H), 1.86 – 1.60 (m, 3H), 1.32 (d, \( J = 6.4 \) Hz, 3H). \(^1\)C NMR (126 MHz, CD₃OD): \( \delta \) 150.81, 150.66, 148.26, 134.80, 125.97, 124.31, 122.93, 117.29, 98.34, 61.23, 47.98, 32.12, 28.76, 18.78. FTIR (thin film) \( \lambda \) (cm⁻¹): 3319, 2937, 1578. HRMS (ESI) C₁₄H₁₇Cl₁N₂O: Calculated for [M+H] C₁₄H₁₈Cl₁N₂O, 265.1108; found: 265.1101.

THF (4.7 mL) was added to a flame-dried round bottom flask under an atmosphere of argon. Freshly distilled oxalyl chloride (190 uL, 2.3 mmol 1.2 eq.) was added to the round bottom flask, and the solution was cooled to -78°C. While stirring at -78°C, DMSO (320 uL, 4.5 mmol, 2.4 eq.) was added via a syringe, causing the evolution of gas. In a separate flame-dried round bottom flask, compound 152, under an argon atmosphere was dissolved in THF (20 mL) by heating to reflux and cooling to 25°C. After 30 minutes, the solution of 152 was added dropwise via a syringe to the oxalyl chloride/DMSO solution. The combined reaction was then stirred for 30 minutes at -78°C. At this time, triethylamine (1.3 mL, 9.45 mmol, 5.0 eq.) was added to the reaction via a syringe. The reaction was stirred at -78°C for 60 min before being moved to an ice bath.
at 0°C. The reaction was stirred at 0°C for 4 hours, until the consumption of the starting alcohol was observed by TLC ($R_f = 0.10$, 2:1:1 Hex:THF:CH$_2$Cl$_2$ with 3 drops of triethylamine). The reaction was then concentrated under reduced pressure to afford a crude paste. The structure of the aldehyde was confirmed by $^1$H NMR of the crude reaction mixture, with ~10% of starting alcohol remaining. Previous experiments demonstrated purification the aldehyde caused decomposition. The crude mixture of 155 was carried on to the next step without further purification. TLC ($R_f = 0.15$, 2:1:1 Hex:THF:CH$_2$Cl$_2$ with 1% v/v triethylamine). $^1$H NMR (500 MHz, CDCl$_3$): δ 9.74 (s, 1H), 8.37 (d, $J = 5.3$ Hz, 1H), 7.82 (d, $J = 2.3$ Hz, 1H), 7.73 (d, $J = 9.0$ Hz, 1H), 7.28 – 7.21 (m, 1H), 6.32 (d, $J = 5.5$ Hz, 1H), 5.48 (d, $J = 7.2$ Hz, 1H), 3.70 – 3.64 (m, 3H), 1.78 (t, $J = 3.6$ Hz, 2H), 1.26 (d, $J = 6.4$ Hz, 3H).

Crude 155, (450mg, 1.9 mmol, 1.0 eq.) was added to a round bottom flask with a stir bar and placed under an atmosphere of argon. The flask was then charged with CH$_2$Cl$_2$ (7mL) and allowed to stir at 23°C until the solution was homogenous. A solution of ethylamine (70% w/v) (230 uL, 2.8 mmol, 1.5 eq.) was prepared in CH$_2$Cl$_2$ (2 mL) and added via a syringe to the reaction vessel. The reaction was stirred at 23°C for 5 minutes, and sodium triacetoxyborohydride (1.20 g, 5.63 mmol, 3.0 eq.) was added in one portion. The reaction was stirred at 23°C for two hours until the aldehyde starting material was
consumed as observed by TLC ($R_f = 0.25$, 2:1:1 Hex:CH$_2$Cl$_2$:THF with 2% triethylamine (v/v)). The reaction was then diluted with CH$_2$Cl$_2$ (5mL) and an equal volume of 2N NaOH (5mL). The reaction was stirred for 60 minutes at 23°C to quench any remaining sodium triacetoxyborohydride. The layers were then separated in a separatory funnel, and the aqueous layer was extracted with CH$_2$Cl$_2$ (2 x 15 mL). The combined organic layers were dried over anhydrous sodium sulfate. The organic layers were then concentrated under reduced pressure to afford a translucent green paste (151) (400 mg). The green paste was purified by flash chromatography (SiO$_2$, 25mm x 100 mm, 10:90:1 MeOH:CH$_2$Cl$_2$:NH$_4$OH) to afford (155 mg, 0.53 mmol, 28% over 2 steps) of a green paste. TLC ($R_f = 0.3$, 10:90:1 MeOH:CH$_2$Cl$_2$:NH$_4$OH). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 8.48 (dd, $J = 5.4$, 0.8 Hz, 1H), 7.94 – 7.91 (m, 1H), 7.70 (d, $J = 8.9$ Hz, 1H), 7.31 (ddd, $J = 8.9$, 2.2, 0.9 Hz, 1H), 6.38 (dd, $J = 5.5$, 0.7 Hz, 1H), 5.54 (d, $J = 7.0$ Hz, 1H), 3.73 – 3.64 (m, $J = 5.9$ Hz, 1H), 2.69 – 2.60 (m, 4H), 1.83 – 1.58 (m, 3H), 1.30 (dd, $J = 6.4$, 0.9 Hz, 3H), 1.12 (td, $J = 7.2$, 0.9 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 152.13, 149.52, 149.29, 134.88, 128.88, 125.07, 121.46, 117.54, 99.30, 49.45, 48.50, 44.36, 34.27, 26.64, 20.29, 15.37. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2966, 2927, 1577. HRMS (ESI) C$_{16}$H$_{22}$ClN$_3$: Calculated for [M+H] C$_{16}$H$_{22}$ClN$_3$, 292.1581; found: 292.1575.
CQ-Alkyne

The aldehyde 144 (57 mg, 0.13 mmol, 1.0 eq.) was added to a flame-dried screwcap vial, followed by desethyl chloroquine 151 (33 mg, 0.1 mmol, 0.8 eq.) in a CH$_2$Cl$_2$ solution (500 uL). The reaction was stirred at 23°C for 5 minutes before sodium triacetoxyborohydride was added in one portion (85 mg, 0.40 mmol, 3.0 eq.). The reaction was complete in 30 minutes when the aldehyde was consumed as observed by TLC ($R_f = 0.5$, EtOAc, UV 254 nm). The reaction was diluted with 1 mL of CH$_2$Cl$_2$ and 1 mL 2N NaOH. The reaction was vortexed for 1 minute, and the resulting emulsion was placed into a freezer set to -20 °C, where after two hours the emulsion had separated. The layers were then separated, and the aqueous layer was extracted with CH$_2$Cl$_2$ (2 x 1 mL). The combined CH$_2$Cl$_2$ layers were washed with 3 mL of brine. The resulting organic layer was then dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a white paste (89 mg). The white paste was dissolved in a solution of 10:20:70 DMSO:CH$_2$Cl$_2$:MeOH and purified by preparative HPLC ($C_{18}$, 19 mm x 250 mm, gradient elution: 10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 40 min). Lyophilization of the collected fractions yields a white film on a vial (21 mg). The vials contents were then dissolved in an immiscible mixture of CHCl$_3$:NH$_4$OH (aq), vortexed for 1 min, the layers were separated, and the resulting organic layer was dried over anhydrous sodium sulfate. The dried organic solution was concentrated under reduced pressure to yield a film on a vial (150) (19 mg, 24%), the structure of which was verified by $^1$H NMR. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.50 (d, $J = 5.4$ Hz, 1H), 7.93 (d, $J = 2.1$ Hz, 1H), 7.81 – 7.71 (m, 5H), 7.33 (dd, $J = 8.9, 2.2$ Hz, 1H),
7.04 (d, $J = 8.9$ Hz, 1H), 6.94 (d, $J = 8.9$ Hz, 1H), 6.40 (d, $J = 5.4$ Hz, 1H), 5.29 (d, $J = 7.3$ Hz, 1H), 4.77 (d, $J = 2.4$ Hz, 2H), 4.16 (t, $J = 4.8$ Hz, 2H), 3.83 (t, 2H), 3.72 – 3.51 (m, 11H), 2.64 (hept, $J = 6.6$, 6.1 Hz, 2H), 2.58 – 2.51 (m, 3H), 2.48 (t, $J = 6.8$ Hz, 2H), 1.78 – 1.53 (m, 4H), 1.29 (d, $J = 6.4$ Hz, 3H), 0.98 (t, $J = 7.2$ Hz, 3H). $^{13}$C NMR (126 MHz, CDCl₃): δ 11.5, 20.5, 24.1, 34.5, 48.4, 48.5, 52.9, 53.7, 56.0, 67.8, 69.7, 69.9, 70.6, 70.8, 70.9, 71.0, 76.3, 78.1, 99.4, 114.3, 114.5, 117.5, 121.6, 125.1, 129.0, 130.9, 131.7, 132.3, 132.4, 134.9, 149.3, 149.6, 152.2, 160.8, 162.3, 194.5. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2922.59, 2869.56, 1646.91, 1599.66, 1576.52, 1538.92 HRMS (ESI) $C_{40}H_{48}ClN_{3}O_{6}$: Calculated for [M+H] $C_{40}H_{49}ClN_{3}O_{6}$, 702.3310; found: 702.3304.
CQ Pulldown

Alkyne 150 (13mg, 0.019 mmol, 1.0 eq.) was added to a screwcap vial and placed under an atmosphere of argon. Desthiobiotin azide 140 was dissolved in MeOH (12 mg/100uL). The whole solution (100 uL) was added to the reaction vessel. A separate aqueous CuSO4 solution was made (100mM) and was used to dissolve the copper (I) ligand THPTA (10mg/100 uL). An aliquot of the CuSO4/THPTA solution (20uL, 0.0018 mmol of Cu and 0.0036 mmol of THPTA) were added to the reaction vessel. Sodium ascorbate (10mg, 0.05 mmol, 2.7 eq.) was then added to the reaction vessel as a solid, and the reaction was stirred for 16 hours at 23 °C. The reaction was monitored by HPLC and was complete when consumption of the alkyne containing starting material was observed (C18, 19 mm x 250 mm, gradient elution: 10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 40 min). The completed reaction was then diluted in 1 mL of methanol and filtered through a 0.45 uM filter. The reaction was then concentrated under reduced pressure to afford 33 mg of a brown film. The film was dissolved in 300 uL of methanol and purified by HPLC (C18, 19 mm x 250 mm, gradient
elution: 10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 40 min, retention time = 21.7 min). The product containing fractions were combined and their pH was brought to 12 by the addition of NH₄OH (aq) (1 mL). NaCl was then added to saturate the aqueous solution. The aqueous solution was then extracted with CHCl₃ (3 x 5 mL). The CHCl₃ extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a film on a vial 156 (8mg, 39%), the structure of which was confirmed by ¹H NMR. ¹H NMR (500 MHz, CDCl₃): δ 0.98 (t, J = 7.1 Hz, 3H), 1.11 (d, J = 6.5 Hz, 3H), 1.21 – 1.52 (m, 6H), 1.60 (dt, J = 22.0, 7.1 Hz, 4H), 1.76 (d, J = 22.5 Hz, 8H), 2.15 (t, J = 7.5 Hz, 2H), 2.48 (t, J = 6.8 Hz, 2H), 2.55 (q, J = 7.1 Hz, 2H), 2.58 – 2.71 (m, 2H), 3.42 (q, J = 5.3 Hz, 2H), 3.48 – 3.74 (m, 20H), 3.78 – 3.85 (m, 3H), 3.87 – 3.92 (m, 2H), 4.13 – 4.18 (m, 2H), 4.38 (s, 1H), 4.54 – 4.60 (m, 2H), 4.86 (s, 1H), 5.29 (s, 2H), 5.38 (d, J = 7.4 Hz, 1H), 6.26 (s, 1H), 6.40 (d, J = 5.5 Hz, 1H), 6.94 (d, J = 8.8 Hz, 1H), 7.06 (d, J = 9.0 Hz, 1H), 7.33 (dd, J = 9.0, 2.2 Hz, 1H), 7.73 – 7.80 (m, 4H), 7.86 (s, 1H), 7.92 (d, J = 2.2 Hz, 1H), 8.49 (d, J = 5.4 Hz, 1H). ¹³C NMR (126 MHz, CDCl₃): δ 194.53, 173.11, 163.24, 162.32, 161.58, 152.16, 149.56, 149.34, 143.50, 134.90, 132.39, 131.41, 130.82, 128.86, 125.10, 124.39, 121.70, 117.54, 114.47, 114.26, 99.35, 71.02, 70.85, 70.77, 70.73, 70.64, 70.61, 70.59, 70.30, 70.11, 69.78, 69.69, 69.57, 67.76, 62.26, 56.14, 53.70, 52.90, 51.54, 50.57, 48.54, 48.43, 39.30, 36.36, 34.52, 29.65, 29.11, 26.26, 25.49, 24.09, 20.46, 15.98, 11.49. FTIR (thin film) λ (cm⁻¹): 3433.64, 1644.98, 1604.48, 1578.45. HRMS (ESI) C₅₈H₈₂ClN₉O₁₁: Calculated for [M+2H]/2 C₅₈H₈₄ClN₉O₁₁, 558.7977; found: 558.7990. [α]D²² −20.3 (c 1.0, CH₂Cl₂).
Acid\textsuperscript{141} 189 (306 mg, 0.64 mmol, 1.0 eq.) was added to a round bottom flask, followed by EDC-HCl (185 mg, 0.96 mmol, 1.5 eq.), and HOBT (130 mg, 0.96 mmol, 1.5 eq.). In a separate flask, amine\textsuperscript{159} 204 (102 mg, 0.706 mmol, 1.1 eq.) was dissolved in DMF (1.5 mL). Triethylamine (90 uL, 0.64 mmol, 1.0 eq.) was added to the DMF solution. The DMF solution was then added to the reaction vessel via a syringe. The reaction was stirred for 2.5 hours, until the acid was no longer detected by LC/MS. The reaction was concentrated to an oil, which was diluted with Et\textsubscript{2}O (10 mL). The solution was washed with water (2 x 5 mL) to remove any remaining DMF. The organic layer was dried over anhydrous MgSO\textsubscript{4} and concentrated under reduced pressure to a yield a crude white foam (384 mg). The foam was then purified via column chromatography (SiO\textsubscript{2}, 25 mm x 120 mm, gradient of increasing MeOH, 3%-5%, in CH\textsubscript{2}Cl\textsubscript{2}, maintaining a constant 1% of NH\textsubscript{4}OH (aq)). The product was isolated as a white foam 205 (259 mg, 67% yield), the structure of which was verified by \textsuperscript{1}H NMR. TLC (R\textsubscript{f} = 0.5, 5:94:1; MeOH:CH\textsubscript{2}Cl\textsubscript{2}:NH\textsubscript{4}OH). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\ 1.45\ (s,\ 9H),\ 1.59\ (m,\ 2H),\ 2.36\ (m,\ 7H),\ 2.58\ (s,\ 3H),\ 2.74\ (s,\ 1H),\ 3.16 – 3.34\ (m,\ 2H),\ 3.69\ (s,\ 4H),\ 4.32\ (d,\ J = 94.4\ Hz,\ 1H),\ 6.55\ (d,\ J = 55.6\ Hz,\ 1H),\ 7.17 – 7.23\ (m,\ 3H),\ 7.24 – 7.31\ (m,\ 6H),\ 7.40 – 7.45\ (m,\ 6H). \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}) \(\delta\ 144.77,\ 129.75,\ 128.09,\ 126.86,\ 66.88,\ 53.85,
30.50, 28.53. FTIR (thin film) λ (cm⁻¹): 3434.6, 1662.34. HRMS (ESI) C₃₅H₄₅N₃O₄S:
Calculated for [M+H] C₃₅H₄₆N₃O₄S, 604.3209; found: 604.3205.

The carbamate 205 (195 mg, 0.32 mmol, 1.0 eq.) was added to a round bottom flask and dissolved in CH₂Cl₂ (1.2 mL). Trifluoroacetic acid (230 uL) was added to the flask, and the reaction was stirred for 24 hours until the starting carbamate was consumed as observed via TLC (Carbamate Rᶠ = 0.45, 5:1:94 MeOH:NH₄OH:CH₂Cl₂) (Product Rᶠ = 0.35, 5:1:94 MeOH:NH₄OH:CH₂Cl₂). The reaction is diluted with CH₂Cl₂(10 mL) then concentrated under reduced pressure to afford a viscous yellow oil. The oil was flashed through a plug of SiO₂ (5:1:94, MeOH:NH₄OH:CH₂Cl₂) and the filtrate was concentrated to a viscous white paste 206 (153 mg, 94%), the structure of which was verified by ¹H NMR. The ¹H NMR of the molecule presents as a mixture of rotamers at a temperature of 300 K. However, ¹H NMR at an elevated temperature observes the coalescence of the rotameric mixture. TLC (Rᶠ = 0.35, 5:1:94 MeOH:NH₄OH:CH₂Cl₂). ¹H NMR (500 MHz, CDCl₃); δ 1.64 (ddt, J = 9.7, 6.9, 3.0 Hz, 2H), 2.16 (s, 3H), 2.32 – 2.43 (m, 6H), 2.49 (dd, J = 12.3, 8.2 Hz, 1H), 2.63 – 2.74 (m, 2H), 3.20 – 3.35 (m, 2H), 3.69 (t, J = 4.7 Hz, 4H), 7.18 – 7.24 (m, 3H), 7.29 (dd, J = 8.5, 6.9 Hz, 6H), 7.40 – 7.47 (m, 6H), 7.57 (t, J = 5.8 Hz, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 172.31, 144.64, 129.67, 128.06, 126.88, 67.01, 66.92, 63.50, 57.39, 53.88, 38.28, 35.26, 34.99, 25.83. FTIR (thin film) λ (cm⁻¹):
The amine¹⁶⁰ 211 (388 mg, 3.1 mmol, 1.0 eq.) and aldehyde¹⁶¹ 202 (953 mg, 4.7 mmol, 1.5 eq.) were added to a round bottom flask. The two compounds were dissolved in CH₂Cl₂ (16 mL) and were stirred at 23 °C for 10 minutes. Sodium triacetoxyborohydride was added to the flask in one portion and the reaction was stirred for 48 hours. The reaction was quenched by dilution with CH₂Cl₂ (20 mL), and 2N NaOH (aq) (30 mL). The biphasic mixture was stirred for 60 minutes. The layers of the biphasic mixture were separated, and the aqueous layer was extracted with CH₂Cl₂ (2 x 20 mL). The combined organic fractions were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a crude paste (1.12 g). The crude paste was then purified by column chromatography (SiO₂, 35 mm x 130 mm, 3:1:96, MeOH:NH₄OH:CH₂Cl₂) to afford a solid 212 (820 mg, 84%), the structure of which was verified by ¹H NMR. MP (CH₂Cl₂) 153-155 °C. TLC (R_f = 0.5, 4:95:1, MeOH:NH₄OH:CH₂Cl₂). ¹H NMR (500 MHz, CDCl₃ δ 7.83 (dd, J = 5.4, 3.0 Hz, 2H), 7.70 (dd, J = 5.5, 3.0 Hz, 2H), 3.76 (t, J = 6.9 Hz, 2H), 3.19 (d, J = 2.5 Hz, 2H), 2.42 (t, J
= 6.9 Hz, 8H), 2.18 (t, J = 2.4 Hz, 1H), 1.86 (p, J = 6.9 Hz, 2H). $^{13}$C NMR (126 MHz, CDCl₃) δ 168.63, 133.97, 132.50, 123.32, 79.01, 73.23, 56.15, 53.11, 51.96, 46.93, 36.82, 25.45. FTIR (thin film) λ (cm⁻¹): 3272.61, 2937.06, 2814.60, 2369.48, 1771.30, 1706.69. HRMS (ESI) C₁₈H₂₁N₃O₂: Calculated for [M+H] C₁₈H₂₂N₃O₂, 312.1728; found: 312.1719.

Phthalimide 212 (820 mg, 2.64 mmol, 1.0 eq.) was added to a screwcap vial, and placed under an atmosphere of argon. The phthalimide was dissolved in EtOH (4 mL), and hydrazine (190 µL, 4.0 mmol, 1.5 eq.) was added via a syringe. The reaction was sealed and heated to reflux in an oil bath preset to 94 °C. The reaction was heated for 2 hours, where after 30 minutes a white precipitate begins to form. The reaction was observed to be complete by the loss of phthalimide starting material, as observed via LC/MS. The reaction was cooled to 23 °C and diluted with EtOH (4 mL). The reaction was filtered through a pad of Celite™. The white solid removed by filtration was washed with EtOH (2 mL), and the combined filtrate was concentrated under reduced pressure to yield a paste (213) (429 mg, 90%), the structure of which was verified by $^1$H NMR. MP (EtOH) 100-105 °C TLC (Rf = 0.0, compound immobile on silica). $^1$H NMR (500 MHz, CDCl₃) δ 3.29 (d, J = 2.5 Hz, 2H), 2.74 (t, J = 6.8 Hz, 2H), 2.69 – 2.35 (m, 10H), 2.23 (t, J = 2.4 Hz, 1H), 1.63 (dd, J = 16.1, 9.0 Hz, 2H). $^{13}$C NMR (126 MHz, CDCl₃) δ 79.02,
N-Methyl-N-Boc-S-Trt-Cys-OH\(^{141}\) 189 (250 mg, 0.52 mmol, 1.0 eq.) was added to a round bottom flask, followed by EDC (150 mg, 0.79 mmol, 1.5 eq.) and HOBT (105 mg, 0.79 mmol, 1.5 eq.). The flask was placed under an atmosphere of argon. In a separate flask, the amine 213 (107 mg, 0.58 mmol, 1.1 eq.) was placed under an argon atmosphere and dissolved in DMF (1.5 mL). Triethylamine (70 uL, 0.52 mmol, 1.0 eq.) was added to the DMF solution, and vortexed for 30 seconds. The DMF solution was then added via a syringe to the original reaction vessel. The reaction was stirred for 3 hours until the acid was consumed as observed by LC-MS. The reaction was then quenched by pouring onto water (20 mL). Upon addition to the water, a white precipitate was observed. Addition of EtOAc (20 mL) was added to solubilize the precipitate. The biphasic solution was separated, and the aqueous fraction was extracted with EtOAc (2 x 20 ml). The combined organic fractions were washed with water (2 x 20 mL) and brine (1 x 60 mL). The organic layer was then dried over anhydrous sodium sulfate and concentrated under reduced pressure yielding crude white foam. The crude product was then purified by
column chromatography (SiO₂, 35 mm x 150 mm, gradient elution 2%, 3%, 4%, and 5% MeOH in CH₂Cl₂, increased every 100 mL), yielding a white foam (214) (279 mg, 83%), the structure of which was verified by ¹H NMR. The ¹H NMR of the molecule presents as a mixture of rotamers at a temperature of 300 K. However, ¹H NMR at an elevated temperature observes the coalescence of the rotameric mixture. TLC (Rᵋ = 0.4, 4:95:1, MeOH:NH₄OH:CH₂Cl₂). ¹H NMR (500 MHz, CDCl₃) δ 8.22 (s, 1H), 7.33 (d, J = 6.5 Hz, 12H), 7.28 – 7.21 (m, 3H), 4.28 (s, 1H), 3.67 (s, 2H), 3.23 (d, J = 2.4 Hz, 2H), 3.11 – 2.88 (m, 9H), 2.68 (dd, J = 12.3, 5.4 Hz, 1H), 2.61 (s, 3H), 2.48 – 2.21 (m, 11H), 1.53 (d, J = 8.8 Hz, 2H), 1.39 (s, 9H). ¹³C NMR (126 MHz, CDCl₃) δ 144.71, 129.68, 128.02, 126.80, 79.06, 73.26, 66.82, 57.67, 56.69, 56.13, 53.20, 52.00, 46.90, 38.10, 36.55, 31.12, 30.37, 28.50, 26.37. FTIR (thin film) λ (cm⁻¹): 2933.20, 1683.55, 1507.1. HRMS (ESI) C₃₈H₄₈N₄O₃S: Calculated for [M+H] C₃₈H₄₉N₄O₃S, 641.3525; found: 641.3510.

The carbamate 214 (200 mg, 0.31 mmol, 1 eq.) is added to a round bottom flask and placed under an atmosphere of argon. CH₂Cl₂ (3 mL) is added to the flask via a syringe to dissolve the carbamate. While stirring, TFA (3 mL) is added to the flask via a syringe, and the reaction is stirred at 23 °C until the carbamate starting material has disappeared as observed via LC-MS. Once the reaction was complete, the solvent was removed under reduced pressure to afford a crude paste. The paste was dissolved in CH₂Cl₂ (10 mL) and conc. NH₄OH (2mL) was added. The reaction was vortexed for 30
seconds, and brine (10 mL) was added to the reaction mixture. The now biphasic mixture was separated, and the resulting aqueous phase was extracted with CH$_2$Cl$_2$ (2 x 10 mL). The resulting organic layers were combined and dried over anhydrous sodium sulfate.

The organic layer was then concentrated under reduced pressure to afford the product as a translucent gel (215) (152mg, 90%). TLC (R$_f$ = 0.1, 4:95:1, MeOH:NH$_4$OH:CH$_2$Cl$_2$). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.53 (s, 1H), 7.44 – 7.39 (m, 6H), 7.28 (dd, $J$ = 8.4, 6.8 Hz, 6H), 7.24 – 7.19 (m, 3H), 3.34 – 3.20 (m, 4H), 2.72 – 2.41 (m, 12H), 2.26 (d, $J$ = 2.4 Hz, 1H), 2.15 (s, 3H), 1.68 (s, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 172.42, 144.70, 129.74, 128.12, 128.08, 126.93, 73.46, 66.97, 63.51, 56.78, 53.18, 51.68, 46.92, 38.23, 35.27, 35.04, 26.03. FTIR (thin film) λ (cm$^{-1}$): 3302.5, 2936.09, 2359.48, 1669.09, 1519.63. HRMS (ESI) C$_{33}$H$_{40}$N$_4$OS: Calculated for [M+H] C$_{33}$H$_{41}$N$_4$OS, 541.3001; found: 541.3026

CyOH$^{139}$ (173) (50 mg, 0.1 mmol, 1.0 equiv). is added to a round bottom flask and placed under an atmosphere of argon. In a separate flame dried round bottom flask solid trisphosgene (30 mg, 0.1 mmol, 1.0 equiv.) is added and placed under an atmosphere of argon. The trisphosgene containing flask is cooled in an ice bath, and CH$_2$Cl$_2$ (1 mL) is added via a syringe. CH$_2$Cl$_2$ (4 mL) is added to the flask containing
CyOH (173), and triethylamine (14 uL, 0.19 mmol, 1.0 equiv.) is added as a solution in CH₂Cl₂. The solution of compound CyOH (173) and triethylamine is vortexed until the solution becomes homogeneous. A syringe pump is used to add the solution of CyOH (173) to the flask of triphosgene over 30 min. The solvent is then removed via a stream of argon and a 40 °C oil bath. The resulting crude mixture is then carried forward to the acylation reaction. The crude reaction mixture is dissolved in CH₂Cl₂ and stirred in an ice bath. The amine 206 (54mg, 1.0 mmol, 0.5 equiv.) is dissolved in CH₂Cl₂ and triethylamine (14 uL, 0.19 mmol, 1.0 equiv.) is added to the solution. The amine containing solution is then added dropwise to the crude reaction, while stirring in an ice bath. The reaction is then stirred for 30 minutes and monitored via LC-MS, where consumption of the crude triphosgene reaction intermediates can be monitored. Once the reaction is complete, the solvent is removed under reduced pressure to afford a crude mixture containing acylated intermediate as observed via LC-MS. The solvent is removed under reduced pressure to afford a crude purple paste.

Crude intermediate (0.095 mmol, 1.0 eq.) was dissolved with CH₂Cl₂ (5mL) in round bottom flask under an atmosphere of argon. To the reaction TFA(1.5 mL), and triisopropylsilane (31 uL, mmol, 5.0 eq) were added to the reaction with a syringe. The reaction was stirred for ten minutes and verified complete via LC/MS (Mass -243 for loss of trityl). The reaction was then concentrated under reduced pressure to remove excess TFA. The crude reaction mixture was then dissolved in CH₂Cl₂ (5 mL) and stirred at 23 °C. Palmitoyl chloride was added to the flask via a syringe, and the reaction was stirred for two hours and monitored via LC/MS. Observe the product mass, the starting dye
CyOH, and palmitoylated CyOH, therefore the reaction was quenched and worked up.

The reaction was concentrated under reduced pressure to a blue paste and dissolved in CH$_2$Cl$_2$ (20 mL). The organic solution was washed with 50% saturated NaHCO$_3$ (20 mL). The layers are separated, and organic layer is collected. The remaining aqueous phase is extracted with CH$_2$Cl$_2$ (1x20 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford a blue purple solid (240 mg). The blue solid was triturated with diethyl ether, which removes the palmitoylated CyOH side product from the crude reaction mixture. The remaining solid was solubilized in methanol and 50% MeCN: 50% Water (0.1% TFA). The solution is filtered through a 0.22 um filter, and purified via HPLC (RP-HPLC, C$_{18}$, 19 mm X 250 mm, 45%MeCN to 99% MeCN in water (0.1% TFA) over 30 minutes). Pure product is obtained as a purple film (198) (22 mg, 20% over three steps). The structure of which was verified by $^1$H NMR and HRMS. The compound is a mixture of rotamers at 300 K which coalesce at 350 K. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.63 (d, $J = 15.2$ Hz, 1H), 8.15 (s, 1H), 7.73 (t, $J = 7.9$ Hz, 2H), 7.62 – 7.48 (m, 3H), 7.42 (s, 1H), 7.34 (s, 1H), 7.15 (d, $J = 8.4$ Hz, 1H), 6.67 (d, $J = 15.2$ Hz, 1H), 4.71 (s, 1H), 4.43 (d, $J = 7.3$ Hz, 2H), 3.79 (s, 4H), 3.61 – 3.31 (m, 12H), 3.28 – 3.16 (m, 10H), 3.10 – 2.65 (m, 7H), 2.59 (s, 2H), 1.95 – 1.74 (m, 13H), 1.58 (s, 2H), 1.34 – 1.13 (m, 29H), 1.02 (d, $J = 8.3$ Hz, 3H), 0.91 – 0.80 (m, 3H). $^{13}$C NMR (126 MHz, MeOD) $\delta$ 200.55, 180.55, 172.00, 161.59, 156.07, 154.97, 154.56, 147.73, 143.92, 142.98, 132.78, 131.40, 130.54, 129.50, 129.27, 124.01, 121.15, 120.70, 116.11, 114.74, 110.96, 106.85, 65.15, 62.56, 56.04, 53.24, 52.59, 48.09, 44.93, 37.42, 35.22, 33.23, 30.95, 30.92, 30.63, 30.45, 30.12, 28.41, 28.39, 27.09, 25.15, 25.07, 23.89, 22.64, 21.66, 14.60, 11.71. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2925, 2853, 1719, 1685, 311
1578, 1535, 1458, 1371, 1285, 1255, 1205, 1186, 1173, 1122, 1064. HRMS (ESI)

CyOH\textsuperscript{139} (173) (100mg, 0.2 mmol, 1.0 equiv.) is added to a round bottom flask and placed under an atmosphere of argon. In a separate flame dried round bottom flask solid trisphosgene (60 mg, 0.2 mmol, 1.0 equiv.) is added and placed under an atmosphere of argon. The trisphosgene containing flask is cooled in an ice bath, and THF (1 mL) is added via a syringe. A 1:1 mixture of CH\textsubscript{2}Cl\textsubscript{2}:THF (8.5 mL) is added to the flask containing CyOH (173), and triethylamine 28 \textmu L, 0.4 mmol, 2.0 equiv.) is added as a solution in THF. The solution of compound CyOH (173) and triethylamine is vortexed until the solution becomes homogeneous. A syringe pump is used to add the solution of CyOH (173) to the flask of trisphosgene over 2 hours. The solvent is then removed via a stream of argon and a 40 °C oil bath. The resulting crude mixture is then carried forward to the acylation reaction.

The crude reaction mixture is dissolved in CH\textsubscript{2}Cl\textsubscript{2} and stirred in an ice bath. The amine (215) (54mg, 1.0 mmol, 0.5 equiv.) is dissolved in CH\textsubscript{2}Cl\textsubscript{2} and triethylamine (14 \textmu L, 0.19 mmol, 1.0 equiv.) is added to the solution. The amine containing solution is then added dropwise to the crude reaction, while stirring in an ice bath. The reaction is then stirred for 30 minutes and monitored via LC-MS, where consumption of the crude trisphosgene reaction intermediates can be monitored. Once the reaction is complete, the
solvent is removed under reduced pressure to afford a crude mixture containing acylated intermediate as observed via LC-MS. The solvent is removed under reduced pressure to afford a crude purple paste.

Crude intermediate is dissolved in CH$_2$Cl$_2$ (2 mL). Trifluoroacetic acid (2 mL) is then added to the reaction mixture, and triisopropylsilane (100 uL, 0.5 mmol, 5.0 equiv.) is added as well. The reaction mixture is stirred for 30 minutes, and monitored via LC-MS. Once the trityl-protected intermediate is consumed, the reaction is diluted with CH$_2$Cl$_2$ (10 mL) and concentrated under reduced pressure to afford a crude purple paste. The paste is dissolved in CH$_2$Cl$_2$ (1.5 mL) and stirred in at room temperature. To the solution, palmitoylchloride (2.5 mL) is added via a syringe, and the reaction is stirred until the free thiol is mostly consumed after stirring for 16 hours. The reaction is diluted with methanol and stirred for 30 minutes to quench excess palmitoyl chloride. The reaction is then concentrated to a crude purple solid. Trituration of the purple solid with Et$_2$O removed methyl palmitate and CyOH which reacted with palmitoyl chloride. The remaining solid (125 mg) is then dissolved in acetonitrile (500 uL), diluted in half with a solution of (50% acetonitrile, 50% water, containing 1% TFA) and filtered through a 0.45 um filter. This mixture is then purified via RPHPLC (C18, 19 mm x 250 mm, 45% MeCN to 99%MeCN over 30 minutes). The product 218 is isolated as a purple film (25 mg, 19% over 3 steps), the structure of which was determined by NMR and HRMS. The molecule is observed by NMR as a mixture of rotamers, which coalesce at 350 K. $^1$H NMR (500 MHz, DMSO) δ 8.64 (d, J = 15.2 Hz, 1H), 8.13 (s, 1H), 7.72 (t, J = 6.8 Hz, 2H), 7.57 (dd, J = 11.1, 8.1 Hz, 2H), 7.50 (t, J = 7.5 Hz, 1H), 7.41 (s, 1H), 7.33 (d, J =
2.2 Hz, 1H), 7.15 (dd, J = 8.4, 2.2 Hz, 1H), 6.67 (d, J = 15.1 Hz, 1H), 4.71 (s, 1H), 4.43 (t, J = 7.3 Hz, 2H), 3.52 (dd, J = 14.0, 5.4 Hz, 1H), 3.45 – 3.34 (m, 3H), 3.31 – 3.15 (m, 6H), 3.10 – 2.94 (m, 6H), 2.87 – 2.68 (m, 8H), 2.60 (t, J = 7.3 Hz, 2H), 2.09 (s, 2H), 1.91 (q, J = 7.5 Hz, 6H), 1.80 (s, 6H), 1.59 (q, J = 7.4 Hz, 2H), 1.35 – 1.15 (m, 25H), 1.04 (t, J = 7.4 Hz, 3H), 0.87 (t, J = 6.7 Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) δ 200.44, 180.43, 171.94, 162.58, 161.41, 155.92, 154.82, 154.43, 154.41, 147.59, 143.80, 142.85, 132.64, 131.27, 130.42, 129.40, 129.16, 123.89, 121.03, 120.51, 119.26, 115.98, 114.63, 110.89, 106.77, 77.99, 76.02, 66.93, 62.50, 60.97, 55.36, 53.02, 52.48, 47.95, 46.65, 44.79, 37.33, 35.21, 33.10, 30.83, 30.80, 30.75, 30.60, 30.55, 30.51, 30.32, 30.00, 28.25, 26.96, 26.11, 25.39, 25.01, 23.76, 22.52, 21.53, 15.46, 14.47, 11.58. FTIR (thin film) λ (cm$^{-1}$): 3308, 2924, 2853, 1716, 1682, 1534, 1458, 1442, 1426, 1371, 1285, 1254, 1206, 1186, 1286, 1064. HRMS (ESI) $\text{C}_{59}\text{H}_{84}\text{N}_{5}\text{O}_{5}\text{S}$: Calculated for [M$^+$] $\text{C}_{59}\text{H}_{84}\text{N}_{5}\text{O}_{5}\text{S}$, 974.6193.5877; found: 974.6185.
The alkyne 218 (8 mg, mmol, 1.0 equiv.) was added to a screwcap vial with a stir bar. BHQ3 221 (6 mg, mmol, 1.5 equiv.) was added to the screwcap vial. The vial contents were placed under an atmosphere of argon and dissolved in DMSO (200 uL). Water was added to the screwcap vial, and the vial contents were vortexed for 30 seconds. Cu(II)SO₄ (40 uL of 0.1 M aqueous solution, mmol, 0.2 equiv.) was added via the use of a pipet. Sodium ascorbate (40 uL of 0.1 M aqueous solution, mmol, 0.2 equiv.) was added to the reaction via a pipet and the screwcap vial was vortexed for 30 seconds. The reaction was stirred for two hours until the alkyne was consumed as observed by LC-MS. The reaction was diluted 1:1 with a mixture of water and acetonitrile (1 mL). The resulting solution was filtered through a 0.45 um filter, and purified by reverse phase HPLC (C₁₈, 19 mm x 250 mm, 30 min gradient of increasing MeCN in water (0.1% TFA): 45%-99% over 30 minutes, 13 mL/min flow rate). The product 222 was isolated as a purple-blue film, the structure of which was determined by ¹H NMR. The molecule at 300 K, as observed by NMR, was found to be a mixture of rotamers, which coalesce at a
temperature of 340 K. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.62 (d, $J = 15.2$ Hz, 1H), 8.43 (d, $J = 9.0$ Hz, 1H), 8.25 – 8.09 (m, 3H), 8.06 – 7.85 (m, 6H), 7.82 – 7.68 (m, 6H), 7.57 (t, $J = 8.8$ Hz, 2H), 7.49 (t, $J = 7.4$ Hz, 1H), 7.41 (s, 1H), 7.30 (t, $J = 4.2$ Hz, 2H), 7.13 (d, $J = 8.5$ Hz, 1H), 6.81 (d, $J = 8.9$ Hz, 2H), 6.67 (d, $J = 15.1$ Hz, 1H), 5.80 (s, 1H), 4.64 (t, $J = 6.1$ Hz, 3H), 4.43 (t, $J = 7.4$ Hz, 2H), 4.00 (t, $J = 6.2$ Hz, 2H), 3.80 (s, 2H), 3.62 (s, 3H), 3.51 – 3.42 (m, 1H), 3.33 (dd, $J = 14.0$, 9.8 Hz, 1H), 3.15 (d, $J = 6.5$ Hz, 2H), 3.08 – 2.66 (m, 19H), 2.58 (d, $J = 7.2$ Hz, 2H), 1.90 (dt, $J = 13.8$, 7.1 Hz, 4H), 1.79 (s, 9H), 1.56 (s, 2H), 1.34 – 1.11 (m, 39H), 1.02 (t, $J = 7.4$ Hz, 3H), 0.86 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (126 MHz, CD$_3$CN) $\delta$ 199.95, 179.81, 169.61, 161.33, 157.23, 156.36, 154.96, 154.35, 154.33, 153.96, 147.37, 145.51, 144.62, 143.49, 143.47, 142.64, 140.55, 139.33, 136.95, 135.52, 135.15, 133.79, 133.69, 132.78, 132.75, 132.58, 132.38, 131.03, 130.07, 129.10, 128.95, 128.70, 128.54, 127.26, 126.75, 125.25, 123.66, 121.20, 120.96, 120.59, 120.32, 120.03, 115.76, 115.61, 114.32, 112.53, 112.42, 112.37, 110.63, 106.16, 92.93, 92.89, 62.35, 60.51, 52.36, 52.05, 49.88, 48.58, 47.85, 44.67, 44.55, 38.35, 36.76, 35.31, 32.68, 30.44, 30.41, 30.37, 30.35, 30.22, 30.20, 30.12, 30.11, 30.08, 29.95, 29.53, 28.28, 28.16, 28.10, 28.05, 26.57, 26.38, 24.85, 23.44, 22.06, 21.06, 14.45, 13.95, 11.59, 11.57. FTIR (thin film) $\lambda$ (cm$^{-1}$): 2921, 1680, 1535, 1460, 1284, 1186. HRMS (ESI) C$_{90}$H$_{116}$N$_{14}$O$_{5}$S: Calculated for [M$^{2+}$+H$^+$] C$_{90}$H$_{117}$N$_{14}$O$_{5}$S$^{2+}$, 501.9684; found: 501.9672.
NMR Spectra
H$_2$N\[\begin{array}{c}
\text{O} \\
\text{N} \\
\text{CO$_2$Me} \end{array}\]
\[\begin{array}{c}
\text{J}_3 \\
\text{J}_3 \\
\text{NH}_2 \\
\text{96}
\end{array}\]

2HCl
NMR taken at 350 K
432 105
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