NOVEL ARTIFICIAL CELL SURFACE RECEPTORS

AND RELATED MOLECULAR PROBES

A Thesis in
Chemistry
by
Sutang Cai

© 2008 Sutang Cai

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

December 2008
The thesis of Sutang Cai was reviewed and approved* by the following:

Blake R. Peterson
Regents Distinguished Professor of Medicinal Chemistry
University of Kansas
Thesis Advisor

Raymond L. Funk
Professor of Chemistry

Mary Beth Williams
Associate Professor of Chemistry

Ayusman Sen
Professor of Chemistry
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

Many receptors on the surface of mammalian cells actively promote the uptake of specific cell impermeable ligands via receptor-mediated endocytosis. To mimic this process, we synthesized artificial cell surface receptors that comprise \(N\)-alkyl derivatives of \(3\beta\)-cholesterylamine linked to ligand binding motifs. These synthetic receptors enable the uptake of proteins and other cell-impermeable molecules, a strategy termed “synthetic receptor targeting”. To improve the efficiency of synthetic receptors and extend their utility as molecular probes, tools for drug delivery, and agents for cellular targeting, we report here studies of structure-activity relationships (SAR). Receptors with different linkers between \(3\beta\)-cholesterylamine and a ligand binding motif were designed, synthesized, and evaluated. It was found that installation of glutamic acid subunits in the linker region of synthetic receptors greatly enhances their cellular activities. Receptors with glutamic acids associate with plasma membrane of living mammalian cells much faster and to a greater extent and can deliver more ligands compared to receptors lacking these subunits.

Although synthetic receptors can promote the internalization of many cell impermeable bioactive molecules, trapping of this cargo in endosomes is a major limitation for many applications. This limitation is also commonly observed with many other endocytic delivery systems. To circumvent this issue, we synthesized two different \(3\beta\)-cholesterylamine derivatives: one was linked to a pH-dependent membrane-lytic dodecapeptide, and the second comprised a disulfide-linked
fluorophore coupled through glutamic acid subunits. When added to living cells, membranes of early / recycling endosomes were selectively disrupted, resulting in cleavage of the disulfide and release of the fluorophore into the cytosol and nucleus. The ability of this system to deliver cargo into and release disulfide-linked cargo from relatively nonhydrolytic early / recycling endosomes may be useful for the delivery of a variety of sensitive molecules to diverse intracellular targets.

We also studied the feasibility of using small artificial cell surface receptors as a generic drug delivery system. The studies showed that a synthetic receptor comprising 3β-cholesterylamine linked to the antibiotic vancomycin and bearing two glutamic acid residues in the linker region significantly enhanced the uptake of a fluorophore linked to a L-Lys-D-Phe-D-Ala tripeptide. The high efficiency of this delivery system may have potential application in the delivery of drugs and other cell impermeable molecules.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................vii

ACKNOWLEDGEMENTS ....................................................................................xi

Chapter 1 Modification of the Linker Region of Synthetic Receptors and Cellular Probes Improves Delivery Efficacy .................................................1

1.1 Introduction ........................................................................................................ 1
1.2 Design and Synthesis of Novel Pennsylvania Green-Derived Probes .................. 6
1.3 Biological Evaluation of Synthetic Pennsylvania Green Probes ................. 9
1.4 Design, Synthesis and Evaluation of Novel NBD-Derived Probes ............ 11
1.5 Conclusions ........................................................................................................ 16
1.6 Experimental section .......................................................................................... 17
  1.6.1 General ........................................................................................................ 17
  1.6.2. Synthetic Procedures and Compound Characterization Data .......... 19
  1.6.3. Biological Assays and Protocols .............................................................. 35
    1.6.3.1 Cell culture ......................................................................................... 35
    1.6.3.2 Microscopy ........................................................................................ 35
    1.6.3.3 Flow Cytometry ................................................................................. 36
    1.6.3.4 Cellular Association Assays ............................................................... 37
    1.6.3.5 Antibody Uptake Assays ................................................................ 37
    1.6.3.6 Colocalization of Green Fluorescent NBD Derivatives and Red Fluorescent Proteins .............................................. 38
1.7 References ........................................................................................................ 38

Chapter 2 Selective Disruption of Early/Recycling Endosomes: Release of Disulfide-Linked Cargo Mediated by a N-Alkyl-3β-Cholesterylamine-Capped Peptide .............................................................................41

2.1 Introduction ........................................................................................................ 41
2.2 Design, Synthesis and Evaluation of a Disulfide Probe as an Endosome Disruption Indicator ................................................................. 42
2.3 Design, Synthesis and Optimization of PC4 Lipopeptides ......................... 46
2.4 Mechanism of Action of PC4 Endosome Lytic Peptides ............................... 50
  2.4.1 PC4 Lipopeptides and the Disulfide Probe Selectively Target Early / Recycling Endosomes ...................................................... 52
  2.4.2 Requirements for Cargo Release: Cholesterylamine Anchor, Disulfide Linkage, and Endosomal Acidity ........................................ 54
2.5 Conclusions ....................................................................................................... 57
2.6 Experimental section ......................................................................................... 57
  2.6.1 General ..................................................................................................... 57
  2.6.2. Synthetic Procedures and Compound Characterization Data ................................. 59
Chapter 3  Synthetic Cell Surface Receptors that Employ Small Molecule Recognition for Delivery of Drugs and Probes .........................................85

3.1 Introduction ..................................................................................85
3.2 Design and Synthesis of Vancomycin-Based Receptors and L-Lys-D-Phe-D-Ala Ligands .........................................................................88
3.4 Evaluation of the Delivery of L-Lys-D-Phe-D-Ala Conjugate by Vancomycin-Based Receptors ..........................................................92
3.5 Conclusions ....................................................................................96
3.6 Experimental section .........................................................................96
3.6.1 General .....................................................................................96
3.6.2 Synthetic Procedures and Compound Characterization Data...98
3.6.3 Biological Assays and Protocols...............................................109
3.6.3.1 Cell culture ......................................................................109
3.6.3.2 Microscopy ......................................................................109
3.6.3.3 Flow Cytometry ...............................................................110
3.6.3.4 Fluorescence Polarization Assay ....................................110
3.6.3.5 Assays of Cellular Uptake of Fluorescent Ligands ........111
  3.6.3.5.1 Preloading Conditions............................................111
  3.6.3.5.2 Preequilibration Conditions ....................................111
3.6.3.6 Tissue Distribution in vivo................................................112
3.7 References.........................................................................................112
LIST OF FIGURES

Figure 1-1: A simple model of synthetic receptor-mediated endocytosis......2

Figure 1-2: Structures of previously reported synthetic DNP and NBD receptors.................................................................4

Figure 1-3: Structures of previously reported Oregon Green, Tokyo Green and Pennsylvania Green probes.................................5

Figure 1-4: Structures of novel Pennsylvania Green probes. ...............6

Figure 1-5: Synthesis of compounds 6-8..............................................8

Figure 1-6: Confocal laser scanning and differential interference contrast (DIC) micrographs of living Jurkat lymphocytes treated with 5-8 (1 µM) for 5 min (left) and 1 hour (right) at 37 °C. Cells were washed with media and analyzed by microscopy .................................................................9

Figure 1-7: Quantification of cellular fluorescence of Jurkat lymphocytes treated with 5-8 (1 µM) in media for 5 min and 1 h at 37 °C. Cells were washed with media and analyzed by flow cytometry. Error bars represent the standard deviation (n = 3). .................................................10

Figure 1-8: Structures of previously reported NBD receptor 2 and novel NBD receptors 19-21. ..............................................................12

Figure 1-9: Confocal laser scanning/DIC micrographs and flow cytometric analysis (panel D) of fluorescence of Jurkat lymphocytes treated with 2, or 19-21 (10 µM) for 5 min or 1 h at 37 °C. The confocal detector gain was set to 800 for cells treated with compounds for 5 min and was set to 650 for cells incubated with compounds for 1 hour. ................13

Figure 1-10: Synthetic receptor-mediated uptake of anti-DNP IgG by Jurkat lymphocytes. Cells were treated with 2 (Panel A) and 20 (Panel B) (5 µM) respectively at 37 °C for 1 h, washed to remove unincorporated receptors, and incubated with red fluorescent anti-DNP-AF633 (0.16 mg/mL) for 4 h. Cells were washed and analyzed by confocal laser scanning microscopy....................................................14

Figure 1-11: Confocal laser scanning and differential interference contrast (DIC) micrographs of living Jurkat lymphocytes treated with green fluorescent NBD derivative 20 (10 µM) for 1 h followed by (A) red fluorescent Texas Red transferrin (500 nM) or (B) DiI-LDL (8 nM) for 5
min at 37 °C. Colocalization of red and green fluorescence is shown as yellow pixels in the DIC overlay images. .............................................16

Figure 2-1: Structure of a disulfide probe 22 as an endosome disruption indicator and a strategy for the selective release of disulfide-tethered cargo from membranes of early/recycling endosomes. Panel A: products of cleavage of 22 by glutathione; panel B: mechanism of release of fluorophore 23 into the cytosol and nucleus of mammalian cells.................................................................43

Figure 2-2: Synthesis of compound 22. ..........................................................44

Figure 2-3: Confocal laser scanning and differential interference contrast (DIC) microscopy of living J-774 macrophages. Panels A and C: Cells were treated with 10 µM of probe 22 (panel A) or 30 (panel C) for 1 h at 37 °C, washed to remove excess receptors, and treated with L. monocytogenes in fresh media for 2 h. Panels B and D: Cells were treated with 10 µM of 22 or 30 for 3 h respectively ........................................45

Figure 2-4: Structures of first generation PC4 lipopeptides. ............................47

Figure 2-5: Confocal laser scanning and DIC micrographs of living Jurkat lymphocytes treated with 22 (2.5 µM) only (panel A) or 22 and 31, 32, 33, 34, or 35 (2 µM) (panels B-F) for 12 h at 37 °C.................................48

Figure 2-6: Structures of second generation PC4 lipopeptides ......................49

Figure 2-7: Structures of red fluorescent PC4 lipopeptide 36, PC4 control peptide 37, a new disulfide probe 38 and its noncleavable control 39 ......50

Figure 2-8: Synthesis of compounds 38 and 39. ........................................51

Figure 2-9: Confocal laser scanning and differential interference contrast (DIC) micrographs of living CHO cells treated with green fluorescent 38 (5 µM) for 12 h at 37 °C followed by (A) red fluorescent Texas Red transferrin (500 nM) or (B) Dil-LDL (8 nM) for 5 min. Panel C: CHO cells were treated with red fluorescent 36 (5 µM) for 12 h followed by green fluorescent transferrin, Alexa Fluor 488 conjugate (610 nM) for 5 min. Panel D: CHO cells were treated with 36 (2 µM) and 38 (5 µM) for 12 h at 37 °C under conditions that minimally disrupt endosomes. Colocalization of red and green fluorescence is shown as yellow pixels in the DIC overlay images. .......................................................................53

Figure 2-10: Panel A: dose dependence of endosomal release quantified by flow cytometry. CHO cells were treated with 38 (5 µM) and 35 (0 to 16 µM) for 24 h at 37 °C and trypsinized for analysis. Panel B: cellular
viability assay. CHO cells were treated with 35 (0 to 16 \( \mu \)M) for 48 h at 37 °C and analyzed by Cell-Titer-Glo luminescent viability assay.

Figure 2-11: Confocal fluorescence and DIC micrographs of living CHO cells treated with 38 or 39 (5 \( \mu \)M) and 35, 36, or 37 (8 \( \mu \)M) for 24 h at 37 °C. In panel F, [chloroquine] = 5 \( \mu \)M.

Figure 2-12: Analytical HPLC profile of peptide 31 after purification by preparative HPLC. Retention time = 17.4 min. Purity by HPLC > 99%.

Figure 2-13: Analytical HPLC profile of peptide 32 after purification by preparative HPLC. Retention time = 17.2 min. Purity by HPLC > 99%.

Figure 2-14: Analytical HPLC profile of peptide 33 after purification by preparative HPLC. Retention time = 16.7 min. Purity by HPLC > 99%.

Figure 2-15: Analytical HPLC profile of peptide 34 after purification by preparative HPLC. Retention time = 17.1 min. Purity by HPLC > 99%.

Figure 3-1: Structures of a D-Phe-D-Ala derived receptor 45 and a fluorescent derivative of the antibiotic vancomycin (46).

Figure 3-2: Structures of vancomycin derived receptors 47 and 48, a fluorescent derivative of L-Lys-D-Phe-D-Ala 49, and negative control compound 50.

Figure 3-3: Synthesis of compounds 47 and 48.

Figure 3-4: Synthesis of compound 49.

Figure 3-5: Determination of the binding affinity of L-Lys-D-Phe-D-Ala for vancomycin by fluorescence polarization. The concentration of 49 (50 nM) was held constant and the concentration of vancomycin was varied (0 to 100 \( \mu \)M) in phosphate buffered saline (PBS) (200 \( \mu \)L, pH 7.4). Experiments were run in triplicate.

Figure 3-6: Panels A-C: Confocal laser scanning and DIC micrographs of living Jurkat lymphocytes treated with 47 (panel B) or 48 (panel C) (10 \( \mu \)M) for 1 h at 37 °C, washed, and further treated with 49 (5 \( \mu \)M) for 4 h at 37 °C. Panel A: Cells were treated with 49 (5 \( \mu \)M) for 4 h at 37 °C. Panel D: flow cytometric analysis of uptake of 49 (5 \( \mu \)M) by 47 or 48 (10 \( \mu \)M).

Figure 3-7: Panel A: Flow cytometric analysis of dose dependent uptake of 49 by Jurkat lymphocytes mediated by 48. PL = Preloading conditions: cells were treated with 48 for 1 h at 37 °C, washed and...
treated with 49 (5 µM) for 4 h at 37 °C. PE = Preequilibration conditions: 48 was mixed with 49 (5 µM) for 10 min and added to cells for 4 h at 37 °C. Panel B: Flow cytometric analysis of uptake of 49 by tissues of Balb/c mice in vivo. Mice were injected intraperitoneally with compound 48 (50 mg/kg) for 1 h, followed by injection with compound 49 or 50 (25 mg/kg). Mice were sacrificed after 10 h, and fluorescence of cells from tissues was analyzed. Error bars represent the standard deviation (n = 3). The in vivo experiments were run in collaboration with Jianfang Hu, Avery August lab.

Figure 3-8: Analytical HPLC profile of vancomycin-derived receptor 47 after purification by preparative HPLC. Retention time = 11.2 min. Purity by HPLC > 99%. .................................................105

Figure 3-9: Analytical HPLC profile of vancomycin-derived receptor 48 after purification by preparative HPLC. Retention time = 10.7 min. Purity by HPLC > 99%. .................................................108
ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Professor Blake R. Peterson for his support and guidance during my studies. I would also like to thank my committee: Prof. Raymond L. Funk, Prof. Mary Beth Williams and Prof. Avery August. I give special thanks to my coworkers Qi Sun and Dr. Siwarutt Boonyarattanakalin. I have learned a lot from them. I also thank Dr. Jocelyn Edathil, Dr. Sheryl A. Rummel, Dr. Laurie Mottram, Ms. Ewa Maddox, Ms. Runzhi Wu.

I owe my thanks to my family. To my mother, Li Zhang, and father, Guotuan Cai, who have been teaching me how to behave and supporting me all these years. To my brother Weiping Cai who always encourages me. Last but not the least, I thank my husband, Zhiwu Lin, for all the support.
Chapter 1

Modification of the Linker Region of Synthetic Receptors and Cellular Probes Improves Delivery Efficacy

1.1 Introduction

The plasma membrane of mammalian cells contains proteins, carbohydrates and lipids that form a hydrophobic barrier that protects the intracellular environment from toxins and many extracellular molecules. Only small hydrophobic molecules can diffuse across the plasma membrane. Many hydrophilic macromolecules, such as proteins, DNA and drugs require active cellular transportation mechanisms to facilitate their cellular uptake. To overcome this barrier, impermeable macromolecules have been modified with lipids, peptides or polymers to enhance their cellular uptake. However, the mechanisms of these methods are not well understood and their applications in cellular delivery are often limited by variability and cell type specificity.

In nature, many receptors on the surface of mammalian cells mediate the uptake of nutrients from the extracellular environment via receptor-mediated endocytosis. These receptors typically span the plasma membrane or are linked to lipids that insert into the plasma membrane outer leaflet. These
receptors project a ligand-binding domain from the cell surface that recognizes specific ligands, enabling their uptake. This process is also employed by many toxins and viruses to invade living cells.\textsuperscript{7} Macromolecules or drugs of interest have been conjugated to ligands that target cell surface receptors to achieve delivery through receptor-mediated endocytosis.\textsuperscript{8} However, these methods can be limited by low expression of target receptors on specific cell lines. In the past few years, our laboratory has been investigating synthetic mimics of cell surface receptors designed to enable the delivery of many cell impermeable molecules.\textsuperscript{9-16} These artificial receptors are particularly advantageous for delivery of ligands that are not recognized by natural receptors.

Figure 1-1: A simple model of synthetic receptor-mediated endocytosis.\textsuperscript{15}
Our synthetic receptors generally comprise three components: a ligand binding motif, a linker region, and $N$-alkyl derivatives of 3β-cholesterylamine as a membrane anchor. Treatment of mammalian cells with these compounds can greatly enhance cellular uptake of specific target molecules, a method termed “synthetic receptor targeting” (SRT, Figure 1-1). In SRT, synthetic receptors are loaded into the plasma membrane of mammalian cells. Ligand-binding headgroups project from the cell surface, bind to target ligands, and result in the internalization of these macromolecules via endocytosis. When the complexes enter endosomes, synthetic receptors can dissociate from their ligands and cycle back to the cell surface to deliver more ligands.

Recently, Boonyarattanakalin et al.\textsuperscript{15} reported a series of receptors bearing dinitrophenyl (DNP) and structurally similar fluorescent 7-nitrobenz-2-oxa-1, 3-diazole (NBD) headgroups that efficiently internalize anti-DNP IgG antibodies. Interestingly, it was found that the cellular localization of synthetic receptors and their delivery activities are related to the length and structure of the linkers between the membrane anchors and headgroups. It was demonstrated that $\beta$-alanine subunits increase the localization of receptors on the cell surface, which facilitates cellular uptake of target antibodies. Receptors 1 and 2 bearing two $\beta$-alanines (Figure 1-2) were found to significantly enhance cellular uptake of anti-DNP antibodies by equally distributing between the cell surface and intracellular compartments compared with analogues receptors lacking these linker subunits. Moreover, when the $N$-alkyl-3β-cholesterylamine anchor is replaced with $N$-acyl analogues, the synthetic receptors were found to become
primarily associated with membranes of the golgi apparatus and nucleus of cells. These results indicated that the structure and length of linker region is critical in the cellular association of receptors and their ligand delivery efficiency.

Figure 1-2: Structures of previously reported synthetic DNP and NBD receptors.\textsuperscript{15}

More recently, Mottram et al.\textsuperscript{17} reported the synthesis of a novel fluorophore, Pennsylvania Green, a hybrid of Oregon Green and Tokyo Green. In this study, corresponding synthetic probes 3-5 were synthesized to compare Pennsylvania Green to Oregon Green and Tokyo Green in a cellular environment (Figure 1-3). It was found that the Pennsylvania Green probe was more photostable and less pH-sensitive than Tokyo Green Probe. However, even though the quantum yields of these fluorophores were very similar, the cellular association activity of the Pennsylvania Green probe was not as good as the Oregon Green probe. These two molecules are structurally similar except that a
methyl group in the Pennsylvania Green substitutes for a carboxyl anion in Oregon Green. The extra negative charge on the Oregon Green probe was thought to contribute to its improved cellular association. Based on this idea, we hypothesized that adding an extra negative charge to the Pennsylvania Green probe might significantly improve its cellular activity. Therefore, synthetic cellular probes or receptors bearing negative charges in the linker region were designed, synthesized and evaluated.

Figure 1-3: Structures of previously reported Oregon Green, Tokyo Green and Pennsylvania Green probes.17
1.2 Design and Synthesis of Novel Pennsylvania Green-Derived Probes

Previous studies indicated the importance of the linker on trafficking and localization of synthetic receptors and a correlation between better cellular association of synthetic probes and a negative charge in the probe structure. Based on these studies, it was hypothesized that adding extra negative charges in the linker may enhance the cellular activity of the Pennsylvania Green probe. Therefore, a new generation of Pennsylvania Green probes (6-8) were designed.

![Figure 1-4: Structures of novel Pennsylvania Green probes.](image)
(Figure 1-4). One of the β-alanine subunits in the Pennsylvania Green probe 5 was replaced with L-glutamic acid (6) to add a negative charge. Compound 7 with two glutamic acids was designed to examine whether increasing the number of negative charges would enhance cellular activity. Compound 8 bearing a glycine subunit was also designed as a linker length control for probe 6.

To construct synthetic receptors 6-8, the Boc protected 3β-cholesterylamine anchor 14 was first prepared according to a new route developed by Qi Sun from our lab (Figure 1-5). This method converted cholesterol 9 to 3β-cholesterylamine 12 via methanesulfonyl intermediate in 90% yield over three steps. Monoalkylation of 3-bromopropyl phthalimide followed by Boc protection and ammonolysis of phthalimide gave the desired anchor 14 in good yield. β-Alanine, glycine or t-buty protected glutamic acid were subsequently coupled to anchor 14 using EDC and HOBT. After the Fmoc group at the end of each anchor was cleaved by piperidine, the free amines were coupled with a succinimidyl ester derivative of 4-carboxy-Pennsylvania Green to afford the protected Pennsylvania Green probes 16, 17 and 18. Final deprotection of the Boc and t-buty groups and purification by HPLC afforded the desired probes 6, 7 and 8.
Figure 1-5: Synthesis of compounds 6-8. Reagents and conditions: (a) MsCl, DIEA, CH₂Cl₂, 4 °C; (b) TMSN₃, BF₃·Et₂O, CH₂Cl₂; (c) LiAlH₄, Et₂O, 4 °C to 22 °C; (d) 3-bromopropyl phthalimide, K₂CO₃, DMF, 64 °C; (e) (Boc)₂O, DIEA, CH₂Cl₂; (f) NH₂NH₂, EtOH, 54 °C; (g) Fmoc-β-Ala-OH, EDC, HOBt, CH₂Cl₂, 4 °C to 22 °C; (h) 20% piperidine, DMF; (i) Fmoc-Gly-OH, EDC, HOBt, CH₂Cl₂, 4 °C to 22 °C; (j) Fmoc-Glu(t-Bu)-OH, EDC, HOBt, CH₂Cl₂, 4 °C to 22 °C; (k) 4-Carboxy-Pennsylvania Green succinimidyl ester, DIEA, DMF; (l) 15% TFA in CH₂Cl₂.
1.3 Biological Evaluation of Synthetic Pennsylvania Green Probes

Figure 1-6: Confocal laser scanning and differential interference contrast (DIC) micrographs of living Jurkat lymphocytes treated with 5-8 (1 µM) for 5 min (left) and 1 hour (right) at 37 °C. Cells were washed with media and analyzed by microscopy.
To test the cellular association ability of the Pennsylvania Green probes 6-8 and compare them with 5, confocal laser scanning microscopy and flow cytometry were used to obtain qualitative and quantitative data. In these experiments, Jurkat lymphocytes were treated with Pennsylvania Green probes 5-8 (1 µM) for 5 minutes or 1 hour and washed with fresh media to remove unincorporated probes. Cellular green fluorescence was analyzed by confocal microscopy (Figure 1-6) and further quantified by flow cytometry (Figure 1-7).

![Fluorescence intensity graph](image)

**Figure 1-7**: Quantification of cellular fluorescence of Jurkat lymphocytes treated with 5-8 (1 µM) in media for 5 min and 1 h at 37 °C. Cells were washed with media and analyzed by flow cytometry. Error bars represent the standard deviation (n = 3).

Confocal laser scanning micrographs revealed that the Pennsylvania Green probes 6 and 7 bearing negative charges load into cells much faster and to a greater extent than those without negative charges (5 and 8). However, probe 6 bearing one glutamic acid was more active than probe 7 bearing two
glutamic acids in terms of the rate of cell association, indicating that a single glutamic acid likely mimics the carboxyl anion in the Oregon Green headgroup to maximize the cellular uptake of Pennsylvania Green probes. Moreover, these two probes showed different subcellular localization. Probe 7, bearing two glutamic acids, was found predominantly associated with intracellular compartments while the single glutamic acid containing probe 6 localized both on the cell surface and internal membranes (Figure 1-6, compare panels B and C), indicating that the addition of an extra glutamic acid in the linker also enhances the endosomal localization of Pennsylvania Green probes. Quantification data measured by flow cytometry were consistent with results observed from confocal micrographs. These experiments revealed that the addition of negative charges in the linker enhances the ability of Pennsylvania Green probes to rapidly associate with cells.

1.4 Design, Synthesis and Evaluation of Novel NBD-Derived Probes

Based on the results obtained from the Pennsylvania Green probes, a series of corresponding green fluorescent NBD receptors 19-21 (Figure 1-8) were designed to test whether negative charges could enhance the capacity of synthetic receptors to deliver proteins into mammalian cells. The previously reported synthetic NBD receptor 2 bearing two β-alanine subunits in the linker region, which promotes cellular uptake of anti-DNP antibodies, was used as a
control molecule. The NBD receptors 19-21 (detailed synthesis and compound data are shown in experimental section) were prepared according to routes similar to that used for the Pennsylvania Green probes 6-8.

The activities of these compounds were evaluated by confocal laser scanning microscopy and flow cytometry (Figure 1-9). The results indicated that synthetic receptors 19 and 20 with negative charges were better than the receptors without the negative charges. Unlike the Pennsylvania Green probes, 

![Structures of previously reported NBD receptor 2 and novel NBD receptors 19-21.](image-url)
(A) Cellular localization of 2

(B) Cellular localization of 19

(C) Cellular localization of 20

(D) Figure 1-9: Confocal laser scanning/DIC micrographs and flow cytometric analysis (panel D) of fluorescence of Jurkat lymphocytes treated with 2, or 19-21 (10 µM) for 5 min or 1 h at 37 °C. The confocal detector gain was set to 800 for cells treated with compounds for 5 min and was set to 650 for cells incubated with compounds for 1 hour.
the NBD receptor 20 bearing two negative charges in the linker region showed faster cellular association than receptor 19 which has only one negative charge.

The cellular uptake of anti DNP IgG by compounds 2 and 20 was qualitatively evaluated by confocal laser scanning microscopy (Figure 1-10). In these experiments, Jurkat lymphocytes were treated with synthetic receptors for 1 hour, and washed with fresh media to remove unincorporated receptors. The cells were subsequently treated with anti-DNP IgG labeled with red fluorescent Alexa fluor 633. Cellular green fluorescence from the synthetic receptors and red fluorescence from the antibodies were imaged after incubation for 4 hours. The

![Figure 1-10: Synthetic receptor-mediated uptake of anti-DNP IgG by Jurkat lymphocytes. Cells were treated with 2 (Panel A) and 20 (Panel B) (5 µM) respectively at 37 °C for 1 h, washed to remove unincorporated receptors, and incubated with red fluorescent anti-DNP-AF633 (0.16 mg/mL) for 4 h. Cells were washed and analyzed by confocal laser scanning microscopy.](image-url)
anti-DNP IgG-AF633 was delivered into cells by these synthetic receptors. Under the same conditions, receptor 20 bearing two glutamic acids was found to greatly enhance the uptake of anti-DNP-AF633 compared to the control receptor 2. Green fluorescent receptors localized on the cell surface and partially colocalized with anti-DNP IgG in intracellular endosomes. This experiment indicated that negative charges in the linker region can greatly enhance the uptake of proteins mediated by synthetic receptors.

To investigate pathways targeted by synthetic receptors, cellular colocalization of green fluorescent NBD receptor 20 and red fluorescent transferrin, a marker of early / recycling endosomes,18 and LDL, which traffics to late endosomes / lysosomes,19 were examined by confocal laser scanning microscopy. In this assay, Jurkat lymphocytes were treated 20 for 1 hour, washed and treated with proteins respectively for 5 minutes. The confocal micrographs showed that synthetic receptor resided on the cell surface and colocalized with transferrin in intracellular compartments but not with LDL, indicating that synthetic receptors are selectively accumulating early / recycling endosomes and do not accumulate in late endosomes or lysosomes.
(A) 20 + Texas Red Transferrin

(A) 20 + Dil-LDL

Figure 1-11: Confocal laser scanning and differential interference contrast (DIC) micrographs of living Jurkat lymphocytes treated with green fluorescent NBD derivative 20 (10 µM) for 1 h followed by (A) red fluorescent Texas Red transferrin (500 nM) or (B) Dil-LDL (8 nM) for 5 min at 37 °C. Colocalization of red and green fluorescence is shown as yellow pixels in the DIC overlay images.

1.5 Conclusions

The modification of the linker region of synthetic receptors by incorporation of glutamic acids proved to be effective in enhancing the association of these compounds with mammalian cells as well as their efficacy as protein delivery agents. This approach may also facilitate the application of N-
alkyl-3\(\beta\)-cholesterylamine based synthetic receptors as cellular probes for studies of the trafficking and sorting of membrane-associated molecules. The enhanced solubility of these synthetic molecules in the aqueous phase due to the addition of hydrophilic glutamic acids subunits may also be advantageous in drug delivery applications.

1.6 Experimental section

1.6.1 General

Chemical reagents and solvents were obtained from Acros, Aldrich and EMD Biosciences. Media and antibiotics were purchased from Mediatech and Gibco BRL. Rabbit polyclonal antidinitrophenyl (anti-DNP) IgG was from Sigma. Texas red Transferrin and Alexa Fluor 633 protein labeling kit for the labeling of anti-DNP IgG antibody were purchased from Molecular Probes. Dil-LDL was from Biomedical Technologies Inc. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All reactions were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EMD
Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of phosphomolybdic acid in ethanol (20%). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 µm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump / gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 µm particle size, 21.5 mm x 25 cm). The HPLC flow rate was maintained at 20 mL/min for the entire run unless otherwise noted. Melting points were measured with a Thomas Hoover capillary melting point apparatus and were uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400 instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm), MeOH-d₄ (¹H 4.80 ppm; ¹³C 49.15 ppm), DMSO-d₆ (¹H 2.50 ppm; ¹³C 39.51 ppm), or (CH₃)₄Si. High-resolution mass spectra were obtained from the Penn State University Mass Spectrometry Facility (ESI and CI) and University of Kansas Mass Spectrometry Facility (ESI). Low-resolution mass spectra were obtained with a Waters ZQ-4000 mass spectrometer. Peaks are reported as m/z.
1.6.2. Synthetic Procedures and Compound Characterization Data

tert-Butyl-3β-cholest-5-en-3-yl[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propyl]carbamate (13). To DMF (10 mL) were added 3β-amino-5-cholestene (12, 386 mg, 1.0 mmol), N-(3-bromopropyl)phthalimide (295 mg, 1.1 mmol), and K₂CO₃ (276 mg, 2.0 mmol). The solution was heated to 60 °C and stirred for 24 h. The reaction was cooled to 22 °C, and the solvent was removed in vacuo. To the resulting residue was added CH₂Cl₂ (10 mL). The insoluble material was removed by filtration and washed with additional CH₂Cl₂ (2 × 5 mL). To the combined filtrate and wash solutions containing the crude secondary amine product was added (Boc)₂O (327 mg, 1.5 mmol) and DIEA (0.5 mL, 3.0 mmol). The reaction was stirred for 4 h at 22 °C and concentrated in vacuo. Flash column chromatography (hexanes / ethyl acetate, 8:1) afforded 13 (465 mg, 69%) as a white solid, mp 59-61 °C; ^1H NMR (400 MHz, CDCl₃) δ 7.83 (m, 2H), 7.69, (m, 2H), 5.30 (d, 1H), 3.68 (t, 2H), 3.13 (br, 3H), 2.01-0.83 (m, 51H), 0.65 (s, 3H); ^13C NMR (100 MHz, CDCl₃) δ 168.2 (× 2), 155.2, 141.3, 133.8 (× 2), 132.0 (× 2), 123.2 (× 2), 79.3, 56.6, 56.1 (× 2), 50.1, 42.6 (× 2), 39.8, 39.4 (× 2), 38.3, 36.8, 36.5, 36.1, 35.9, 35.7, 31.2 (× 2), 28.4 (× 3), 28.2, 27.9, 26.7, 24.2, 23.8 (× 2), 22.8, 22.5, 20.9, 19.3, 18.7, 11.8; IR (film) ν max 2935, 2867, 1772, 1715, 1689,
1467, 1395, 1365, 1238, 1172, 1146, 1031, 888, 756, 720 cm$^{-1}$; HRMS (Cl+) m/z 673.4946 (M+H$^+$, C$_{43}$H$_{65}$N$_2$O$_4$, requires 673.4944).

9H-Fluoren-9-ylmethyl {3-[(3-{{(tert-butoxycarbonyl)[3β-cholest-5-en-3-yl]amino}propyl}amino]-3-oxopropyl}carbamate (15).

To a solution of tert-Butyl-3β-cholest-5-en-3-yl[3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl) propyl]carbamate (13, 1.41 g, 2.1 mmol) in absolute ethanol (50 mL) was added anhydrous hydrazine (350 µL, 11 mmol). The solution was heated to 50 °C and stirred for 4 h. The reaction was cooled to 22 °C, and a white precipitate was removed by filtration. The filtrate was concentrated in vacuo, and the residue was dissolved in CHCl$_3$ (100 mL). After insoluble material was removed by filtration, concentration of the filtrate in vacuo afforded the phthalimide-deprotected primary amine (1.13 g, 99%), a white solid that was carried forward without further purification. To Fmoc-β-Ala-OH (715 mg, 2.3 mmol) in anhydrous CH$_2$Cl$_2$ (50 mL) at 4 °C were added HOBt (340 mg, 2.5 mmol) and EDC (480 mg, 2.5 mmol). This mixture was stirred at 4 °C for 30 min. To this solution was added the phthalimide-deprotected primary amine in
anhydrous CH$_2$Cl$_2$ (25 mL) dropwise. The reaction was allowed to warm to 22 °C and stirred for 12 h. The solution was diluted with CH$_2$Cl$_2$ (100 mL) and washed with aqueous NaOH (0.1 M, 100 mL) and saturated aqueous NaCl (100 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo.

Flash column chromatography (hexanes / ethyl acetate, 2:1) afforded 15 (1.62 g, 93%) as a glassy solid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.76 (d, J = 7.3 Hz, 2H), 7.58 (d, J = 7.4 Hz, 2H), 7.39 (m, 2H), 7.30 (m, 2H), 7.05 (br, 1H), 5.82 (br, 1H), 5.33 (s, 1H), 4.34 (d, J = 7.2 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 3.51-3.24 (m, 6H), 2.60-0.86 (m, 54H), 0.67 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.0, 156.5, 144.0 (× 3), 143.9, 141.2 (× 2), 127.6, 127.6, 127.0 (× 2), 125.1, 125.0, 121.4, 119.9 (× 2), 77.1, 66.7, 56.7 (× 2), 56.1 (× 2), 50.0, 47.2, 42.3, 39.7, 39.5, 37.2, 36.8, 36.5, 36.1, 35.9, 35.7, 31.8 (× 2), 28.5 (× 3), 28.2, 27.9 (× 2), 26.8, 24.2, 23.8, 22.8 (× 2), 22.5 (× 2), 20.9, 19.4, 18.7, 11.8; IR (film) $\nu$ max 3323, 2949, 1717, 1668, 1539, 1449, 1412, 1366, 1251, 1169, 1081, 908, 737 cm$^{-1}$; HRMS (ESI+) m/z 835.5819 (M+H$,^+$, C$_{53}$H$_{78}$N$_3$O$_5$ requires 835.5863).

H-fluoren-9-ylmethyl \{3-[(3-\((\text{tert-butoxycarbonyl})[3\beta\text{-cholest}-5\text{-en}-3\text{-yl}]\text{amino})\)propyl]amino]-3-oxopropyl\}carbamate (15, 303 mg, 0.36 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo. To a solution of Fmoc-Glu (t-Bu)-OH (157 mg, 0.37 mmol) in anhydrous CH₂Cl₂ (10 mL) at 4 °C were added HOBt (55 mg, 0.41 mmol) and EDC (79 mg, 0.41 mmol) and the solution was stirred for 30 min. The primary amine derived from 15 was dissolved in anhydrous CH₂Cl₂ (5 mL) and added. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH₂Cl₂ (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Flash column chromatography (CH₂Cl₂ / MeOH, 50:1) afforded 16 (361 mg, 98%) as a white solid, mp 120-122 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.51 (br, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.09 (br, 1H), 5.88 (d, J = 8.0 Hz, 1H), 5.31 (d, J = 4.0 Hz, 1H), 4.34 (t, J = 7.1 Hz, 2H), 4.21 (t, J = 7.1 Hz, 2H), 3.57-3.16 (m, 6H), 2.60-0.85 (m, 67H), 0.66 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 172.7, 171.4, 171.4, 156.2, 143.9, 143.8, 141.3 (× 3), 127.7 (× 2), 127.9 (× 2), 125.2 (× 2), 121.4, 119.9 (× 2), 80.9, 79.9, 67.1, 56.7 (× 2), 56.1 (× 2), 54.6, 50.1, 47.2, 42.3, 39.7, 39.8, 39.6, 38.4, 37.1, 36.7, 36.2, 36.0, 35.8, 31.9 (× 2), 31.5, 28.6 (× 3), 28.2, 28.1 (× 3), 28.0, 27.9, 27.0, 24.3, 23.8, 22.8 (× 2), 22.6 (× 2), 21.0, 19.5, 18.7, 11.8; IR (film) ν max 3295, 3066, 3005, 2935, 2863, 1726, 1652, 1540, 1465, 1451, 1415, 1366, 12523, 1163,
1046, 849, 757, 666 cm\(^{-1}\); HRMS (ESI+) m/z 1043.6853 (M+Na\(^+\), C\(_{62}\)H\(_{92}\)N\(_4\)O\(_8\)Na requires 1043.6813).

**tert-Butyl-3\(\beta\)-cholest-5-en-3-yl[14-(9\(H\)-fluoren-9-yl)-5,9,12-trioxo-13-oxa-4,8,11-triazatetradec-1-yl]carbamate (17).**

9\(H\)-fluoren-9-ylmethyl \{3-[[3-{(tert-butoxycarbonyl)[3\(\beta\)-cholest-5-en-3-yl]amino} propyl]amino]-3-oxopropyl]carbamate (15, 150 mg, 0.18 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed *in vacuo*. To a solution of Fmoc-Gly-OH (60 mg, 0.20 mmol) in anhydrous CH\(_2\)Cl\(_2\) (10 mL) at 4 °C were added HOBt (30 mg, 0.22 mmol) and EDC (42 mg, 0.22 mmol) and the solution was stirred for 30 min. The primary amine derived from 15 was dissolved in anhydrous CH\(_2\)Cl\(_2\) (5 mL) and added. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH\(_2\)Cl\(_2\) (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated *in vacuo*. Flash column chromatography (CH\(_2\)Cl\(_2\) / MeOH, 20:1) afforded 17 (153 mg, 97%) as a white solid, mp 112-114 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.75 (d, \(J = 7.4\) Hz, 2H), 7.60 (d, \(J = 7.0\) Hz, 2H), 7.39 (t, \(J = 7.3\) Hz, 2H), 7.30 (t, \(J = 7.3\) Hz, 2H), 6.99 (br, 1H), 5.78 (br, 1H), 5.31
(s, 1H), 4.38 (d, J = 6.9 Hz, 2H), 4.22 (t, J = 7.0 Hz, 1H), 3.86 (d, J = 4.5 Hz, 2H), 3.56 (d, J = 5.0 Hz, 2H), 3.22 (m, 2H), 2.52-0.86 (m, 56H), 0.67 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.5, 169.0, 156.5, 148.3, 143.8 (× 2), 141.2 (× 3), 127.7 (× 2), 127.0 (× 2), 125.1 (× 2), 121.4, 119.9 (× 2), 80.0, 67.1, 56.7, 56.1 (× 2), 50.0, 47.1, 44.3, 42.3, 41.5, 39.7, 39.5, 38.4, 37.1, 36.7, 36.1, 35.9, 35.7, 31.8 (× 2), 28.5 (× 3), 28.2, 27.9 (× 2), 26.8, 24.2, 23.8, 22.8 (× 2), 22.5 (× 2), 20.9, 19.4, 18.7, 11.8; IR (film) ν max 3311, 2936, 2868, 1717, 1667, 1543, 1465, 1450, 1413, 1365, 1168, 1048, 758 cm$^{-1}$; HRMS (ESI+) m/z 893.6122 (M+H$^+$, C$_{55}$H$_{81}$N$_4$O$_6$ requires 893.6156).


**tert-Butyl**-(15S)-5-[3β-cholest-5-en-3-yl]-15-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-2,2-dimethyl-4,10,14-trioxo-3-oxa-5,9,13-triazaoctadecan-18-oate (16, 150 mg, 0.15 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo. To a solution of Fmoc-Glu(t-Bu)-OH (70 mg, 0.16 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL) at 4 °C were added HOBt (24 mg, 0.18 mmol) and EDC (35 mg, 0.18 mmol) and the
solution was stirred for 30 min. The primary amine derived from 16 was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) and added. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH$_2$Cl$_2$ (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) followed by saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. Flash column chromatography (CH$_2$Cl$_2$ / MeOH, 30:1) afforded 18 (162 mg, 92%) as a white solid, mp 138-144 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.78 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.2 Hz, 2H), 7.59 (br, 1H), 7.41 (t, J = 7.4 Hz, 2H), 7.36 (br, 1H), 7.33 (t, J = 7.4 Hz, 2H), 7.19 (br, 1H), 5.92 (br, 1H), 5.32 (d, J = 6.4 Hz, 1H), 4.36 (d, J = 7.2 Hz, 3H), 4.24 (t, J = 7.0 Hz, 2H), 3.58-3.20 (br, 7H), 2.60-0.86 (m, 79H), 0.67 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 172.9, 172.8, 171.4, 171.1 (× 2), 156.3, 156.3, 143.9, 143.7, 141.2 (× 3), 127.7 (× 2), 127.1 (× 2), 125.2 (× 2), 121.3, 120.0 (× 2), 81.0, 80.9, 77.2, 67.2, 56.7, 56.2 (× 2), 53.1 (× 2), 50.1 (× 2), 47.1, 42.3, 39.8, 39.5 (× 2), 38.4, 36.7, 36.2 (× 2), 35.8 (× 2), 31.9 (× 2), 28.6 (× 3), 28.2, 28.0 (× 9), 27.9 (× 2), 24.3, 23.8, 22.8 (× 2), 22.6 (× 2), 21.0, 19.5, 18.7, 11.8; IR (film) ν max 3289, 3066, 3005, 2934, 2868, 1729, 1693, 1679, 1636, 1539, 1450, 1413, 1388, 1367, 1281, 1254, 1158, 1044, 757, 667 cm$^{-1}$; HRMS (ESI+) m/z 1206.8032 (M+H$^+$, C$_{71}$H$_{108}$N$_5$O$_{11}$ requires 1206.8045).
(4S)-5-\{3-\{3-[\beta]-Cholest-5-en-3-ylamino\}propyl\}amino\}-3-oxopropyl]amino\} -4-\{4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl\] amino\} -5-oxopentanoic acid (6).

\[\text{tert-Butyl-(15S)-5-[3\beta]-cholest-5-en-3-yl]-15-\{[(9H-fluoren-9-ylmethoxy)carbonyl] amino\}-2,2-dimethyl-4,10,14-trioxo-3-oxa-5,9,13-triazaoctadecan-18-oate (16, 75 mg, 0.074 mmol) was dissolved in DMF (1 mL) containing piperidine (20\%) and stirred for 30 min at 22 °C. The solvent was removed \textit{in vacuo}. The resulting residue was dissolved in anhydrous DMF (5 mL). To this solution was added 1-\{4-(2,7-Difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl\}oxy\} pyrrolidine-2,5-dione (4-carboxy-Pennsylvania Green succinimidy ester, 30 mg, 0.062 mmol) followed by diisopropylethylamine (50 \(\mu\)L, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated \textit{in vacuo}. The resulting orange residue was treated with \(\text{CH}_2\text{Cl}_2\) (5 mL) containing TFA (15\%) and stirred at 22 °C for 12 h. The reaction was concentrated \textit{in vacuo}, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90\% H\_2O, 9.9\% MeCN, and 0.1\% TFA to 99.9\% MeCN and 0.1\% TFA over 20 min; retention time = 15.4 min (254 nm) to afford 6 (23 mg, 36.4\%) as an orange solid, mp 196-198 °C; \textsuperscript{1}H NMR (300 MHz, DMSO-d\textsubscript{6}) \(\delta\) 8.76-8.72 (m, 3H),
8.19 (s, 2H), 8.14 (s, 1H), 8.05 (d, J = 5.4 Hz, 1H), 7.49 (d, J = 5.7 Hz, 1H), 6.98
(s, 2H), 6.72 (d, J = 8.2 Hz, 2H), 5.45 (s, 1H), 4.53 (d, J = 2.7 Hz, 1H), 3.23 (m,
2H), 3.14 (m, 2H), 3.01 (m, 3H), 2.46-0.92(m, 51H), 0.72 (s, 3H); ¹³C NMR (75
MHz, DMSO-d₆) δ 174.0 (x 2), 172.7, 171.2, 170.8, 165.9, 158.0, 149.3, 138.6,
135.9, 135.2, 134.5, 129.8, 129.0, 125.5, 122.4, 105.2, 56.6, 56.1, 55.5, 53.1,
49.3, 41.8 (x 2), 41.6, 36.4, 36.2, 35.6, 35.3, 35.2, 34.3, 31.2 (x 2), 30.5, 27.7,
27.4 (x 2), 26.9, 26.2, 24.3, 23.8, 23.2, 22.6 (x 2), 22.4 (x 2), 20.5, 19.2, 18.7,
18.5, 11.6; IR (film) ν max 3324, 3071, 2950, 2863, 1671, 1646, 1610, 1539,
1503, 1465, 1373, 1310, 1193, 1136, 836, 759 cm⁻¹; HRMS (ESI+) m/z
1007.5679 (M+H⁺, C₅₉H₇₇F₂N₄O₈Na requires 1007.5710).

(4S)-5-\{[(1S)-3-Carboxy-1-\{[(3-\{3-[3β\-cholest-5-en-3-ylamino]propyl\}amino]-
3-oxopropyl]amino\}carbonyl]propyl\}amino\}-4-\{[4-(2,7-difluoro-6-hydroxy-3-
oxo-3H-xanthen-9-yl)-3-methylbenzoyl]amino\}-5-oxopentanoic acid (7)

tert-Butyl (15S,18S)-15-(3-tert-butoxy-3-oxopropyl)-5-[3β-cholest-5-en-3-yl]-18-
{[(9H-fluoren-9-ylmethoxy)carbonyl]amino}-2,2-dimethyl-4,10,14,17-tetraoxo-3-
osa-5,9,13,16-tetraazahenicosan-21-oate (18, 30 mg, 0.025 mmol) was
dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22
°C. The solvent was removed in vacuo. The resulting residue was dissolved in dry DMF (2 mL). To this solution was added 1-[(4-(2, 7-Difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl)oxy]pyrrolidine-2,5-dione (4-carboxy-Pennsylvania Green succinimidyl ester, 13 mg, 0.027 mmol) followed by diisopropylethylamine (50 µL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated in vacuo. The resulting orange residue was treated with CH₂Cl₂ (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated in vacuo, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H₂O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 15 min; retention time = 13.5 min (254 nm) to afford 7 (6.2 mg, 22%) as an orange solid, mp 145-148 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.65 (d, J = 7.8 Hz, 1H), 8.37 (s, 2H), 8.07-7.92 (m, 4H), 7.39 (d, J = 8.0 Hz, 1H), 6.85 (s, 1H), 6.62 (d, J = 10.9 Hz, 1H), 5.37 (s, 1H), 4.46 (m, 1H), 4.21 (m, 1H), 3.29-3.20 (m, 4H), 2.90 (m, 3H), 2.46-0.92 (m, 55H), 0.63 (s, 3H); ¹³C NMR (100 MHz, DMSO-d₆) δ 174.4 (x 2), 172.6, 171.8, 171.7, 129.8, 129.0, 125.5, 122.4, 105.2, 57.0, 56.5, 56.0, 49.8, 42.3, 42.1, 39.3, 39.2, 39.1, 36.8, 36.6, 36.1, 36.0, 35.8, 35.6, 33.8 (x 2), 31.7 (x 2), 31.0, 30.6, 27.9 (x 2), 25.8, 24.9 (x 2), 24.8, 24.3, 23.6, 23.1 (x 2), 22.8 (x 2), 19.5, 19.2, 19.0, 12.1; IR (film) ν max 3419, 2951, 1682, 1643, 1540, 1438, 1375, 1310, 1205, 1142, 1015, 801, 721.6 cm⁻¹; HRMS (ESI⁺) m/z 1136.6179 (M+H⁺, C₆₄H₈₄F₂N₁₀O₁₁ requires 1136.6136).

tert-Butyl-3β-cholest-5-en-3-yl[14-(9H-fluoren-9-yl)-5,9,12-trioxo-13-oxa-4,8,11-triazatetradec-1-yl]carbamate (17, 25 mg, 0.028 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo. The resulting residue was dissolved in dry DMF (2 mL). To this solution was added 1-[[4-(2,7-Difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylbenzoyl]oxy]pyrrolidine-2,5-dione (4-carboxy-Pennsylvania Green succinimidyl ester, 11 mg, 0.023 mmol) followed by diisopropylethylamine (50 µL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated in vacuo. The resulting orange residue was treated with CH₂Cl₂ (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated in vacuo, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H₂O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 15 min; retention time = 13.0 min (254 nm) to afford 8 (18 mg, 82%) as an orange solid, mp 200-203 °C; ¹H NMR (300 MHz, DMSO-d₆) δ 8.91 (s, 1H), 8.56 (s, 2H), 8.0-7.91 (m, 4H), 7.40 (d, J = 7.6 Hz, 1H), 6.86 (s, 2H), 6.60 (d, J = 11.1 Hz, 2H), 5.38 (s, 1H), 3.87 (m, 2H), 3.14 (m, 4H), 2.93 (m,
(4S)-5-\{[3-\{3-\beta\text{-cholest-5-en-3-ylamino}\}propyl]amino\}-3-oxopropyl]amino\} -4-\{6-\{7-nitro-2,1,3-benzoxadiazol-4-yl\}amino\}hexanoyl]amino\} -5-oxopentanoic acid (19).

*tert*-Butyl-(15S)-5-\[3\beta\text{-cholest-5-en-3-yl}\]-15-\{[(9H-fluoren-9-ylmethoxy)carbonyl]\} amino\}-2,2-dimethyl-4,10,14-trioxo-3-oxa-5,9,13-triazaoctadecan-18-oate (16, 32 mg, 0.031 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed *in vacuo*. The resulting residue was dissolved in dry THF (2 mL). To this solution was added 6-(\text{-N}-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid succinimidyl ester (10 mg, 0.026 mmol) followed by diisopropylethylamine (50 µL, 0.24 mmol). The reaction
was stirred at 22 °C for 12 h and concentrated in vacuo. The resulting yellow residue was treated with CH$_2$Cl$_2$ (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated in vacuo, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H$_2$O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 10 min; retention time = 9.3 min (254 nm) to afford 19 (15.3 mg, 64.3%) as a yellow solid, mp 174-176 °C; $^1$H NMR (300 MHz, DMSO-d$_6$) δ 12.2 (s, 1H), 9.56 (s, 1H), 8.52-8.44 (m, 3H), 8.03-7.93 (m, 3H), 6.40 (d, J = 7.9 Hz, 1H), 5.37 (s, 1H), 4.17 (d, J = 4.4 Hz, 1H), 3.50-2.91 (m, 9H), 2.33-0.83 (m, 56H), 0.61 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) δ 174.2, 172.5, 171.6, 171.2, 145.5, 144.6, 138.9, 138.9, 138.3, 122.9 (x 2), 99.4, 57.0, 56.4, 55.9, 52.2, 49.7, 43.7, 42.2, 42.0, 36.7, 36.5 (x 2), 36.0 (x 2), 35.7, 35.5 (x 2), 35.3, 34.7, 31.6 (x 2), 30.6, 28.1, 27.7 (x 2), 26.6, 26.4, 25.2, 24.8, 24.2, 23.6, 23.0 (x 2), 22.8 (x 2), 20.9, 19.1, 18.9, 12.0; IR (film) ν max 3391, 2946, 2868, 1651, 1588, 1440, 1299, 1270, 1202, 1130, 757, 721 cm$^{-1}$; HRMS (ESI+) m/z 919.6008 (M+H$^+$, C$_{50}$H$_{79}$N$_8$O$_8$ requires 919.6021).

tert-Butyl (15S,18S)-15-(3-tert-butoxy-3-oxopropyl)-5-[3β-cholest-5-en-3-yl]-18-\((\text{[9H-fluoren-9-ylmethoxy]carbonyl]amino})-2,2\text{-dimethyl-4,10,14,17-tetraoxo-3-oxa-5,9,13,16-tetraazahenicosan-21-oate (18, 61 mg, 0.050 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed \textit{in vacuo}. The resulting residue was dissolved in dry THF (2 mL). To this solution was added 6-(\text{N-7-nitrobenz-2-oxa-1,3-diazol-4-yl}amino)hexanoic acid succinimidyl ester (15 mg, 0.038 mmol) followed by diisopropylethylamine (50 µL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated \textit{in vacuo}. The resulting yellow residue was treated with \text{CH}_2\text{Cl}_2 (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated \textit{in vacuo}, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H$_2$O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 10 min; retention time = 9.0 min (254 nm) to afford 20 (27 mg, 68%) as a yellow solid, mp 198-204 °C; \textsuperscript{1}H NMR (300 MHz, DMSO-d$_6$) \(\delta\) 9.56 (s, 1H), 8.52-8.43 (m, 3H), 8.04-7.87(m,
33

4H), 6.40 (d, J = 8.6 Hz, 1H), 5.38 (s, 1H), 4.17 (m, 2H), 3.50-2.91 (m, 9H), 2.33-
0.83 (m, 60H), 0.61 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 174.0, 173.9, 172.5,
171.3, 170.9, 170.8, 145.1, 144.5, 138.5, 138.5, 137.8, 122.5, 120.5, 99.5, 56.6,
56.1, 55.5, 52.0, 51.8, 49.3, 41.8 (x 2), 41.6, 36.4, 36.2 (x 2), 35.6 (x 2), 35.4,
35.2 (x 2), 35.0, 34.3, 31.3 (x 2), 30.2, 30.0, 27.8, 27.4 (x 2), 27.0, 26.3, 26.0,
24.8, 24.4, 23.8, 23.2, 22.7 (x 2), 22.4 (x 2), 20.5, 18.8, 18.5, 11.6; IR (film) $\nu$
max 3297, 3077, 2934, 1666, 1586, 1528, 1440, 1300, 1267, 1201, 1182, 1130,
760 cm$^{-1}$; HRMS (ESI+) m/z 1048.6400 (M+H$^+$, C$_{55}$H$_{86}$N$_9$O$_{11}$ requires 1048.6447).

$^N$-(2-{[3-{3-[3β-Cholest-5-en-3-ylamino]propyl}amino]-3-oxopropyl]amino}-
2-oxoethyl)-6-{[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanamide (21).

tert-Butyl-3β-cholest-5-en-3-yl[14-(9H-fluoren-9-yl)-5,9,12-trioxo-13-oxa-4,8,11-
triazatetradec-1-yl]carbamate (17, 41 mg, 0.046 mmol) was dissolved in DMF (1
mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was
removed in vacuo. The resulting residue was dissolved in dry THF (2 mL). To this
solution was added 6-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid
succinimidyl ester (15 mg, 0.038 mmol) followed by diisopropylethylamine (50 µL, 0.24 mmol). The reaction was stirred at 22 °C for 12 h and concentrated in vacuo. The resulting yellow residue was treated with CH$_2$Cl$_2$ (5 mL) containing TFA (15%) and stirred at 22 °C for 12 h. The reaction was concentrated in vacuo, and the crude product was dissolved in methanol (2 mL) and purified by preparative reverse-phase HPLC (gradient: 90% H$_2$O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 10 min; retention time = 10.1 min (254 nm) to afford 21 (28 mg, 88%) as a yellow solid, mp 138-141 °C; $^1$H NMR (300 MHz, DMSO-d$_6$) δ 9.57 (s, 1H), 8.49 (d, J = 7.5 Hz, 3H), 8.04 (s, 2H), 7.86 (s, 1H), 6.40 (d, J = 8.7 Hz, 1H), 5.36 (s, 1H), 3.61 (d, J = 4.8 Hz, 2H), 3.45 (s, 2H), 3.25 (d, J = 5.1 Hz, 2H), 3.12 (d, J = 4.4 Hz, 2H), 2.91 (m, 3H), 2.33-0.83 (m, 52H), 0.61 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) δ 172.4, 170.8, 168.9, 145.1, 144.5, 138.5, 138.5, 137.9, 122.4, 120.5, 99.5, 56.6, 56.1, 55.5, 49.3, 43.3, 41.9, 41.8, 41.6, 36.3, 36.1 (x 2), 35.6 (x 2), 35.3 (x 2), 35.2, 35.0, 34.3, 31.2 (x 2), 27.7, 27.4 (x 2), 26.3, 26.1, 24.8, 24.4, 23.2, 22.7 (x 2), 22.4 (x 2), 20.5, 18.7, 18.5, 11.6; IR (film) ν max 3296, 3077, 2937, 2863, 1668, 1643, 1590, 1528, 1443, 1297, 1201, 1133, 757, 721 cm$^{-1}$; HRMS (ESI+) m/z 869.5621 (M+Na$^+$, C$_{47}$H$_{74}$N$_8$O$_6$Na requires 869.5629).
1.6.3. Biological Assays and Protocols

1.6.3.1 Cell culture

Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were propagated in a humidified 5% CO₂ incubator at 37 °C. Media used for cell culture and wash steps contained antibiotics and FBS unless otherwise noted.

1.6.3.2 Microscopy

A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. 7-nitrobenz-2-oxa-1, 3-diazole (NBD) and the Pennsylvania Green were excited with a 488 nm Argon ion laser (25 mW, 1% laser power) and emitted photons were collected through 505 nm long pass filter. Alexa Fluor 633 was excited with a 633nm HeNe laser and emitted photons were collected through a 650 nm long pass filter. Excitation of Texas Red, and Dil employed a 543 nm HeNe laser and emitted photons were collected through a 560 nm long pass filter. Slides used for live-cell imaging were prepared by sealing media (30 µL) containing suspension cells between a micro cover glass (22 x 22 mm, VWR) and a micro slide (1.0 mm thick, 25 x 75 mm, VWR) via Press-to-seal silicone isolator (22 mm diameter, 0.5 mm deep,
Invitrogen). To maintain cell viability, imaging of suspension cells should be completed within 30 min after the slide is prepared. For imaging of adherent cell lines, slides were prepared by first pressing the silicone isolator onto a micro slide and adding media (180 µL) in the space created by the micro slide and isolator. The cover glass used to cultivate the cells (the side with cells face down) was sealed using the silicone isolator. To maintain cell viability, imaging of adherent cells should be performed immediately after preparation of the slide.

1.6.3.3 Flow Cytometry

Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10,000 cells were counted. For studies of cellular association of synthetic receptors, NBD and Pennsylvania Green were excited at 488 nm with a 15 mW air-cooled argon-ion laser, the emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT voltage for this instrument was set to 724 for detection of NBD and Pennsylvania Green.
1.6.3.4 Cellular Association Assays

Synthetic NBD receptors 2, 19-21 and Pennsylvania Green Probes 5-8 in DMSO were added to RPMI media (500 µL, final DMSO concentration = 1%) containing Jurkat lymphocytes (1 x 10^6). The cells were incubated at 37 °C for 5 minutes and 1 hour respectively, washed with RPMI media (2 x 500 µL) to remove unincorporated receptors, resuspended in media, and analyzed by confocal microscopy or flow cytometry.

1.6.3.5 Antibody Uptake Assays

Synthetic NBD receptors 2 and 20 in DMSO were added to RPMI media (500 µL, final DMSO concentration = 1%) containing Jurkat lymphocytes (1 x 10^6). The cells were incubated for 1 hour at 37 °C, washed with RPMI media (2 x 500 µL) to remove unincorporated receptors, and resuspended in fresh media (100 µL) containing red fluorescent anti-DNP-AF633 (final concentration = 0.16 mg/mL). Cells were maintained at 37 °C for 4 hours, washed with fresh media (2 x 500 µL) prior to analysis by confocal microscopy.
1.6.3.6 Colocalization of Green Fluorescent NBD Derivatives and Red Fluorescent Proteins

Jurkat lymphocytes (1 × 10^6) in media (500 µL) were treated with 20 (final concentration = 10 µM, final DMSO concentration = 1%) at 37 °C for 1 hour, washed with fresh media (2 x 500 µL), and resuspended in media (100 µL) containing Texas Red-Transferrin (final concentration = 500 nM) or Dil-LDL (final concentration = 8 nM). Cells were incubated at 37 °C for 5 min, washed with fresh media (2 x 500 µL), and analyzed by confocal microscopy.

1.7 References


2.1 Introduction

In the past few years, synthetic receptor targeting (SRT) has achieved significant progress toward the goal of delivery of bioactive molecules into living cells.\textsuperscript{1-5} However, trapping of cargo molecules in endosomal compartments remains a major limitation for delivery of molecules to targets. This problem also exists in many other carrier delivery systems, such as liposomes,\textsuperscript{6,7} polymers,\textsuperscript{8,9} and cell penetrating peptides (CPPs).\textsuperscript{10-12} To overcome this barrier using SRT, we have preliminarily investigated conjugation of endosome-disruptive polymers and peptides to the 3\(\beta\)-cholesterylamine anchor, tethering laser-assisted membrane inactivation dyes to the 3\(\beta\)-cholesterylamine anchor, and decorating synthetic receptors with membrane disruptive cationic functionalities. However, prior efforts did not show significant cargo release with low toxicity.

In nature, many viruses enter host cells through endocytic pathways,\textsuperscript{13} but they elude endosome entrapment using fusion proteins (FPs),\textsuperscript{14} such as influenza hemagglutinin (HA),\textsuperscript{15} on their surface. It has been elucidated that the
N-terminal domain of HA, (HA2, 20 amino acids) inserts into the host membrane, undergoes a conformational change upon the pH change in endosomes, and induces mixing of viral and host cell membranes.16 When HA protein and the HA2 peptide are reconstituted into liposome membranes17 or linked to CPPs18, they facilitate their escape from endosomes, resulting in significant biological activities. More recently, Weber et al.19 identified a pH-dependent lytic peptide, PC4, by phage display technology. PC4 exhibited membrane leakage activity comparable to HA2, but the smaller size of PC4 (12 amino acids, SSAWWSYWPPVAC) facilitated chemical conjugation to the 3β-cholesterylamine anchor, simplifying the design of targeted endosomal disruption agents.

2.2 Design, Synthesis and Evaluation of a Disulfide Probe as an Endosome Disruption Indicator

To monitor the microenvironment of endosomes and the effectiveness of potential endosome disrupting agents, a novel synthetic probe 22 (Figure 2-1) bearing a disulfide bond between N-alkyl-3β-cholesterylamine and a green fluorescent fluorescein headgroup was designed. When added to mammalian cells, derivatives of N-alkyl-3β-cholesterylamine associate with cellular plasma membranes and traffic between the cell surface and early / recycling endosomes.4,20 Because these endosomes are oxidizing,21 the disulfide of 22
should remain intact in these compartments. However, if 22 were exposed to reduced glutathione (GSH) in the cytosol, the disulfide bond would be cleaved (Figure 2-1). 22,23 Therefore, it was proposed that disruption of endosomes loaded with 22 would enable GSH to access these compartments, reduce the disulfide of 22, and release the soluble fluorophore 23 into the cytoplasm and nucleus of cells, a process that can be easily visualized by confocal laser scanning microscopy.

Figure 2-1: Structure of a disulfide probe 22 as an endosome disruption indicator and a strategy for the selective release of disulfide-tethered cargo from membranes of early/recycling endosomes. Panel A: products of cleavage of 22 by glutathione; panel B: mechanism of release of fluorophore 23 into the cytosol and nucleus of mammalian cells.
Figure 2-2: Synthesis of compound 22. Reagents and conditions: (a) cysteamine hydrochloride, H₂O₂, Et₃N, Fe₂SO₄·7H₂O, 27%; (b) Fmoc-OSu, NaHCO₃, 2, 6-dioxane, H₂O, 68%; (c) 20% piperidine, DMF (d) Fmoc-β-Ala-OH, EDC, HOBT, CH₂Cl₂, 4 °C to 22 °C; (e) 27, EDC, HOBT, CH₂Cl₂, 4 °C to 22 °C; (f) 5-Carboxyfluorescein succinimidyl ester, DIEA, DMF; (g) 15% TFA in CH₂Cl₂.

To synthesize 22, Fmoc protected 3-(2-aminoethylthio)-propanoic acid 27 was prepared by oxidative coupling of 3-mercaptopropionic acid and cysteamine hydrochloride, followed by Fmoc protection. Compound 27 was further coupled to the N-alkyl-3β-cholesterylamine anchor 28 containing two β-alanines to give the disulfide derivative 29. The Fmoc protecting group was cleaved and the amine coupled with fluorescein succinimidyl ester. Subsequent deprotection afforded 22 in good yield.

The release of fluorescein was initially evaluated by treating J-774 mouse macrophage cells with the disulfide probe 22 for 1 hour, followed by incubation
with *Listeria monocytogenes*, a bacterium known to disrupt phagosomes / endosomes by secreting the protein *listeriolysin* O (LLO). LLO is activated by the decreased pH in these intracellular compartments, forms pores in membranes and allows the bacteria to escape into the cytoplasm.\(^{25}\)

(A) *L. monocytogenes* and 22  
(B) 22 only  
(C) *L. monocytogenes* and 30  
(D) 30 only

Figure 2-3: Confocal laser scanning and differential interference contrast (DIC) microscopy of living J-774 macrophages. Panels A and C: Cells were treated with 10 µM of probe 22 (panel A) or 30 (panel C) for 1 h at 37 °C, washed to remove excess receptors, and treated with *L. monocytogenes* in fresh media for 2 h. Panels B and D: Cells were treated with 10 µM of 22 or 30 for 3 h respectively. (Experiments were run in collaboration with Boonyarattanakalin, S.)

Compared to 22 alone, J-774 cells treated with 22 and *L. monocytogenes* showed significantly different patterns of intracellular fluorescence. Release of the green fluorophore into the cytoplasm and nucleus by *L. monocytogenes* was observed as shown in Figure 2-3. Control experiments using probe 30 without
the disulfide bond showed no release of the fluorophore upon treatment of \textit{L. monocytogenes}. These experiments indicated that the disulfide probe 22 is effective in monitoring disruption of endosomes / phagosomes by staining the cytoplasm and nucleus of cells with green fluorescence.

2.3 Design, Synthesis and Optimization of PC4 Lipopeptides

PC4 was identified as a pH-dependent membrane lytic dodecapeptide. This peptide demonstrated greater membrane leakage under acidic conditions than at a physiological pH. We hypothesized that conjugation of the PC4 peptide to \textit{N-alkyl-3β-cholesterylamine} would cause it to cycle between the cell surface and early / recycling endosomes. Once PC4 peptides enter endosomes, the acidic conditions (pH ~ 6) in these intracellular compartments, maintained by the activation of proton pumps, would activate PC4 and cause membrane leakage and escape of contents from endosomes.

To prove this concept, a series of PC4 peptides 31-33 capped with derivatives of \textit{N-alkyl-3β-cholesterylamine} and bearing different linkers were designed (Figure 2-4). Based on previous studies indicating that negative charges in the linker region greatly enhance the cellular activities of synthetic receptors, peptides 32 and 33 bearing two or three glutamic acid subunits between the PC4 domain and 3β-cholesterylamine anchor were designed to compare with peptide 31 bearing two β-alanines in this region. These
lipopeptides were synthesized on solid phase using standard Fmoc methodology and Rink amide Novagel resin.

Figure 2-4: Structures of first generation PC4 lipopeptides.
Figure 2-5: Confocal laser scanning and DIC micrographs of living Jurkat lymphocytes treated with 22 (2.5 μM) only (panel A) or 22 and 31, 32, 33, 34, or 35 (2 μM) (panels B-F) for 12 h at 37 °C.

In a preliminary bioassay, Jurkat cells were incubated with the disulfide probe 22 alone or combined with the three PC4 peptides at 37 °C. Cells were imaged at 12 h by confocal laser scanning microscopy (Figure 2-5). The disulfide probe 22 alone, as expected, accumulated in defined intracellular compartments. Whereas 31 bearing two β-alanines showed similar results, 32 and 33 bearing
glutamic acids resulted in significant fluorescence in the cytosol and nucleus, indicating the cleavage of disulfide bond and release of contents due to endosome disruption. These results support the hypothesis that the trafficking and accumulation of PC4 peptides in acidic endosomes could cause the leakage of these compartments. It also established that adding glutamic acids in the linker region improves cellular association of these compounds, enhancing the concentration of the PC4 peptides in endosomes, which facilitates the membrane disruption. Therefore, the glutamic acids are critical for the endosome disruption activity of these PC4 lipopeptides. Although peptide 33 caused similar fluorescence release, peptide 32 was used as a prototype molecule for further studies due to the observation that peptide 33 showed cellular toxicity at higher concentrations whereas 32 was non-toxic at the same concentration.

Figure 2-6: Structures of second generation PC4 lipopeptides (characterization data for compound 35 is reported in Qi Sun’s Ph.D. dissertation, Penn State University, 2008).
Second generation PC4 peptides 34 and 35 (Figure 2-6) with either a shorter or longer linker between the glutamic acid residue and the PC4 peptide were designed to examine structure-activity relationships (SAR). It was found that linker length is also critical for the activity of PC4 peptide as peptide 35 with two amino hexanoic acids showed the most effective fluorescence release (Figure 2-5, panel F).

2.4 Mechanism of Action of PC4 Endosome Lytic Peptides

Figure 2-7: Structures of red fluorescent PC4 lipopeptide 36, PC4 control peptide 37, a new disulfide probe 38 and its noncleavable control 39 (characterization data of 36 and 37 is reported in Qi Sun's Ph.D. dissertation, Penn State University, 2008).
To examine the trafficking of PC4 lipopeptides, a red fluorescent PC4 peptide (36) conjugated to 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein (6-Hex) as well as the unmodified N-acetyl capped PC4 peptide (37) was designed and synthesized (Figure 2-7). A new disulfide probe 38 bearing two glutamic acids and a non-cleavable amide bond control compound 39 were also designed. The synthesis of 38 and 39 (Figure 2-8) was similar to the synthesis of the disulfide probe 22.

Figure 2-8: Synthesis of compounds 38 and 39. Reagents and conditions: (a) NH₂NH₂, EtOH, 50 °C; (b) EDC, HOBt, Fmoc-Glu(OtBu)-OH, 4 °C to 22 °C; (c) 20 % piperidine, DMF; (d) EDC, HOBt, 27 / 42, 4 °C to 22 °C; (e) 5-Carboxyfluorescein succinimidyl ester, DIEA, DMF; (f) 15 % TFA, CH₂Cl₂.
2.4.1 PC4 Lipopeptides and the Disulfide Probe Selectively Target Early / Recycling Endosomes

To confirm the hypothesis that PC4 lipopeptides target early / recycling endosomes, the subcellular localization of the green fluorescent disulfide probe and a red fluorescent PC4 peptide was examined in Chinese hamster ovary (CHO) cells by confocal laser scanning microscopy. In these experiments, CHO cells were treated with disulfide probe 38 or PC4 peptide 36 and fluorescent markers of intracellular compartments. As shown in Figure 2-9, disulfide probe 38 was found localized in defined intracellular compartments, which were identified as early / recycling endosomes by colocalization with red fluorescent transferrin (panel A). As a control, cells were treated with red fluorescent Dil-labeled low density lipoprotein (LDL), a protein that accumulates in late endosomes and lysosomes, showed distinct red fluorescence (panel B), indicating that N-alkyl-3β-cholesterylamine membrane anchor selectively delivered the disulfide probe 38 to early / recycling endosomes. Complete colocalization of red fluorescent PC4 peptide 36 with green fluorescent transferrin (panel C) as well as with disulfide probe 38 (panel D) confirmed the selective delivery of both PC4 peptide and disulfide probe to early / recycling endosomes and established that their colocalization in these compartments is required for efficient cargo release.
Figure 2-9: Confocal laser scanning and differential interference contrast (DIC) micrographs of living CHO cells treated with green fluorescent 38 (5 µM) for 12 h at 37 °C followed by (A) red fluorescent Texas Red transferrin (500 nM) or (B) Dil-LDL (8 nM) for 5 min. Panel C: CHO cells were treated with red fluorescent 36 (5 µM) for 12 h followed by green fluorescent transferrin, Alexa Fluor 488 conjugate (610 nM) for 5 min. Panel D: CHO cells were treated with 36 (2 µM) and 38 (5 µM) for 12 h at 37 °C under conditions that minimally disrupt endosomes. Colocalization of red and green fluorescence is shown as yellow pixels in the DIC overlay images.
2.4.2 Requirements for Cargo Release: Cholesterylamine Anchor, Disulfide Linkage, and Endosomal Acidity

![Graph A: Dose dependence of endosomal release quantified by flow cytometry. CHO cells were treated with 38 (5 µM) and 35 (0 to 16 µM) for 24 h at 37 °C and trypsinized for analysis.]

![Graph B: Cellular viability assay. CHO cells were treated with 35 (0 to 16 µM) for 48 h at 37 °C and analyzed by Cell-Titer-Glo luminescent viability assay.]

Figure 2-10: Panel A: dose dependence of endosomal release quantified by flow cytometry. CHO cells were treated with 38 (5 µM) and 35 (0 to 16 µM) for 24 h at 37 °C and trypsinized for analysis. Panel B: cellular viability assay. CHO cells were treated with 35 (0 to 16 µM) for 48 h at 37 °C and analyzed by Cell-Titer-Glo luminescent viability assay.
To determine the optimal effective concentration of PC4 peptides in CHO cells, a dose-dependent endosome disruption assay was conducted. In this assay, CHO cells were treated with disulfide 38 and different concentrations of compound 35 for 24 h and the fluorescence of CHO cells was quantified by flow cytometry. The dose dependent augmentation of cellular fluorescence (Figure 2-11, panel A) is due to the release of fluorescein, the fluorescence of which is partially quenched in acidic endosomes. A cellular viability assay (panel B) indicated that PC4 peptide 35 is nontoxic to CHO cells up to concentration of 8 \( \mu \text{M} \). This concentration was used for further studies.

At the same concentration, the unmodified PC4 peptide 37 only minimally affected fluorescence release, whereas the lipopeptide 35 and its red fluorescent analogue 36 showed significant fluorescence staining of the cytosol and nucleus of CHO cells (Figure 2-11). When the disulfide of 38 was replaced with non-cleavable amide bond of 39, the release of fluorophore was completely blocked. These results indicated that the cholesterylamine anchor and disulfide linkage are essential for cargo release. In another bioassay, when the endosomal pH was increased by treatment of CHO cells with an endosome acidification inhibitor, chloroquine,\(^{28}\) the release of fluorophore was completely blocked (Figure 2-11, compare panels E and F). This observation confirmed the pH-dependent membrane lytic activity of PC4 peptide and proved that the mechanism of action of PC4 lipopeptide is endosome targeting and membrane destabilization at the acidic pH of the endosome.
(A) 38 alone

(B) 38 + 37

(C) 38 + 35

(D) 39 + 35

(E) 38 + 36

(F) 38 + 36 + chloroquine

Figure 2-11: Confocal fluorescence and DIC micrographs of living CHO cells treated with 38 or 39 (5 µM) and 35, 36, or 37 (8 µM) for 24 h at 37 °C. In panel F, [chloroquine] = 5 µM.
2.5 Conclusions

A short synthetic PC4 peptide capped with N-alkyl-3β-cholesterylamine enables the release of a disulfide-linked fluorophore from entrapment in early / recycling endosomes. This endosome disruption system may be advantageous for delivery of drugs that need to reach their targets in the cytosol or nucleus. Although other synthetic vehicles that disrupt endosomes have been reported,\textsuperscript{17,18,30} the high specificity of N-alkyl-3β-cholesterylamines to target early / recycling endosomes, which are relatively nonhydrolytic compared with lysosomes, may have a wide range of cellular delivery applications.

2.6 Experimental section

2.6.1 General

Chemical reagents and solvents were obtained from Acros, Aldrich and EMD Biosciences. Media and antibiotics were purchased from Mediatech and Gibco BRL. The CellTiter Glo reagent was from Promega. Texas red Transferrin was purchased from Molecular Probes. Dil-LDL was from Biomedical Technologies Inc. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after
passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All reactions were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EMD Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of phosphomolybdic acid in ethanol (20%). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 µm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump/gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 µm particle size, 21.5 mm x 25 cm). The HPLC flow rate was maintained at 20 mL/min for the entire run unless otherwise noted. Melting points were measured with a Thomas Hoover capillary melting point apparatus and were uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400 instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm), MeOH-d₄ (¹H 4.80 ppm; ¹³C 49.15 ppm), DMSO-d₆ (¹H 2.50 ppm; ¹³C 39.51 ppm), or (CH₃)₄Si. High-resolution mass spectra were obtained from the Penn State University Mass Spectrometry Facility (ESI and CI) and University of Kansas Mass Spectrometry Facility (ESI). Low-resolution mass spectra were obtained with a Waters ZQ-4000 mass spectrometer. Peaks are reported as m/z.
3-[(2-{[(9H-Fluoren-9-ylmethoxy)carbonyl]amino}ethyl)dithio]propanoic acid (27)

To cysteamine hydrochloride (2.0 g, 17.5 mmol) were added 3-mercaptopropionic acid (1.52 mL, 17.5 mmol), H₂O (18 mL) and triethylamine (3.7 mL). The reaction was cooled to 4 °C, and one crystal of FeSO₄·7H₂O was added. Aqueous H₂O₂ solution (16%) was added dropwise until the reaction turned yellow. To the reaction was added concentrated HCl until pH = 2.0. The mixture was stirred for 30 min. The resulting white precipitate was removed by vacuum filtration. The filtrate was made basic by addition of aqueous NaOH (2.0 M) and washed with CH₂Cl₂ (2 x 20 mL). The aqueous phase was neutralized by addition of aqueous HCl (2.0 M) and concentrated in vacuo. Flash column chromatography (CH₂Cl₂ / CH₃OH, 10:1) afforded 3-(2-aminoethyldithio)propanoic acid (26)²⁴ (848 mg, 27%) as a white solid. To a solution of 26 (500 mg, 2.8 mmol) and NaHCO₃ (700 mg, 8.3 mmol) in H₂O (50 mL) was added a solution of 9-fluorenylmethyl chloroformate (870 mg, 3.3 mmol) in dioxane (25 mL). The reaction was stirred for 16 h at 22 °C. The solution was washed with diethyl ether (2 × 50 mL) and adjusted to pH = 1.0 with aqueous HCl (2.0 M). The aqueous phase was extracted with EtOAc (2 × 50 mL). The combined organic
extracts were washed with saturated aqueous NaCl (30 mL), dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. Flash column chromatography (CH$_2$Cl$_2$ / CH$_3$OH, 30:1) afforded 27 (766 mg, 68%) as a white solid, mp 104-106°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.69 (br, 1H), 7.75 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.38 (t, J = 7.5 Hz, 2H), 7.30 (t, J = 7.5 Hz, 2H), 5.25 (br, 1H), 4.39 (d, J = 7.1 Hz, 2H), 4.20 (t, J = 7.1 Hz, 1H), 3.50 (m, 2H), 2.90 (t, 2H), 2.76 (m, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 176.7, 156.5, 143.8 (× 2), 141.3 (× 2), 127.7 (× 2), 127.1 (× 2), 125.0 (× 2), 120.0 (× 2), 66.8, 47.2, 39.7, 37.9, 33.9, 32.9; IR (film) ν max 3600-2500 (br), 3331, 3045, 2954, 2915, 1697, 1544, 1442, 1412, 1279, 1146, 1021, 987, 938, 739, 648 cm$^{-1}$; LRMS (ESI+) 426.0 m/z (M+Na$^+$, C$_{20}$H$_{21}$NO$_4$S$_2$Na requires 426.1).


tert-Butyl-3β-cholest-5-en-3-yl[15-(9H-fluoren-9-yl)-5,9,13-trioxo-14-oxa-4,8,12-triazapentadec-1-yl]carbamate (28, 143 mg, 0.149 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo, and the primary amine derived from 28 was dissolved in
anhydrous CH$_2$Cl$_2$ (5 mL). To a solution of 3-[(2-[(9H-fluoren-9-ylmethoxy)carbonyl]amino)ethyl]dithio]propanoic acid (27) (70 mg, 0.174 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL) at 4 °C were added HOBt (26 mg, 0.192 mmol) and EDC (57 mg, 0.192 mmol) and the solution was stirred for 30 min. To this solution was added the solution of primary amine derived from 28. The reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH$_2$Cl$_2$ (10 mL) and washed with aqueous NaOH (0.1 M, 20 mL) and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. Flash column chromatography (CH$_2$Cl$_2$ / CH$_3$OH, 20:1) afforded 29 (152 mg, 90%) as a white solid; mp 123-125 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.78 (d, J = 7.1 Hz, 2H), 7.60 (d, J = 7.1 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.3 Hz, 2H), 7.14 (br, 1H), 6.93 (br, 1H), 6.85 (br, 1H), 5.80 (br, 1H), 5.32 (d, J = 6.9 Hz, 2H), 4.60 (d, J = 6.8 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 3.55-3.22 (m, 9H), 2.98 (t, J = 6.0 Hz, 1H), 2.82 (t, J = 6.0 Hz, 1H), 2.60-0.86 (m, 61H), 0.68 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.7, 171.0 (× 2), 156.5 (× 2), 143.9 (× 2), 141.3 (× 2), 141.2, 127.7 (× 2), 127.1 (× 2), 125.1 (× 2), 121.5, 120.0 (× 2), 80.1, 66.7, 56.7, 56.2 (× 2), 50.1, 47.3 (× 2), 42.3 (× 2), 39.8 (× 2), 39.5 (× 2), 38.4, 37.2, 36.7, 36.2 (× 2), 35.8 (× 2), 35.6, 34.0, 31.9 (× 2), 28.6 (× 3), 28.2, 28.0 (× 2), 24.3, 23.8, 22.8 (× 2), 22.6 (× 2), 21.0, 19.5, 18.7, 11.9; IR (film) ν max 3305, 3071, 2936, 2862, 1654, 1545, 1465, 1450, 1410, 1365, 1168, 1139, 757 cm$^{-1}$; HRMS (ESI+) m/z 1092.6290 (M+Na$^+$, C$_{61}$H$_{91}$N$_5$O$_7$S$_2$Na requires 1092.6258).
5-\{21-\[3\beta\text{-Cholest-5-en-3-ylamino}\]-9,13,17-trioxo-5,6-dithia-2,10,14,18-tetraazahenicosan-1-oyl\}-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (22)

tert-Butyl-3\beta\text{-cholest-5-en-3-yl}\[23-(9H-fluoren-9-yl)-5,9,13,21-tetraoxo-22-oxa-16,17-dithia-4,8,12,20-tetraazatricos-1-yl\]carbamate (29, 28 mg, 0.026 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed \textit{in vacuo}, and the primary amine derived from 29 was dissolved in anhydrous DMF (2 mL). To this solution was added 5-carboxyfluorescein, succinimidyl ester (18 mg, 0.039 mmol) and DIEA (50 µL, 0.24 mmol). The reaction was stirred for 4 h at 22 °C followed by concentration \textit{in vacuo}. The residue was dissolved in CH$_2$Cl$_2$ (5 mL) containing TFA (20%) and stirred for 2 h at 22 °C. The reaction was concentrated \textit{in vacuo}, and the crude product was purified by preparative reverse-phase HPLC (gradient: 9.95% MeCN, 89.95% H$_2$O, and 0.1% TFA to 99.9% MeCN, 0% H$_2$O, and 0.1% TFA over 20 min; retention time = 17.3 min (215 nm), which afforded 22 (27 mg, 93%) as a yellow solid, mp 182-185 °C; $^1$H NMR (400 MHz, MeOH-d$_4$) δ 8.42 (s, 1H), 8.09-8.02 (m, 2H), 7.20 (d, J = 8.0 Hz, 1H), 6.72-6.56 (m, 6H), 5.42 (d, J = 4.2 Hz, 1H), 3.76 (m, 1H), 3.59 (t, J = 6.7 Hz, 2H), 3.28-3.02 (m, 5H), 2.89-2.71 (m, 7H), 2.48-
0.89 (m, 48H), 0.72 (s, 3H); $^{13}$C NMR (100 MHz, MeOH-d$_4$) δ 173.5 (× 2), 172.4 (× 2), 172.3 (× 2), 167.3, 167.2, 161.0, 153.1 (× 2), 141.2, 138.4, 138.0, 136.4, 130.0, 129.5 (× 2), 128.0, 125.0, 124.3, 123.4, 113.1 (× 2), 110.2, 102.2, 63.4, 57.9, 56.6, 56.1, 50.0, 42.1, 41.9, 39.6, 39.3, 39.1, 37.1, 36.8, 36.4, 36.0, 35.8, 35.7, 35.6, 35.4, 35.2, 34.9, 33.9, 31.6, 31.5, 27.9, 27.7, 26.5, 24.9, 23.8, 23.5, 21.8, 21.5, 20.7, 20.1, 18.2, 17.8, 10.9; IR (film) ν max 3297, 2934, 1739, 1630, 1531, 1443, 1383, 1202, 1177, 842, 757 cm$^{-1}$; HRMS (ESI+) m/z 1128.5573 (M+Na$^+$, C$_{62}$H$_{83}$N$_5$O$_9$S$_2$Na requires 1128.5530).


To a solution of *tert*-Butyl-3β-cholest-5-en-3-yl[3-({1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl}) propyl]carbamate (13, 202 mg, 0.3 mmol) in absolute ethanol (10 mL) was added anhydrous hydrazine (50 µL, 1.56 mmol). The solution was heated to 50 °C and stirred for 4 h. The reaction was cooled to 22 °C, and a white precipitate was removed by filtration. The filtrate was concentrated *in vacuo,*
and the residue was dissolved in CHCl₃ (20 mL). After insoluble material was removed by filtration, concentration of the filtrate in vacuo afforded the phthalimide-deprotected primary amine (156 mg, 97%), a white solid that was carried forward without further purification. To Fmoc-Glu(t-Bu)-OH (130 mg, 0.3 mmol) in anhydrous CH₂Cl₂ (10 mL) at 4 °C were added HOBt (40 mg, 0.3 mmol) and EDC (58 mg, 0.3 mmol). This mixture was stirred at 4 °C for 30 min. To this solution was added the phthalimide-deprotected primary amine in anhydrous CH₂Cl₂ (5 mL) dropwise. The reaction was allowed to warm to 22 °C and stirred for 12 h. The solution was diluted with CH₂Cl₂ (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) and saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Flash column chromatography (hexanes / ethyl acetate, 2:1) afforded 40 (203 mg, 91%) as a white solid, mp 83-85 °C; \(^1\)H NMR (300 MHz, CDCl₃) δ 7.74 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.5 Hz, 2H), 7.38 (t, J = 7.5 Hz, 2H), 7.29 (t, J = 7.5 Hz, 2H), 5.88 (d, 1H), 5.31 (d, 1H), 4.37 (d, J = 7.1 Hz, 2H), 4.16 (br, 1H), 4.15 (t, J = 7.1 Hz, 1H), 3.27 (m, 4H), 2.60-0.85 (m, 65H), 0.66 (s, 3H); \(^13\)C NMR (75 MHz, CDCl₃) δ 172.6, 171.0, 156.3, 143.9, 143.8, 141.2 (× 3), 127.6 (× 2), 125.2 (× 2), 121.4, 119.9 (× 2), 80.7, 79.8, 67.0, 58.2, 56.7, 56.1 (× 2), 54.5, 50.1, 47.1, 42.3, 41.3, 39.7, 39.5, 38.4, 37.1, 36.7, 36.2, 35.8, 31.9 (× 2), 31.7, 28.5 (× 3), 28.4, 28.2, 28.1 (× 4), 28.0, 26.8, 24.3, 23.8, 22.8, 22.6, 21.0, 19.4, 18.7, 11.8; IR (film) ν max 3310, 2935, 2868, 1728, 1668, 1531, 1450, 1412, 1366, 1249, 1163, 1048, 757 cm⁻¹; HRMS (ESI+) m/z 950.6640 (M+H⁺, C₅⁹H₈₈N₃O₇ requires 950.6622).

tert-Butyl(4R)-5-[[3-{(3-tert-butoxycarbonyl)-3β-cholest-5-en-3-yl]amino}propyl]-4-[[9H-fluoren-9-ylmethoxy]carbonyl]amino]-5-oxopentanoate (40, 160 mg, 0.17 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo, and the primary amine derived from 40 was dissolved in anhydrous CH₂Cl₂ (5 mL). To a solution of Fmoc-Glu(t-Bu)-OH (80 mg, 0.19 mmol) in anhydrous CH₂Cl₂ (10 mL) at 4 °C were added HOBt (27 mg, 0.2 mmol) and EDC (40 mg, 0.2 mmol) and the solution was stirred for 30 min. The solution of crude primary amine derived from 40 was added. The reaction was allowed to warm to 22 °C and was stirred for 12 h. This solution was diluted with CH₂Cl₂ (30 mL) and washed with aqueous NaOH (0.1 M, 30 mL) and saturated aqueous NaCl (30 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Flash column chromatography (hexanes / ethyl acetate, 2:1) afforded 41 (172 mg, 89%) as a white solid, mp 90-92 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.75 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.38 (t, J = 7.5 Hz, 2H), 7.30 (t, J = 7.5 Hz, 2H), 5.90 (br, 1H), 5.29 (d, 1H), 4.41 (br, 1H) 4.36 (d, J = 7.1 Hz, 2H), 4.20 (t, J = 7.1 Hz, 1H).
3.60 (br, 1H), 3.24 (br, 4H), 2.60-0.86 (m, 76H), 0.67 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.9, 172.2, 171.3, 170.6, 156.4, 156.2, 143.9, 143.7, 141.3 ($\times$ 3), 127.7 ($\times$ 2), 127.1 ($\times$ 2), 125.1 ($\times$ 2), 121.3, 119.9 ($\times$ 2), 81.0, 80.5, 79.3, 67.1, 56.7, 56.1 ($\times$ 2), 53.0, 50.1 ($\times$ 2), 47.2, 47.1, 46.5, 42.3 ($\times$ 2), 39.7, 39.5 ($\times$ 2), 38.4, 36.7, 36.2, 35.8, 31.9 ($\times$ 2), 31.7, 28.5 ($\times$ 3), 28.3, 28.2, 28.1, 28.0 ($\times$ 10), 26.8, 24.2, 23.8, 22.8 ($\times$ 2), 22.6 ($\times$ 2), 21.0, 19.4, 19.1, 18.7, 11.8; IR (film) $\nu$ max 3306, 2934, 2868, 1727, 1650, 1531, 1450, 1412, 1392, 1367, 1249, 1156, 1049, 960, 848, 757 cm$^{-1}$; HRMS (ESI+) 1135.7745 m/z (M+H$^+$, C$_{68}$H$_{103}$N$_4$O$_{10}$ requires 1135.7674).


tert-Butyl(11S,14S)-11-(3-tert-butoxy-3-oxopropyl)-5-(3$\beta$-cholest-5-en-3-yl)-14-{{(9H-fluoren-9-ylmethoxy)carbonyl]amino}-2,2-dimethyl-4,10,13-trioxo-3-oxa-5,9,12-triazaheptadecan-17-oate (41, 100 mg, 0.088 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo, and the primary amine derived from 41 was
dissolved in anhydrous CH$_2$Cl$_2$ (5 mL). To a solution of 3-[(2-[(9H-fluoren-9-ylmethoxy)carbonyl]amino)ethyl]dithio]propanoic acid (27) (40 mg, 0.1 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL) at 4 °C were added HOBt (15 mg, 0.11 mmol) and EDC (21 mg, 0.11 mmol) and the solution was stirred for 30 min. To this solution was added the solution of primary amine derived from 41. The reaction was allowed to warm to 22 °C and was stirred for 12 h. This solution was diluted with CH$_2$Cl$_2$ (20 mL) and washed with aqueous NaOH (0.1 M, 20 mL) and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated \textit{in vacuo}. Flash column chromatography (hexanes / ethyl acetate, 1:1) afforded 43 (92 mg, 81%) as a glassy solid; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.75 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.5 Hz, 2H), 7.39 (t, J = 7.1 Hz, 2H), 7.30 (t, J = 7.5 Hz, 2H), 5.31 (d, 1H), 4.50 (br, 2H), 4.39 (d, J = 7.1 Hz, 2H), 4.22 (t, J = 7.1 Hz, 1H), 3.50 (br, 2H), 3.30 (br, 1H), 3.22 (br, 4H), 2.98 (t, 2H), 2.81 (t, 2H), 2.66-0.86 (m, 79H), 0.67 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 173.2, 172.7, 171.3, 171.1, 170.7, 156.5, 156.1, 143.9 (× 2), 141.3 (× 3), 127.7 (× 2), 127.0 (× 2), 125.1 (× 2), 121.3, 120.0 (× 2), 81.1, 80.8, 79.8, 66.7, 58.3, 56.7, 56.1 (× 2), 52.8 (× 2), 50.1, 47.2, 42.3, 41.7, 39.8, 39.7, 39.5, 38.4, 38.0, 37.2, 36.7, 36.2, 35.8, 33.9, 31.9 (× 3), 29.7, 28.5 (× 3), 28.3, 28.2, 28.1 (× 6), 28.0, 27.7, 26.8, 24.3, 23.8, 22.8, 22.6, 21.0, 19.5, 18.7, 11.9; IR (film) ν max 3292, 2934, 2868, 1729, 1683, 1635, 1538, 1450, 1411, 1366, 1253, 1156, 903, 848, 780, 757 cm$^{-1}$; HRMS (ESI+) 1298.7854 m/z (M+H+, C$_{73}$H$_{112}$N$_5$O$_{11}$S$_2$ requires 1298.7800).

tert-Butyl(11S,14S)-11-((tert-butoxy-3-oxopropyl)-5-(3β-cholest-5-en-3-yl)-14-{{(9H-fluoren-9-ylmethoxy)carbonyl}amino}-2,2-dimethyl-4,10,13-trioxo-3-oxa-5,9,12-triazauhtadecan-17-oate (41, 80 mg, 0.070 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 20 min at 22 °C. The solvents were removed in vacuo, and the primary amine derived from 41 was dissolved in anhydrous CH₂Cl₂ (5 mL). To a solution of 3-[[3-((9H-fluoren-9-ylmethoxy)carbonyl]amino)propanoyl]amino]propanoic acid (42)²⁹ (30 mg, 0.077 mmol) in anhydrous CH₂Cl₂ (5 mL) at 4 °C were added HOBt (11 mg, 0.077 mmol) and EDC (15 mg, 0.077 mmol). The solution was stirred for 30 min at 4 °C. The solution of the crude primary amine derived from 41 was added and the reaction was allowed to warm to 22 °C and stirred for 12 h. This solution was diluted with CH₂Cl₂ (20 mL), washed with aqueous NaOH (0.1 M, 20 mL), and washed with saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. Flash column chromatography
(hexanes / ethyl acetate, 1:1) afforded **44** (75 mg, 89%) as a white solid, mp 136-137 °C; ^1^H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 7.76 (d, \( J = 7.5 \) Hz, 2H), 7.61 (d, \( J = 7.4 \) Hz, 2H), 7.39 (t, \( J = 7.4 \) Hz, 2H), 7.31 (t, \( J = 7.4 \) Hz, 2H), 5.32 (d, \( J = 5.8 \) Hz, 1H), 4.40 (br, 2H), 4.34 (d, \( J = 7.0 \) Hz, 2H), 4.20 (t, \( J = 7.0 \) Hz, 1H), 3.61 (br, 2H), 3.52-3.30 (m, 5H), 3.2 (br, 2H), 2.66-0.86 (m, 81H), 0.68 (s, 3H); ^1^C NMR (100 MHz, CDCl\textsubscript{3}) \( \delta \) 173.0 (\( \times \) 2), 172.8, 172.5, 172.1, 170.0, 157.0, 156.8, 144.0 (\( \times \) 3), 141.4 (\( \times \) 2), 127.7 (\( \times \) 2), 127.0 (\( \times \) 2), 125.1 (\( \times \) 2), 121.3, 120.0 (\( \times \) 2), 81.1, 80.8, 79.8, 66.9, 66.8, 56.8, 56.2, 53.5, 52.6, 50.2, 47.3 (\( \times \) 2), 46.7, 43.0, 42.4, 39.8, 39.6, 38.5, 37.4, 37.2, 36.8, 36.3, 36.1, 35.9, 33.2, 32.9, 32.0, 31.9, 31.7, 28.5 (\( \times \) 3), 28.3, 28.1, 28.0 (\( \times \) 6), 27.8, 26.9, 26.4, 25.6, 24.4, 23.9, 22.9, 22.6, 21.1, 19.4, 18.7, 11.9; IR (film) \( \nu \) max 3290, 3068, 2935, 2868, 1727, 1688, 1634, 1538, 1449, 1416, 1367, 1253, 1157, 1026, 960, 903, 850, 757, 666 cm\textsuperscript{-1}; HRMS (ESI\textsuperscript{+}) 1277.8502 m/z (M+H\textsuperscript{+}, \( \text{C}_{74}\text{H}_{113}\text{N}_6\text{O}_{12} \) requires 1277.8416).

![Chemical Structure](image)

(11S,14S)-11-(2-Carboxylatoethyl)-1-[3-carboxylato-4-(6-oxido-3-oxo-3H-xanthen-9-yl)phenyl]-14-[(3-(3β-cholest-5-en-3-ylammonio)propyl)amino] carbonyl]-1,9,12-trioxo-5,6-dithia-2,10,13-triazahexadecan-17-oate (38).
tert-Butyl(13S,16S)-16-[(3-((tert-butoxycarbonyl)(3β-cholest-5-en-3-yl)amino)propyl)amino]-13-(3-tert-butoxy-3-oxopropyl)-1-(9H-fluoren-9-yl)-3,11,14-trioxo-2-oxa-7,8-dithia-4,12,15-triazanonadecan-19-oate (43, 29 mg, 0.022 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo, and the resulting residue was dissolved in anhydrous DMF (2 mL). To this solution was added 5-carboxyfluorescein, succinimidyl ester (20 mg, 0.045 mmol) and DIEA (50 µL, 0.24 mmol). The reaction was stirred for 12 h at 22 °C followed by concentration in vacuo. The residue was dissolved in CH₂Cl₂ (10 mL) containing TFA (20%) and stirred for 12 h at 22 °C. The reaction was concentrated in vacuo, and the crude product was purified by preparative reverse-phase HPLC (gradient: 9.95% MeCN, 89.95% H₂O, and 0.1% TFA to 99.9% MeCN, 0% H₂O, and 0.1% TFA over 25 min; retention time = 18.5 min (215 nm), which afforded 38 (23 mg, 85%) as a yellow solid, mp 194-197 °C; ¹H NMR (400 MHz, MeOH-d₄) δ 8.52-7.68 (m, 3H), 7.38 (d, 1H), 6.75-6.58 (m, 6H), 5.42 (s, 1H), 4.35-4.25 (m, 2H), 3.78 (t, 1H), 3.35 (t, 1H), 3.08-2.89 (m, 7H), 2.80-2.67 (m, 2H), 2.48-2.37 (m, 6H), 2.20-0.89 (m, 46H), 0.72 (s, 3H); ¹³C NMR (100 MHz, MeOH-d₄) δ 176.5, 176.4, 176.3, 174.7, 174.6, 174.3 (× 2), 170.4, 168.3, 168.2, 162.0, 154.4 (× 2), 142.0, 139.4, 137.6, 135.4, 130.5, 130.4 (× 2), 128.9, 126.0, 125.3, 124.8, 114.2 (× 2), 111.0, 103.7, 59.4, 58.0, 57.5, 55.1, 55.0, 54.9, 51.3, 43.4 (× 2), 43.2, 41.0, 40.7 (× 2), 40.6, 38.5, 38.1, 37.8, 37.3, 37.1, 36.9, 36.4, 35.4, 33.0, 32.9, 31.2, 29.3, 29.1, 27.7, 27.6, 26.2, 25.3, 24.9, 23.2, 22.9, 22.0, 19.6, 19.2, 12.3; IR (film) ν max 3700-2500 (br), 3301, 3068, 2940, 2847, 1725, 1707, 1661, 1644, 1550, 1538,
1498, 1453, 1311, 1247, 1195, 1180, 1136, 1113, 846, 793, 756, 718 cm\(^{-1}\);

HRMS (ESI+) m/z 1222.5847 (M+H\(^+\), C\(_{66}\)H\(_{88}\)N\(_5\)O\(_{13}\)S\(_2\) requires 1222.5820).

(11S,14S)-11-(2-Carboxylatoethyl)-1-[3-carboxylato-4-(6-oxido-3-oxo-3H-xanthen-9-yl)phenyl]-14-[(3-(3β-cholest-5-en-3-ylammonio)propyl]amino)carbonyl]-1,5,9,12-tetraoxo-2,6,10,13-tetraazaheptadecan-17-oate (39).

tert-Butyl(13S,16S)-16-[(3-((tert-butoxycarbonyl)(3β-cholest-5-en-3-yl)amino)propyl]amino]carbonyl]-13-(3-tert-butoxy-3-oxopropyl)-1-(9H-fluoren-9-yl)-3,7,11,14-tetraoxo-2-oxa-4,8,12,15-tetraazanonadecan-19-oate (44, 41 mg, 0.032 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed \textit{in vacuo}, and the primary amine derived from 44 was dissolved in anhydrous DMF (2 mL). To this solution was added 5-carboxyfluorescein, succinimidyl ester (26 mg, 0.06 mmol) and DIEA (50 µL, 0.24 mmol). The reaction was stirred for 12 h at 22 °C followed by concentration \textit{in vacuo}. The residue was dissolved in CH\(_2\)Cl\(_2\) (10 mL) containing TFA (20%) and stirred for 12 h at 22 °C. The reaction was concentrated \textit{in vacuo}, and the crude product was purified by preparative reverse-phase HPLC (gradient: 9.9% MeCN, 90% H\(_2\)O, and 0.1% TFA to 99.9% MeCN, 0% H\(_2\)O, and 0.1% TFA
over 25 min; retention time = 17.6 min (215 nm) to afford 39 (37 mg, 88%) as a yellow solid, mp 202-205 °C; $^1$H NMR (300 MHz, MeOH-d$_4$) $\delta$ 8.47-7.64 (m, 3H), 7.38 (d, 1H), 6.74-6.54 (m, 6H), 5.45 (s, 1H), 4.31-4.23 (m, 2H), 3.72 (t, 1H), 3.63-3.40 (m, 3H), 3.34 (t, 1H), 3.14 (t, 3H), 3.05 (m, 2H), 2.93 (m, 1H), 2.58-2.34 (m, 6H), 2.11-0.85 (m, 46H), 0.72 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 174.0 ($\times$ 2), 173.9 ($\times$ 2), 171.4 ($\times$ 2), 171.0, 170.4, 168.2, 159.8, 158.4, 158.1, 151.8, 149.8, 140.7, 138.8, 134.6, 129.0, 124.2, 123.4, 123.0, 122.2, 120.8, 117.9, 115.0, 112.7, 112.1, 109.0, 102.3, 56.4, 56.1, 55.6, 52.3, 52.1, 49.1, 47.4, 43.5 ($\times$ 2), 41.8, 41.0, 36.4, 36.2, 35.7, 35.3, 35.2, 33.3 ($\times$ 2), 31.3, 30.6, 30.5 ($\times$ 2), 27.4, 27.0, 25.9, 25.5, 25.4, 24.5, 24.2, 23.2, 22.7, 22.4, 22.1 ($\times$ 2), 21.7, 18.8, 18.6, 11.7; IR (film) $\nu$ max 3700-2500 (br), 3266, 3068, 2930, 2847, 1713, 1667, 1632, 1591, 1537, 1453, 1384, 1307, 1274, 1245, 1200, 1177, 1133, 920, 845, 799 cm$^{-1}$; HRMS (ESI+) 1201.6511 m/z (M+H$^+$, C$_{67}$H$_{89}$N$_6$O$_{14}$ requires 1201.6437).

**General strategy for peptide synthesis**

Peptide synthesis was constructed with Rink amide Novagel resin (0.62 mmol/g, 50 mg, 0.032 mmol) by a Burrell Wrist-Action Laboratory Shaker and standard N-Fmoc methodology. The following Fmoc protected amino acids: Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Trp-OH, Fmoc-Tyr(t-Bu)-OH, Fmoc-Ser(t-Bu)-OH, Fmoc-Glu(t-Bu)-OH, Fmoc-β-Ala-OH, Fmoc-ε-Ahx-OH, were coupled to the resin by addition of a DMF solution (2 mL) of amino acid (4.0 eq.), HOBt (3.8 eq.), HBTU (3.8 eq.) and DIEA (8.0 eq.) with shaking at 22 °C for 2 h. Deprotection of Fmoc carbamates on the resin was conducted by addition of
piperidine (20%) in DMF (2 mL for 5 min followed by 2 mL for 15 min and 2 mL for 5 min \( \times 2 \)). After removal of the \( N\text{-}\alpha\text{-Fmoc} \) group of the N-terminal amino acid, the free amine was capped by addition of 5-[(tert-butoxycarbonyl)[(3\( \beta \)]-cholest-5-en-3-yl]amino]pentanoic acid\(^{29} \) and the amino acid coupling reagents. The product was cleaved from the resin by shaking with TFA/TIPS/H\( _2 \)O (90:8:2) for 2 h and purified by preparative reverse phase-HPLC.

\[
N\{6-[(N\{5-[3\( \beta \)]-Cholest-5-en-3-ylamino]pentanoyl\}-b-alanyl-b-alanyl]amino\}
\]

\[\text{hexanoyl]}\text{-}L\text{-}seryl-L\text{-}seryl-L\text{-}alanyl-L\text{-}tryptophyl-L\text{-}tryptophyl-L\text{-}ser\text{-}L\text{-}

\text{tyrosyl-L\text{-}tryptophyl-L\text{-}prolyl-L\text{-}prolyl-L\text{-}valyl-L\text{-}alaninamide} \text{ (31).}

HPLC (gradient: 90\% H\( _2 \)O, 9.9\% MeCN, and 0.1\% TFA to 99.9\% MeCN and 0.1\% TFA over 20 min). Retention time = 16.4 min (254 nm) to afford 31 as a white solid (12 mg, 17.4\%). LRMS (ESI\(^+\)) \text{m/z} 2158.83 (M+H\(^+\), C\text{116}H\text{165}N\text{20}O\text{20} requires 2158.25).

![Figure 2-12: Analytical HPLC profile of peptide 31 after purification by preparative HPLC. Retention time = 17.4 min. Purity by HPLC > 99%.
](image)
$N$-[6-{$N$-[$3\beta$-Cholest-5-en-3-ylammonio]pentanoyl}-5-oxidanidyl-5-oxidanylidene-L-norvalyl-5-oxidanidyl-5-oxidanylidene-L-norvalyl]amino]


HPLC (gradient: 90% H$_2$O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min). Retention time = 16.8 min (254 nm) to afford 32 as a white solid (9.1 mg, 12.5%). LRMS (ESI+) m/z 2275.00 (M+H$^+$, C$_{120}$H$_{160}$N$_{20}$O$_{24}$ requires 2274.26).

Figure 2-13: Analytical HPLC profile of peptide 32 after purification by preparative HPLC. Retention time = 17.2 min. Purity by HPLC > 99%.
$N\{6-[(N\{-5\{\beta\}-Cholest-5-en-3-ylamino\}pentanoyl)\}L-\alpha-glu-tamyl-L-\alpha-$

$glutamyl-L-\alpha-glu-tamyl\}amino\}hexanoyl\}L-seryl-L-seryl-L-alanyl-L-$

$tryptophyl-L-tryptophyl-L-seryl-L-tyrosyl-L-tryptophyl-L-prolyl-L-prolyl-L-$

$valyl-L-alaninamide (33)$. HPLC (gradient: 90% $\text{H}_2\text{O}$, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min). Retention time = 16.6 min (254 nm) to afford 33 as a white solid (9.1 mg, 12.5%). LRMS (ESI+) m/z 2403.47 (M+H$^+$, C$_{125}$H$_{176}$N$_{21}$O$_{27}$ requires 2403.30).

Figure 2-14: Analytical HPLC profile of peptide 33 after purification by preparative HPLC. Retention time = 16.7 min. Purity by HPLC > 99%.

HPLC (gradient: 90% H₂O, 9.9% MeCN, and 0.1% TFA to 99.9% MeCN and 0.1% TFA over 20 min). Retention time = 17.0 min (254 nm) to afford 34 as a white solid (12.5 mg, 16.7%). LRMS (ESI+) m/z 2231.42 (M+H⁺, C₁₂₃H₁₇₄N₂₁O₂₅ requires 2231.21).

Figure 2-15: Analytical HPLC profile of peptide 34 after purification by preparative HPLC. Retention time = 17.1 min. Purity by HPLC > 99%.
2.6.3. Biological Assays and Protocols

2.6.3.1 Cell culture

Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). CHO-K1 cells (Chinese hamster ovary cells, ATCC# CCL-61) were cultivated in F-12K medium supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). J-774 cells were maintained in DMEM supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). All cell lines were propagated in a humidified 5% CO₂ incubator at 37 °C. Media used for cell culture and wash steps contained antibiotics and FBS unless otherwise noted.

2.6.3.2 Microscopy

A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. Fluorescein and Alexa Fluor-488 were excited with a 488 nm Argon ion laser (25 mW, 1% laser power) and emitted photons were collected through 505 nm long pass filter. Excitation of 6-Hex, Texas Red, and Dil employed a 543 nm HeNe laser and emitted photons were collected through a 560 nm long pass filter. Slides used for live-cell imaging were prepared by sealing media (30 µL) containing suspension cells between a
micro cover glass (22 x 22 mm, VWR) and a micro slide (1.0 mm thick, 25 x 75
mm, VWR) via Press-to-seal silicone isolator (22 mm diameter, 0.5 mm deep,
Invitrogen). To maintain cell viability, imaging of suspension cells should be
completed within 30 min after the slide is prepared. For imaging of adherent cell
lines, slides were prepared by first pressing the silicone isolator onto a micro
slide and adding media (180 µL) in the space created by the micro slide and
isolator. The cover glass used to cultivate the cells (the side with cells face down)
was sealed using the silicone isolator. To maintain cell viability, imaging of
adherent cells should be performed immediately after preparation of the slide.

2.6.3.3 Flow Cytometry

Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow
cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded
cellular physical properties of size and granularity that allowed gating of live cells.
After gating, 10,000 cells were counted. In studies of endosomal release, the
fluorophores were excited at 488 nm with a 15 mW air-cooled argon-ion laser,
the emission was split with a 550 nm dichroic and filtered through a 510 nm long
pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT
voltage for this instrument was set to 524 for CHO cells.
2.6.3.4 Examination of Colocalization of Red Fluorescent and Green Fluorescent Compounds and Proteins by Confocal

CHO cells \((1.2 \times 10^5)\) in media \((2 \text{ mL})\) were cultivated on round collagen-coated coverslips \((22 \text{ mm}, \text{ BD BioCoat})\) in a 6-well plate. After incubation at 37 °C for 24 h, media was replaced with fresh media \((2 \text{ mL})\). Cells were treated with \(36\) (final concentration = 2 µM) and/or \(38\) (final concentration = 5 µM) in DMSO (final [DMSO] = 1%) at 37 °C for 12 h followed by washing with fresh media \((2 \text{ mL})\). Cells treated with \(36\) and \(38\) were immediately analyzed by confocal microscopy after the wash step. Cells treated with \(38\) alone were incubated with media containing transferrin, Texas Red conjugate (final concentration = 500 nM) or Dil-LDL (final concentration = 8 nM). Cells treated with \(36\) alone were incubated with media containing transferrin, Alexa Fluor 488 conjugate (final concentration = 610 nM). Cells treated with these fluorescent protein markers of early/recycling or late endosomal/lysosomal compartments were incubated at 37 °C for 5 min, washed with PBS \((2 \times 2 \text{ mL})\), and analyzed by confocal microscopy.

2.6.3.5 Cytotoxicity Assays

Trypsinized CHO cells \((2 \times 10^3)\) in media \((100 \muL)\) were loaded on a 96-well plate. After incubation for 36 h, the media was replaced with fresh media \((100 \muL)\) containing increasing concentrations of \(35\) in DMSO (final [DMSO] = 1%). After 48 h of incubation, the cells in each well were washed with PBS \((2 \times 100 \muL)\) and detached by treatment with a solution of trypsin \((50 \muL, \text{ 5 min})\). The
cell suspension (10 µL) was transferred to an opaque 96-well plate containing PBS (170 µL per well) and treated with CellTiter-Glo reagent (20 µL, Promega) according to the Promega protocol provided with the reagent. After incubation at ambient temperature (22 °C) for 10 min, the luminescence of the samples was measured with a Packard Fusion microplate reader.

2.6.3.6 Inhibition of Acidification of Endosomes with Chloroquine

Exponentially growing CHO cells were cultivated on round collagen-coated coverslips (22 mm, BD BioCoat) in a six-well plate (1.2 × 10^5 cells/well, 2 mL). After 24 h, the media was replaced with fresh media containing chloroquine (final concentration = 5 µM) in DMSO (final [DMSO] = 1%) and cells were incubated at 37 °C for 1 h. The media was replaced with fresh media containing 38 (5 µM) and 36 (8 µM) in DMSO (final [DMSO] = 1%) with or without chloroquine (final concentration = 5 µM) in DMSO (final [DMSO] = 1%). The cells were incubated at 37 °C for 24 h. The cells were washed with PBS (2 × 2 mL) prior to analysis by confocal microscopy.

2.6.3.7 Dose Dependent Endosome Release Assays

Exponentially growing CHO cells were in a 24-well plate (1.2 × 10^5 cells/well, 0.5 mL). After 12 h, the media was replaced with fresh media
containing 38 (5 µM) and increasing concentrations of 35 (final [DMSO] = 1%) and cells were incubated at 37 °C for 24 h. The cells were washed with PBS (2 × 0.5 mL) and tripsinized by tripsin (200 µL, 5 min). The cell solutions were washed with PBS (2 × 0.5 mL) prior to analysis by flow cytometry.

2.7 References


3.1 Introduction

Many drugs must penetrate hydrophobic lipid bilayers to reach their intracellular targets. Consequently, many drug candidates fail due to poor cellular permeability. To facilitate the delivery of poorly-permeable drugs across membranes, drugs have been extensively modified with cell penetrating peptides, proteins (CPPs), \(^1\) polymers \(^2\) and liposomes. \(^3\) Ligands of natural internalizing cell surface receptors have also been linked to drugs to deliver these conjugates in cells via receptor-mediated endocytosis. \(^4\) Receptors involved in these studies, including transferrin receptors, \(^5\) LDL receptors \(^6\) and folate receptors, \(^7\) have been intensively targeted because of their high delivery efficiency. However, this approach for the delivery of therapeutics is limited by low receptor expression on various cell types.

Synthetic mimics of cell surface receptors studied in our lab enable the cellular delivery of various cell impermeable molecules, such as antibodies and other proteins. \(^8\)-\(^{13}\) This strategy has potential applications in the delivery of drugs that are poorly permeable and not internalized by natural receptors expressed
Figure 3-1: Structures of a D-Phe-D-Ala derived receptor 45 and a fluorescent derivative of the antibiotic vancomycin (46).\textsuperscript{15}

on the cell surface. For example, the glycopeptide vancomycin is an antibiotic drug that inhibits biosynthesis of the bacterial cell wall by binding to peptidoglycan precursors terminating in D-Ala-D-Ala.\textsuperscript{14} However, due to the low cell permeability of vancomycin, this drug is not effective against bacteria that penetrate through the plasma membrane. Boonyarattanakalin\textsuperscript{15} used D-Phe-D-Ala derived synthetic receptor 45 to deliver a fluorescent derivative of vancomycin 46 into mammalian cells in culture and tissues of Balb/c mice. The reason D-Phe-D-Ala was used instead of the natural D-Ala-D-Ala is that D-Phe-D-Ala has a higher affinity for vancomycin (Kd ~ 7 µM) compared with D-Ala-D-
Ala (Kd ~ 100 µM).\textsuperscript{16, 17} This result proved the feasibility of using synthetic receptors for drug delivery. Based on this precedent, we sought to create a generic drug delivery system using similar small molecule recognition properties. In a related area of research, selective vesicle fusion has been achieved by displaying vancomycin and D-Ala-D-Ala in apposing membranes as binding motifs.\textsuperscript{18}

We utilized the vancomycin glycopeptide and the D-Phe-D-Ala dipeptide as a recognition pair for the construction of a novel drug delivery system. In our system, it was proposed that vancomycin-derived synthetic receptors may enable the delivery of drugs conjugated to the easily synthesized D-Phe-D-Ala ligand. Since it has been reported that the binding affinity of diacetyl-L-Lys-D-Ala-D-Ala for vancomycin (Kd ~ 1 µM) is about 100 times better than that of N-acetyl-D-Ala-D-Ala due to extra hydrogen bonding interactions,\textsuperscript{16} we hypothesized that L-Lys-D-Phe-D-Ala tripeptide might have better binding affinity for vancomycin and improve drug delivery efficiency.
3.2 Design and Synthesis of Vancomycin-Based Receptors and L-Lys-D-Phe-D-Ala Ligands

Figure 3-2: Structures of vancomycin derived receptors 47 and 48, a fluorescent derivative of L-Lys-D-Phe-D-Ala 49, and negative control compound 50.
A synthetic receptor 47 comprising vancomycin linked to the $N$-alkyl-$3\beta$-cholesterylamine anchor bearing two $\beta$-alanine subunits was first designed as a prototype compound. Based on the success of modification of the linker region with glutamic acid subunits in improving the cellular association and delivery ability of synthetic receptors, vancomycin-based receptor 48 bearing two glutamic acids was also designed for comparison with 47. To evaluate the ability of these receptors to mediate the uptake of the L-Lys-D-Phe-D-Ala tripeptide ligand, the Pennsylvania Green fluorophore was linked to the tripeptide (49) via amino hexanoic acid. The negative control 50 comprising a L-Lys-L-Phe-L-Ala tripeptide, a sequence that does not bind vancomycin was also designed.

Receptor 47 was synthesized as shown in Figure 3-3. Compound 55 was first synthesized from $3\beta$-cholesterylamine via alkylation with ethyl 5-bromovalerate and Boc protection, followed by coupling with a linker derived from two $\beta$-alanines.\textsuperscript{12} Hydrolysis of the ethyl ester under basic conditions afforded the free carboxylate acid analogue 56. Reaction with $N$-hydroxysuccinimide and deprotection of the Boc carbamate, followed by the coupling with vancomycin, afforded the desired compound 47. To synthesize receptor 48, the $3\beta$-cholesterylamine derivative 18 with two attached glutamic acids was reacted with glutaric anhydride to provide carboxylic acid 57, which was converted to receptor 48 using the same route for preparation of compound 47.
Figure 3-3: Synthesis of compounds 47 and 48. Reagents and conditions: (a) ethyl 5-bromovalerate, K$_2$CO$_3$, DMF; (b) (Boc)$_2$O, DIEA, CH$_2$Cl$_2$; (c) 20% piperidine, DMF; (d) LiOH, MeOH/THF/H$_2$O (3:1:1); (e) 54, EDC, HOBt, 4 °C to 22 °C; (f) N-Hydroxysuccinimide, DDC, THF; (g) 15% TFA, CH$_2$Cl$_2$; (h) vancomycin hydrochloride, K$_2$CO$_3$, DMSO; (i) glutaric anhydride, K$_2$CO$_3$, THF, 50 °C.
The Pennsylvania Green tripeptide conjugate 49 was synthesized via sequential amino acid coupling of t-butyl protected D-alanine 59 with Fmoc-protected D-phenylalanine, lysine, and amino hexanoic acid to afford precursor 62 (Figure 3-4). Coupling with Pennsylvania Green succinimidyl ester, DIPEA, DMF; (f) 15% TFA, CH$_2$Cl$_2$. The negative control compound 50 was prepared similarly.
3.4 Evaluation of the Delivery of L-Lys-D-Phe-D-Ala Conjugate by Vancomycin-Based Receptors

To determine the affinity of the L-Lys-D-Phe-D-Ala tripeptide for vancomycin, the fluorescent compound 49 was evaluated using fluorescence polarization assays. The L-Lys-D-Phe-D-Ala tripeptide binds to vancomycin with $K_d$ value of 174 nM (Figure 3-5). The second binding affinity observed in this experiment was consistent with the divalent back to back interactions of vancomycin with the peptide binding motif.²⁰

Figure 3-5: Determination of the binding affinity of L-Lys-D-Phe-D-Ala for vancomycin by fluorescence polarization. The concentration of 49 (50 nM) was held constant and the concentration of vancomycin was varied (0 to 100 µM) in phosphate buffered saline (PBS) (200 µL, pH 7.4). Experiments were run in triplicate.
Figure 3-6: Panels A-C: Confocal laser scanning and DIC micrographs of living Jurkat lymphocytes treated with 47 (panel B) or 48 (panel C) (10 µM) for 1 h at 37 °C, washed, and further treated with 49 (5 µM) for 4 h at 37 °C. Panel A: Cells were treated with 49 (5 µM) for 4 h at 37 °C. Panel D: flow cytometric analysis of uptake of 49 (5 µM) by 47 or 48 (10 µM).

The uptake of 49 by the vancomycin-derived receptors 47 and 48 was evaluated by both confocal microscopy and flow cytometry (Figure 3-6). In these experiments, Jurkat lymphocytes were treated with receptors for an hour, cells were washed to remove unincorporated receptors and treated with fluorescent tripeptide 49 for 4 hours. Treatment with 49 alone showed no intracellular fluorescence, whereas the treatment with 47 and 48 prior to addition of 49 promoted the accumulation of intracellular fluorescence, indicating that the vancomycin-derived receptors were capable of delivering the fluorescent L-Lys-
D-Phe-D-Ala ligand into Jurkat cells. Receptor 48 bearing two glutamic acids was more effective than receptor 47 lacking these subunits. Quantitative data obtained by flow cytometry indicated that the delivery efficacy of 48 is more than five times better than that of 47. These experiments again established the positive effect of glutamic acids in the linker region of synthetic receptors.

The dose dependent uptake of 49 by receptor 48 in Jurkat lymphocytes was examined by flow cytometry. As shown in Figure 3-7 (panel A), substantial dose dependent enhancement of fluorescence was observed. Preequilibration of 48 and 49 before adding the mixture to cells was more effective than preloading the plasma membrane with receptor, conditions where cells were saturated with receptor 48, washed to remove unincorporated receptor, and subsequently treated with 49.

Based on these promising results, a synthetic receptor targeting assay was conducted in vivo by injecting different groups of mice intraperitoneally with 48 (50 mg/kg) or PBS as a control. One hour later, 49 or the L-Lys-L-Phe-L-Ala control ligand 50 were injected. Fluorescence of cells separated from spleen, liver, skeletal muscle, pancreas, and brain were quantified by flow cytometry (Figure 3-7, panel B). Enhanced uptake of 49 by 48 was observed in pancreas, spleen and liver cells in mice, while the control 50 showed weak uptake and tissue accumulation in vivo indicating the delivery depends on the specific recognition of L-Lys-D-Phe-D-Ala by vancomycin.
Figure 3-7: Panel A: Flow cytometric analysis of dose dependent uptake of 49 by Jurkat lymphocytes mediated by 48. PL = Preloading conditions: cells were treated with 48 for 1 h at 37 °C, washed and treated with 49 (5 µM) for 4 h at 37 °C. PE = Preequilibration conditions: 48 was mixed with 49 (5 µM) for 10 min and added to cells for 4 h at 37 °C. Panel B: Flow cytometric analysis of uptake of 49 by tissues of Balb/c mice in vivo. Mice were injected intraperitoneally with compound 48 (50 mg/kg) for 1 h, followed by injection with compound 49 or 50 (25 mg/kg). Mice were sacrificed after 10 h, and fluorescence of cells from tissues was analyzed. Error bars represent the standard deviation (n = 3). The in vivo experiments were run in collaboration with Jianfang Hu, Avery August lab.)
3.5 Conclusions

A potential drug delivery system based on synthetic mimics of cell surface receptors and small molecule recognition was investigated. By conjugating drugs of interest to a small L-Lys-D-Phe-D-Ala tripeptide ligand, the drug conjugates have the potential to be delivered by the vancomycin-based receptor. This delivery system significantly enhanced the uptake of fluorescent tripeptide ligand, suggesting potential applications for the delivery of various impermeable bioactive molecules.

3.6 Experimental section

3.6.1 General

Chemical reagents and solvents were obtained from Acros, Aldrich and EMD Biosciences. Media and antibiotics were purchased from Mediatech and Gibco BRL. Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) media were purchased from Valley Biomedical. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). All reactions
were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25mm silica gel 60 F254 (EMD Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with a solution of phosphomolybdic acid in ethanol (20%). Flash column chromatography employed ICN SiliTech Silica Gel (32-63 µm). Purification by preparative reverse phase HPLC employed an Agilent 1100 preparative pump/gradient extension instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) reverse phase column (7 µm particle size, 21.5 mm x 25 cm). The HPLC flow rate was maintained at 20 mL/min for the entire run unless otherwise noted. Melting points were measured with a Thomas Hoover capillary melting point apparatus and were uncorrected. Infrared spectra were obtained with a Perkin Elmer 1600 Series FTIR. NMR spectra were obtained with Bruker CDPX-300, DPX-300, AMX-360, or DRX-400 instruments with chemical shifts reported in parts per million (ppm, δ) referenced to either CDCl₃ (¹H 7.27 ppm; ¹³C 77.23 ppm), MeOH-d₄ (¹H 4.80 ppm; ¹³C 49.15 ppm), DMSO-d₆ (¹H 2.50 ppm; ¹³C 39.51 ppm), or (CH₃)₄Si. High-resolution mass spectra were obtained from the Penn State University Mass Spectrometry Facility (ESI and CI) and University of Kansas Mass Spectrometry Facility (ESI). Low-resolution mass spectra were obtained with a Waters ZQ-4000 mass spectrometer. Peaks are reported as m/z.
3.6.2 Synthetic Procedures and Compound Characterization Data


To a solution of Fmoc-D-Phe-OH (387 mg, 1.82 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL) at 4 °C were added HOBt (267 mg, 1.98 mmol) and EDC (380 mg, 1.98 mmol) and the solution was stirred for 30 min. To this solution was added H-D-Ala-O-t-BuHCl (300 mg, 1.65 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL) containing DIEA (0.3 mL, 1.82 mmol). The reaction was allowed to warm to 22°C and stirred for 12 h. This solution was diluted with CH$_2$Cl$_2$ (10 mL) and washed with aqueous NaOH (0.1 M, 20 mL) and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo to afford **tert-Butyl (R)-2-(((R)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-phenylpropanamido)propanoate (60)**, a white solid was carried through without further purification.

Compound **60** (200 mg, 0.39 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo. To a solution of Fmoc-L-Lys(Boc)-OH (200 mg, 0.43 mmol) in anhydrous
CH$_2$Cl$_2$ (5 mL) at 4 °C were added HOBt (63 mg, 0.47 mmol) and EDC (90 mg, 0.47 mmol) and the solution was stirred for 30 min. The primary amine derived from 60 was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) and added dropwise. The reaction was allowed to warm to 22°C and stirred for 12 h. This solution was diluted with CH$_2$Cl$_2$ (10 mL) and washed with aqueous NaOH (0.1 M, 20 mL) and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. Flash column chromatography (Hexanes / Ethyl acetate, 1:1) afforded 61 (283 mg, 92%) as a white solid, mp 101-104 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.74 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.3 Hz, 2H), 7.38 (t, J = 7.3 Hz, 2H), 7.29 (d, J = 7.0 Hz, 2H), 7.22-7.17 (m, 5H), 6.92 (d, J = 7.7 Hz, 1H), 6.77 (d, J = 7.0 Hz, 1H), 5.70 (d, J = 5.4 Hz, 1H), 4.73 (q, J = 6.7 Hz, 1H), 4.65 (s, 1H), 4.40-4.28 (m, 3H), 4.18 (t, J = 6.9 Hz, 1H), 4.10 (q, J = 7.0 Hz, 1H), 3.15 (dd, J = 5.8 Hz, J = 13.8 Hz, 1H), 3.05-3.02 (m, 3H), 1.65 (m, 1H), 1.60-1.30 (m, 3H), 1.43 (s, 9H), 1.41 (s, 9H), 1.30 (d, J = 7.1 Hz, 3H), 1.17 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.7, 171.5, 170.1, 156.2, 156.0, 143.8, 143.7, 141.2, 141.2, 136.4, 129.2 (x 2), 128.5 (x 3), 127.6 (x 2), 127.0 (x 2), 126.9, 125.0, 119.9 (x 2), 81.8, 79.1, 67.1, 54.9, 54.1, 48.7, 47.0, 39.8, 37.9, 32.0, 29.7, 28.4 (x 3), 27.8 (x 3), 22.2, 18.1; IR (film) ν max 3289, 3071, 3011, 2978, 2934, 2863. 1709, 1645, 1531, 1451, 1391, 1367, 1247, 1152, 1043, 849, 758, 741 cm$^{-1}$; HRMS (ESI+) m/z 765.3824 (M+Na$^+$, C$_{42}$H$_{54}$N$_4$O$_8$Na requires 765.3839).

tert-Butyl (10S,13R,16R)-13-benzyl-10-[[9H-fluoren-9-ylmethoxy]carbonyl]amino)-2,2,16-trimethyl-4,11,14-trioxo-3-oxa-5,12,15-triazaheptadecan-17-oate (61, 389 mg, 0.52 mmol) was dissolved in DMF (2 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was removed in vacuo, and the primary amine derived from 61 was dissolved in anhydrous CH2Cl2 (5 mL). To a solution of Fmoc-ε-Ahx-OH (201 mg, 0.57 mmol) in anhydrous CH2Cl2 (5 mL) at 4 °C were added HOBt (84 mg, 0.62 mmol) and EDC (120 mg, 0.62 mmol) and the solution was stirred for 30 min. To the solution was added the solution of primary amine derived from 61. The reaction was allowed to warm to 22°C and stirred for 12 h. This solution was diluted with CH2Cl2 (10 mL) and washed with aqueous NaOH (0.1 M, 20 mL) and saturated aqueous NaCl (20 mL). The organic layer was dried over anhydrous Na2SO4 and concentrated in vacuo. Flash column chromatography (Hexanes / Ethyl acetate, 1:1) afforded 62 (427 mg, 95%) as a white solid, mp 147-149 °C; 1H NMR (400 MHz, CDCl3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.4 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.30 (d, J = 7.4
101 Hz, 2H), 7.25-7.17 (m, 5H), 6.78 (br, 1H), 6.68 (dd, J₁ = 7.5 Hz, J₂ = 18.3 Hz, 1H), 6.24 (s, 1H), 5.12 (s, 1H), 4.69 (q, J = 7.3 Hz, 1H), 4.62 (s, 1H), 4.38-4.20 (m, 5H), 3.18-3.13 (m, 3H), 3.04-2.99 (m, 3H), 2.20 (t, J = 7.2 Hz, 2H), 1.65-1.25 (m, 31H), 1.12 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) δ 173.3, 171.8, 171.6, 170.2, 156.5, 156.1, 144.0 (x 2), 141.2 (x 2), 136.6, 129.3 (x 2), 128.5 (x 2), 127.6 (x 2), 127.0 (x 3), 126.9, 125.0, 119.9 (x 2), 81.8, 79.0, 66.5, 54.2, 53.1, 48.7, 47.3, 40.7, 39.8, 37.8, 36.3, 31.5, 29.5 (x 2), 28.4 (x 3), 27.9 (x 3), 26.2, 24.7, 22.3, 18.1; IR (film) ν max 3297. 3066, 2973, 2935, 2857, 1690, 1635, 1538, 1451, 1368, 1254, 1171, 1147, 757, 741 cm⁻¹; HRMS (ESI+) m/z 878.4623 (M+Na⁺, C₄₈H₆₅N₅O₉Na requires 878.4680).

(10S,13R,16R)-10-(4-Aminobutyl)-13-benzyl-1-[4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl]-3-methylphenyl]-16-methyl-1,8,11,14-tetraoxo-2,9,12,15-tetraazaheptadecan-17-oic acid (49)

tert-Butyl (12S,15R,18R)-15-benzyl-12-{4-[(tert-butoxycarbonyl)amino]butyl}-1-(9H-fluoren-9-yl)-18-methyl-3,10,13,16-tetraoxo-2-oxa-4,11,14,17-tetraazanonadecan-19-oate (62, 15 mg, 0.018 mmol) was dissolved in DMF (1 mL) containing piperidine (20%) and stirred for 30 min at 22 °C. The solvent was
removed in vacuo, and the residue was dissolved in anhydrous DMF (2 mL). To this solution was added 4-carboxy-Pennsylvania Green, succinimidyl ester (6 mg, 0.013 mmol) and DIEA (50 µL, 0.24 mmol). The reaction was stirred for 12 h at 22 °C and concentrated in vacuo. The residue was dissolved in CH₂Cl₂ (10 mL) containing TFA (20%) and stirred for 12 h at 22 °C. The reaction was concentrated in vacuo, and the crude product was purified by preparative reverse-phase HPLC (gradient: 9.95% MeCN, 89.95% H₂O, and 0.1% TFA to 99.9% MeCN, 0% H₂O, and 0.1% TFA over 25 min; retention time = 13.8 min (215 nm)), which afforded 49 (6.5 mg, 62%) as an orange solid, mp 176-178 °C; ¹H NMR (300 MHz, MeOH-d₄) δ 7.88 (s, 1H), 7.82 (d, J = 7.9 Hz, 1H), 7.30 (d, J = 7.9 Hz, 1H), 7.26-7.15 (m, 5H), 6.86 (d, J = 6.9 Hz, 2H), 6.69 (d, J = 10.8 Hz, 2H), 4.64 (dd, J₁ = 4.1 Hz, J₂ = 10.6 Hz, 1H), 4.32 (q, J = 7.1 Hz, 1H), 4.08 (t, J = 7.3 Hz, 1H), 3.38 (m, 1H), 2.85-2.58 (m, 3H), 2.17 (t, J = 6.8 Hz, 2H), 2.06 (s, 3H), 1.61-1.26 (m, 15H), 1.07 (m, 1H), 0.68 (m, 1H); ¹³C NMR (75 MHz, MeOH-d₄) δ 176.6 (x 2), 174.6 (x 2), 173.4, 169.9 (x 2), 156.6, 139.1 (x 2), 138.4 (x 2), 138.1, 136.7, 131.2 (x 2), 130.8 (x 3), 129.9 (x 3), 128.2 (x 2), 126.8 (x 2), 116.5, 113.3, 113.1, 107.0, 55.9, 55.4, 50.4, 41.4, 40.9, 38.9, 36.9, 32.3, 30.6, 28.6, 28.1, 26.9, 23.8, 20.0, 17.9; IR (film) ν max 3412, 2934, 1643, 1536, 1465, 1306, 1191, 1139, 798 cm⁻¹; HRMS (ESI+) m/z 864.3344 (M+Na⁺, C₄₅H₄₉F₂N₅O₉Na requires 864.3396).
(10S,13S,16S)-10-[(4-Aminobutyl)-13-benzyl-1-[4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenyl]-16-methyl-1,8,11,14-tetraoxo-2,9,12,15-tetraazaheptadecan-17-oic acid (50)

The procedure for the synthesis of compound 50 was similar to that of compound 49 and the crude product was purified by preparative reverse-phase HPLC (gradient: 9.95% MeCN, 89.95% H2O, and 0.1% TFA to 99.9% MeCN, 0% H2O, and 0.1% TFA over 20 min; retention time = 10.2 min (215 nm)), which afforded 50 (5.0 mg, 48%) as an orange solid, mp 158-161 °C; 1H NMR (300 MHz, MeOH-d4) δ 7.88 (s, 1H), 7.82 (d, J = 7.8 Hz, 1H), 7.30 (d, J = 7.9 Hz, 2H), 7.20-7.10 (m, 5H), 6.87 (d, J = 7.0 Hz, 2H), 6.68 (d, J = 10.9 Hz, 2H), 4.60 (dd, J1 = 4.9 Hz, J2 = 9.2 Hz, 1H), 4.29 (q, J = 7.1 Hz, 1H), 4.21 (t, J = 7.9 Hz, 1H), 3.37 (t, J = 5.8 Hz, 1H), 2.88-2.80 (m, 3H), 2.17 (m, 2H), 2.07 (s, 3H), 1.64-1.21 (m, 17H); 13C NMR (75 MHz, MeOH-d4) δ 176.5 (x 2), 174.6 (x 2), 173.4, 166.9 (x 2), 154.2, 138.7 (x 2), 138.5 (x 2), 138.3, 131.2 (x 2), 130.9 (x 3), 129.8 (x 3), 128.8 (x 2), 127.2 (x 2), 116.8, 113.3, 113.1, 107.0, 55.9, 54.7, 51.5, 43.0, 40.9, 40.7, 37.0, 32.5, 30.6, 28.2, 27.8, 26.9, 24.0, 20.1, 17.8; IR (film) v max 3411, 2929, 1644, 1539, 1495, 1306, 1191, 1139, 798 cm⁻¹; HRMS (ESI+) m/z 864.3376 (M+Na⁺, C₄₅H₄₉F₂N₅O₉Na requires 864.3396).

Ethyl 5-\{(3\beta\)-cholest-5-en-3-yl\}-2,2-dimethyl-4,10,14-trioxo-3-oxa-5,11,15-triaza-octadecan-18-oate (55, 20 mg, 0.026 mmol) was dissolved in CH$_3$OH (3 mL) and THF (1 mL), followed by aqueous LiOH (1 mL, 0.2 M). The solution was stirred for 12 h at 22 °C and neutralized by the addition of acetic acid. The solution was concentrated \textit{in vacuo} to give compound 56, which was dissolved in anhydrous
THF (2 mL). To this solution, DDC (16 mg, 0.078 mmol) and N-hydroxysuccinimide (6 mg, 0.052 mmol) were added. The reaction was stirred for 12 h at 22 °C. The precipitation was filtered off and the filtrate was concentrated in vacuo. The resulting residue was dissolved in CH₂Cl₂ (5 mL) containing 15% TFA and stirred for 2 h at 22 °C. The solvent was removed in vacuo. The residue was dissolved in anhydrous DMSO (2 mL). To this solution were added vancomycin hydrochloride (7 mg, 0.005 mmol) and K₂CO₃ (4 mg, 0.03 mmol). The reaction was stirred for 12 h at 22 °C. The solution was filtered and purified by preparative reverse phase-HPLC (gradient: 90% H₂O and 10% MeCN to 100% MeCN over 15 min). Retention time = 11.9 min (254 nm) to afford 47 as a white solid (4.9 mg, 51%). LRMS (ESI+) m/z 2057.45 (M+H⁺, C₁₀₄H₁₃₉Cl₂N₁₂O₂₇ requires 2057.92).

**Figure 3-8:** Analytical HPLC profile of vancomycin-derived receptor 47 after purification by preparative HPLC. Retention time = 11.2 min. Purity by HPLC > 99%.
\((15R,18R)\)-15,18-Bis(3-tert-butoxy-3-oxopropyl)-5-\(3\beta\)-cholest-5-en-3-yl]-2,2-dimethyl-4,10,14,17,20-pentaoxo-3-oxa-5,9,13,16,19-pentaazatetracosan-24-oic acid (57).

\(\text{tert-Butyl } (15S,18S)-15-(3\text{-}\text{tert-butoxy-3-oxopropyl})-5-\(3\beta\)-cholest-5-en-3-yl]-18-[[\(9H\)-fluoren-9-ylmethoxy]carbonyl]amino}-2,2-dimethyl-4,10,14,17-tetraoxo-3-oxa-5,9,13,16-tetraazahenicosan-21-oate (18, 220 mg, 0.18 mmol) was dissolved in DMF (2 mL) containing piperidine (20\%) and stirred for 30 min at 22 °C. The solvent was removed \textit{in vacuo}, and the residue was dissolved in anhydrous THF (2 mL). To this solution was added glutaric anhydride (42 mg, 0.365 mmol) and K\(_2\)CO\(_3\) (76 mg, 0.55 mmol). The reaction was stirred for 4 h at 40 °C. The precipitate was removed by pipet filtration and the filtrate was concentrated \textit{in vacuo}. Flash column chromatography (CH\(_2\)Cl\(_2\) / CH\(_3\)OH / CH\(_3\)COOH, 20:1:0.1) afforded 57 (198 mg, 99\%) as an oil; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.83 (d, J = 6.0 Hz, 2H), 7.50 (d, J = 5.4 Hz, 1H), 7.43 (br, 1H), 5.33 (s, 1H), 4.47 (d, J = 3.7 Hz, 2H), 3.61-3.23 (m, 7H), 2.50-0.86 (m, 85H), 0.67 (s, 3H); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 178.0 (× 3), 177.5, 176.8, 172.6, 172.4, 172.1, 141.0, 121.4, 81.2, 80.9, 77.1, 56.7, 56.1, 52.8, 51.8, 50.0, 42.3 (× 2), 39.7, 39.5 (× 2), 38.4, 36.9, 36.6, 36.1, 35.7 (× 2), 34.9, 33.2, 31.8 (× 2), 31.5, 28.5 (× 3), 28.2, 28.0 (× 6), 24.3, 23.7, 22.8 (× 2), 22.5 (× 2), 21.0, 20.8 (× 2), 20.3, 19.8,
19.6 (× 3), 19.4, 18.7, 11.8; IR (film) ν max 3322, 3088, 2937, 2868, 1726, 1652, 1540, 1456, 1413, 1368, 1251, 1204, 1159, 845, 758, 667 cm⁻¹; HRMS (ESI⁺) m/z 1120.7517 (M+Na⁺, C₆₁H₁₀₃N₅O₁₂Na requires 1120.7501).


(15R,18R)-15,18-Bis(3-tert-butoxy-3-oxopropyl)-5-[3β-cholest-5-en-3-yl]-2,2-dimethyl-4,10,14,17,20-pentaoxo-3-oxa-5,9,13,16,19-pentaazatetracosan-24-oic
acid (57, 30 mg, 0.027 mmol) was dissolved in anhydrous THF (2 mL). To this solution, DDC (17 mg, 0.082 mmol) and N-hydroxysuccinimide (6 mg, 0.052 mmol) were added. The reaction was stirred for 12 h at 22 °C. The precipitation was removed by pipet filtration and the filtrate was concentrated in vacuo. The resulting residue was dissolved in CH₂Cl₂ (5 mL) containing 20% TFA and stirred for 12 h at 22 °C. The solvent was removed in vacuo. The residue was dissolved in anhydrous DMSO (2 mL). To this solution were added vancomycin hydrochloride (10 mg, 0.007 mmol) and K₂CO₃ (4 mg, 0.03 mmol). The reaction was stirred for 12 h at 22 °C. The solution was filtered and purified by preparative reverse phase-HPLC (gradient: 90% H₂O and 10% MeCN to 100% MeCN over 15 min). Retention time = 12.3 min (254 nm) to afford 48 as a white solid (11.8 mg, 76%). LRMS (ESI+) m/z 2316.66 (M+H⁺, C₁₁₄H₁₅₃Cl₂N₁₄O₃₃ requires 2316.01).

![Figure 3-9: Analytical HPLC profile of vancomycin-derived receptor 48 after purification by preparative HPLC. Retention time = 10.7 min. Purity by HPLC > 99%.](image)
3.6.3 Biological Assays and Protocols

3.6.3.1 Cell culture

Jurkat lymphocytes (human acute T-cell leukemia, ATCC #TIB-152) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Fetal Bovine Serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were propagated in a humidified 5% CO₂ incubator at 37 °C. Media used for cell culture and wash steps contained antibiotics and FBS unless otherwise noted.

3.6.3.2 Microscopy

A Zeiss LSM 5 Pascal confocal laser-scanning microscope fitted with a Plan Apochromat objective (63X) was employed. The Pennsylvania Green fluorophore were excited with a 488 nm Argon ion laser (25 mW, 1% laser power) and emitted photons were collected through 505 nm long pass filter. Slides used for live-cell imaging were prepared by sealing media (30 µL) containing suspension cells between a micro cover glass (22 x 22 mm, VWR) and a micro slide (1.0 mm thick, 25 x 75 mm, VWR) via Press-to-seal silicone isolator (22 mm diameter, 0.5 mm deep, Invitrogen). To maintain cell viability, imaging of suspension cells should be completed within 30 min after the slide is prepared. For imaging of adherent cell lines, slides were prepared by first pressing the silicone isolator onto a micro slide and adding media (180 µL) in the space...
created by the micro slide and isolator. The cover glass used to cultivate the cells (the side with cells face down) was sealed using the silicone isolator. To maintain cell viability, imaging of adherent cells should be performed immediately after preparation of the slide.

3.6.3.3 Flow Cytometry

Analyses were performed with a Beckman-Coulter XL-MCL bench-top flow cytometer. Forward-scatter (FS) and side-scatter (SSC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After gating, 10,000 cells were counted. In studies of uptake of fluorescent ligands, the fluorophore were excited at 488 nm with a 15 mW air-cooled argon-ion laser, the emission was split with a 550 nm dichroic and filtered through a 510 nm long pass filter and 530/30-nm band pass filter using the XL-MCL cytometer. The PMT voltage for this instrument was set to 724 for these experiments.

3.6.3.4 Fluorescence Polarization Assay

Fluorescence polarization assay used a Packard Fusion microtiterplate reader with a 485 nm excitation filter and a 535 nm polarization emission filter. Experiment was done in triplicate. Preequilibrate 49 (50 nM) and various concentrations of vancomycin in PBS (pH 7.4, 200 µL) in black 96 well plates
(Costar) with gentle shaking for 30 min at 22 °C prior to fluorescence polarization measurements.

3.6.3.5 Assays of Cellular Uptake of Fluorescent Ligands

3.6.3.5.1 Preloading Conditions

Jurkat lymphocytes (1 × 10^6) in media (500 µL) was incubated with 48 (final [DMSO] = 1%) for 1 h at 37 °C, cells were washed with fresh media (2 x 500 µL) and resuspended in fresh media (500 µL) containing 49 (5 µM, final [DMSO] = 1%). Cells were incubated for 4 h at 37 °C. Cells were washed with fresh media (2 x 500 µL) prior to analysis by confocal microscopy or flow cytometry.

3.6.3.5.2 Preequilibration Conditions

Receptor 48 was equilibrated with ligand 49 (5 µM, final [DMSO] = 1%) in RPMI media (500 µL) for 10 min at 22 °C. The mixture was added to Jurkat cells (1 x 10^6) and the cells were incubated for 4 h at 37 °C. Cells were washed with fresh media (2 x 500 µL) prior to analysis by confocal microscopy or flow cytometry.
3.6.3.6 Tissue Distribution in vivo

Mice (Balb/c) were divided into three groups. Each group comprises three mice. Mice were injected intraperitoneally with PBS (300 µL) containing compounds. In group I, each mouse was injected the synthetic receptor 48 (1 mg/mouse). One hour later, the fluorescent L-Lys-D-Phe-D-Ala 49 (0.5 mg/mouse) was injected. Group II mice received the fluorescent L-Lys-L-Phe-L-Ala control compound 50 (0.5 mg/mouse) one hour after receiving 48 (1 mg/mouse). In group III, mice were injected with PBS (300 µL) for 1 h and received 49 (0.5 mg/mouse). Mice were sacrificed 10 h after the injection. The spleen, liver, pancreas, brain, and skeletal muscle were removed by dissection. Cell suspensions from tissues were clarified by forcing through nylon mesh, centrifuged (4300 rpm), and resuspended in PBS (6 mL) for analysis by flow cytometry.

3.7 References


