Synthesis & Pharmacokinetic Evaluation of Cyclotriazadisulfonamide (CADA) Analogs to Target Signal Peptides

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Abstract

The small molecule cyclotriazadisulfonamide (CADA) was found to down-modulate the expression of the human cluster of differentiation 4 (hCD4) glycoprotein and sortilin through a specific interaction with respective signal peptide (SP), a short chain of amino acids that initiates a highly conserved biochemical process called cotranslational translocation. The SP is an unprecedented target in modulating protein expression. Since each protein expressed through cotranslational translocation has a unique SP, small molecules may potentially be designed to target individual SPs and modulate protein expression with high specificity. The goals of this research were to synthesize CADA analogs to study the interactions between CADA and SPs, synthesize CADA analogs with enhanced drug-like properties, and establish a pharmacokinetic profile of various biologically active CADA analogs.

Several CADA analogs were synthesized and investigated for their utility in photoaffinity labeling (PAL) experiments. The aryl azide CADA analog RA018 was synthesized and evaluated in PAL experiments but ultimately did not yield any labeled SP. Additionally, the synthesis of several CADA analogs with diazirines moieties was pursued and ultimately found to be unsuccessful. A series of PEGylated and mPEGylated CADA analogs were synthesized and evaluated for their biological activity and pharmacokinetic properties. These analogs exhibited better or similar potency to CADA. Finally, the pharmacokinetics of PEGylated and mPEGylated CADA analogs were analyzed alongside numerous CADA analogs that exhibit favorable biological activity and/or structural features associated with enhanced drug-like properties.
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CHAPTER 1

Cyclotriazadisulfonamide (CADA): a signal peptide-specific small molecule to modulate protein expression
Introduction

The small molecule cyclotriazadisulfonamide (CADA, Figure 1) certainly tells a story of innovation, decades of research, and a deep fascination and appreciation of the beautiful complexity of nature. From the serendipitous discovery of its biological activity to the realization of its unique mechanism of action, CADA has challenged and inspired scientists of both biological and chemical expertise to explore the feasibility of an unprecedented method of modulating protein expression.

CADA (9-benzyl-3-methylene-1,5-di-p-toluenesulfonyl-1,5,9-triazacyclododecane) was submitted to the U.S. National Cancer Institute (NCI) as part of an anti-HIV screening program in the early 1990’s. CADA was found to exhibit biological activity against several strains of the human immunodeficiency virus (HIV) in several cell lines. Miraculously, CADA was not initially synthesized for anti-HIV purposes.

Research efforts were initially geared towards structure-activity relationship experiments in collaboration with Dr. Dominique Schols and Dr. Kurt Vermeire of the Rega Institute for Medical Research at the Katholieke Universiteit Leuven (KU Leuven) in Leuven, Belgium. In 2002, Vermeire et al. used flow cytometry to discover that T-cells treated with CADA exhibited a significant decrease in the expression of the cluster of differentiation 4 (CD4) glycoprotein (Figure 2a). Notably, no other proteins were found to be affected by CADA, suggesting that CADA was down-modulating CD4 expression.
with high specificity (Figure 2c). Among other functions, CD4 plays a pivotal role in the immune system, signaling to other immune cells to initiate an immune response to foreign agents. However, CD4 is also the primary receptor that HIV and the human herpesvirus-7 (HHV-7) use to enter and infect immune cells. Along with co-receptors CCR5 and CXCR4, a trimer of CD4 interacts with the gp120 receptor on the HIV virion to initiate the entry process of the virus into immune cells (Figure 3), which suggested CADA’s anti-

![Antiviral Activity of CADA Against Different HIV Strains](image)

<table>
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<tr>
<th>HIV strains</th>
<th>MT-4</th>
<th>SupT1</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 IIIB</td>
<td>0.3</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>HIV-1 RF</td>
<td>1.5</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>HIV-1 NL43</td>
<td>0.5</td>
<td>3.2</td>
<td>1.6</td>
</tr>
<tr>
<td>HIV-2 ROD</td>
<td>0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HIV-2 EHO</td>
<td>0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 2. Initial experiments indicated CADA down-modulates CD4 expression. (a) The expression of CD4 in various cell lines is greatly decreased after several days following treatment with CADA. (b) CADA inhibits HIV replication in various cell lines with an IC\(_{50}\) of 0.2-3.2 µM. (c) CADA is specific for down-modulating CD4 expression, other glycoproteins tested mostly unaffected (within experimental error).
HIV activity may be correlated with lower levels of CD4 expression at the surface of immune cells. However, the exact mechanism of CADA’s effect(s) on CD4 expression remained unknown.

A thorough understanding of CADA’s mechanism of action wouldn’t be realized until 2014, when Vermeire et al. reported that CADA down-modulates CD4 expression in a signal peptide-specific manner, preventing the successful cotranslational translocation of CD4 across the membrane of the endoplasmic reticulum (ER).\(^4\) Cotranslational translocation is a highly conserved process of protein expression for a vast number of transmembrane (TM) and secreted proteins in eukaryotic cells.\(^5\) A secondary substrate affected by CADA was identified by Van Pueyenbroeck et al. in 2017. Sortilin, also a type I transmembrane protein, was also to be down-modulated in a signal peptide-specific manner analogous to CD4.\(^6\)
The cluster of differentiation 4 glycoprotein, the human immunodeficiency virus, and the acquired immunodeficiency syndrome

CADA’s mechanism of action results in the down-modulation of the cluster of differentiation 4 (CD4) glycoprotein, a type-I TM glycoprotein found on the surface of T-cells and various other immune cells. T-cells are white blood cells that play a crucial role in signaling various immune responses, such as the activation of cluster of differentiation 8 (CD8) killer cells.²

The structure of CD4 consists of four extracellular N-terminal domains, a transmembrane domain, and a short cytoplasmic domain (Figure 4).³⁷ The D1 domain interacts with receptors on antigen-presenting cells, while the cytoplasmic domain contains a sequence of amino acids that interact with a tyrosine kinase, which transfers a phosphate group from ATP to tyrosine residues to stimulate a biological response.

CD4 is relevant in the treatment the HIV, a virus belonging to a specific family of retroviruses known as lentiviruses.⁸ HIV was discovered in 1983 by Robert Gallo, Françoise Barré-Sinoussi, and Luc Montagnier, who were investigating possible causes of a significant uptick in rare opportunistic infections in homosexual men and drug abusers. It seemed to reason that there must be a cause for the induced immunodeficiency in these patients, of which HIV was eventually found to be the cause.⁸
HIV’s pathology involves the reduction of CD4 on HIV-infected immune cells, which significantly alters the effectivity of the immune system.\(^\text{10,11}\) As a result, pathogens and foreign biological threats that would normally be destroyed by the immune response go unnoticed, rendering the patient prone to severe illness or death by opportunistic infections (Figure 5). HIV infections are said to develop to the acquired immunodeficiency syndrome (AIDS) if an individual’s CD4-positive cell count falls below 200 cells per cubic millimeter of blood; an uninfected individual has a CD4-positive cell count of 500-1400 cells per cubic millimeter of blood.\(^\text{11}\)

![Figure 5. The progression of HIV to AIDS if left untreated. Notably, viral latency is common for HIV and HIV replicates rapidly with declining CD4 counts.](image)

Despite decades of research, HIV/AIDS remains a global health crisis (Figure 6). Approximately 36.9 million humans were estimated to be living with HIV globally as of 2017, with an additional 1.8 million individuals becoming infected.\(^\text{12}\) An estimated 75% of HIV-infected individuals are currently aware of their infection, while the remaining infected population (> 9 million) are unaware of their infection, presumably due to the inability to access affordable HIV testing.
Figure 6. Global HIV statistics (source: UNAIDS) showing new cases and the global distribution of HIV/AIDS in 2017.12
The HIV life cycle is fairly complicated, consisting of several events that are crucial for HIV replication. As shown in Figure 7, the HIV virion contains vital viral components required to hijack the immune cells molecular machinery, which are transferred into the cell upon binding to CD4. HIV belongs to a class of viruses called retroviruses, which insert their DNA into the host genome in order to replicate. Upon entry to the immune cell, this process begins with HIV reverse transcriptase making a DNA copy of the HIV’s RNA, which is integrated by HIV integrase into the host’s genome. HIV’s RNA and proteins are synthesized by the host cell, and HIV protease cleaves the HIV
proteins into functional components, which are packaged into a new virion and released from the immune cell.

**Current methods of HIV/AIDS therapy**

The Federal Drug Administration (FDA) approved the first drug for the treatment of HIV in October of 1990 (zidovudine (AZT)). Access to HIV treatment was not a reality for the 8-10 million people who were thought to be living with HIV globally, and very few treatments were available for those who were fortunate to have access. In fact, HIV was the 9th leading cause of mortality overall in the USA and the 3rd leading cause of mortality for men aged 25-44 years old.9

Though a cure for HIV/AIDS has not been discovered, a thorough understanding of the HIV infection and replication has propelled the development of drugs that inhibit various stages of the HIV life cycle. Indeed, a human infected with HIV can now live a relatively normal and healthy life due to a treatment regimen known as highly active antiretroviral therapy (HAART), a method that employs several different drugs which inhibit HIV replication at different stages of the HIV life cycle.14,15 Although HIV is exceptionally proficient in mutating in response to the inhibition of a single stage of its life cycle, the probability that the virus would simultaneously mutate at multiple points in its genome is quite low; HAART is an ingenious method of preventing resistant strains of HIV. There are currently 54 FDA approved drugs for the treatment of HIV, a few of which are shown in Table 1.
Table 1. Several common drugs used in highly active antiretroviral treatment (HAART). NNRTI: non-nucleoside reverse-transcriptase inhibitor; NRTI: nucleoside reverse-transcriptase inhibitor

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Trade name</th>
<th>Structure</th>
<th>Class</th>
<th>Approved</th>
</tr>
</thead>
<tbody>
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<td>Sustiva</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>NNRTI</td>
<td>1998</td>
</tr>
<tr>
<td>Lamivudine (3TC)</td>
<td>Epivir</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>NRTI</td>
<td>1995</td>
</tr>
<tr>
<td>Tenofovir disoproxil</td>
<td>Viread</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>Protease inhibitor</td>
<td>2001</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>Selzentry</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>Entry inhibitor</td>
<td>2007</td>
</tr>
<tr>
<td>Raltegravir</td>
<td>Isentress</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>Integrase inhibitor</td>
<td>2007</td>
</tr>
</tbody>
</table>
The major classes of HAART drugs are classified as either entry inhibitors (as is the case for CADA), reverse transcriptase inhibitors, protease inhibitors, or integrase inhibitors. A successful treatment regimen requires the inhibition of more than one stage of the HIV life cycle due to its ability to rapidly mutate, resulting in drug resistance. HAART has allowed many individuals infected with HIV to live relatively normal and healthy lives. As of 2017, more than about 60% of individuals (22 million) living with HIV had access to HAART; a remarkable improvement from the 8 million HIV-infected individuals that had access in 2010. Furthermore, AIDS-related mortality has greatly decreased; 940,000 AIDS-related deaths were reported in 2017, compared to 1.9 million AIDS-related deaths in 2004.

An overview of the Sec61 pathway

The secretory pathway (Figure 8) is a multi-organelle network that is responsible for the synthesis, modification, regulation, and trafficking both secreted and transmembrane (TM) proteins, as well as lipids in eukaryotic cells. Functional defects at virtually any level of the pathway often result in disease, such as cystic fibrosis, multiple sclerosis, and rheumatoid arthritis, of which effective treatment is often difficult to acquire. One aspect of the secretory pathway that has, until recently, gained minimal attention as a therapeutic target for modulating protein expression is the translation and translocation of proteins across the ER membrane.

Protein synthesis begins at the ribosome, a remarkable organelle consisting of RNA and proteins. Ribosomes translate messenger RNA (mRNA) into proteins, recruiting transfer RNA (tRNA) with the appropriate amino acid to construct a polypeptide chain.
Ribosomes are either directed to or directly embedded in the endoplasmic reticulum (ER), which consists of a network of branching tubules and flattened sacs, all of which are thought to be interconnected so that the ER membrane forms one continuous membrane enclosing an internal space called the lumen. The ER is production site for all TM proteins and lipids for cell organelles, as well as a vast number of secretory proteins. Proteins are moved from the ER to the Golgi apparatus, where they slowly move closer to the cell surface through a series of vesicles for eventual secretion\textsuperscript{16}.

![Figure 8. An overview of the Sec61 pathway.\textsuperscript{16}](image)

**Cotranslational translocation across the ER membrane: a fundamental and highly conserved process of protein expression**

CD4 and sortilin are type I transmembrane proteins that are expressed through cotranslational translocation\textsuperscript{2,20}, a fundamental mode of protein expression for both secretory and transmembrane proteins in eukaryotic cells. Notably, cotranslational translocation is also a major pathway of protein expression in yeast and bacteria, suggesting that this mode of protein expression has been conserved in the evolution of complex
biological systems.\textsuperscript{5,6} Cotranslational translocation is characterized by the concomitant translation and translocation of a protein across and/or into the ER membrane (the lumen). Once the proteins are inside the ER lumen, they are post-translationally modified and routed to their appropriate destinations through the secretory pathway. The process has been heavily reviewed in recent years and is summarized in Figure 9. Remarkably, the molecular machinery involved in cotranslational translocation is universal to every protein undergoing the process, despite one chemical structure: the signal peptide (SP).

Figure 9. An overview of cotranslational translocation. A signal peptide is recognized by the signal recognition particle (SRP), which arrests translation in the ribosome (1) and directs the ribosome and nascent protein complex to the SR receptor located on the cytosolic side of the ER membrane (2). The complex is delivered to the Sec61 translocon (3), where the SRP and SR dissociate and translation ensues with concomitant translocation of the nascent protein across the ER membrane (4). The signal peptide is cleaved by the SPase and the fully synthesized protein is routed to its destination.\textsuperscript{21}

Cotranslational translocation is initiated by the presence of a signal peptide (SP), a short chain of amino acids (15-30 residues) located on the N-terminus of a nascent protein.\textsuperscript{22} The discovery of the role of signal peptides was the subject of the 1999 Nobel Prize in Physiology, which was awarded to Günter Blöbel. Interestingly, each protein
undergoing cotranslational translocation contains its own unique chain of amino acids that comprise the signal peptide. However, several common structural motifs of signal peptides exist despite their unique chemical compositions (Figure 10). Signal peptides are hydrophobic overall, usually consisting of a positively charged region near the N-terminus (N-region) with the first residue being methionine, a hydrophobic, lysine-rich middle region (H-region), and a C-terminus (C-region) that contains a signal peptide cleavage site; the signal peptide is not present in the mature protein and is subject to proteasomal degradation.22

The primary function of a signal peptide is that of a molecular postal address that directs protein biogenesis to proceed through cotranslational translocation.22 As the ribosome is translating mRNA containing an N-terminal signal peptide, the N-terminus of the signal peptide eventually emerges from the ribosome exit tunnel and is recognized by the signal recognition particle (SRP), a ribonucleoprotein that essentially links a translating ribosome to translocation across the ER membrane.23,24 The SRP is thought to be continuously sampling the ribosome exit tunnel for the presence of a signal peptide.25 Remarkably, the SRP is capable of recognizing an immeasurable amount of different signal peptides, which is thought to be the result of a methionine rich α-helical region that is malleable enough to bind to a large number of hydrophobic structures. Importantly, the SRP halts or slows protein translation after binding to the emerging signal sequence. If, instead, translation
was not interrupted, the nascent protein could eventually be exposed to the hydrophilic environment of the cytosol, causing aggregation and presumable loss of protein function.

The SRP-ribosome complex is routed to the signal recognition particle receptor (SR) located on the surface of the ER. After docking to the SR, the entire SRP-ribosome complex is transferred to the Sec61 translocon (SecY in prokaryotes), causing the SRP and SR to dissociate and resumption of translation through the translocon and into the ER lumen.

The eukaryotic Sec61 translocon is certainly a remarkable protein conducting channel and has been extensively studied utilizing the highly homologous prokaryotic SecY translocon (Figure 11). The Sec61 is a heterotrimeric complex that facilitates the translocation across and the integration into the ER membrane. All co-translationally translated proteins are translocated through this channel. The complex consists of two essential and one non-essential subunits: the Sec61α, Sec61γ, and Sec61β, respectively. The Sec61α is pseudo-symmetric and is comprised of ten transmembrane domains (TMDs) which form a central aqueous pore. The Sec61γ traverses the bilayer diagonally behind the TMDs of the Sec61 α, essentially connecting the two halves of the Sec61α subunit. The
less conserved and non-essential Sec61β makes relatively few contacts with Sec61α and its function in the translocation process is less understood.\textsuperscript{5,26}

Notably, TMD2b and TMD7 of Sec61α form an opening to the lipid bilayer in the channel known as the lateral gate. Conformational changes induced between TMD5 and TMD6 upon binding to a signal peptide are thought to open the lateral gate to allow TM domains to enter the lipid bilayer during co-translational translocation across the translocon. This observation highlights the versatility of the channel to simultaneously translocate proteins across and into the ER membrane. Finally, the signal peptide is cleaved from the nascent protein by signal peptidase (SPase), a serine protease embedded in the ER membrane.\textsuperscript{27}

**Inhibitors of protein translocation across the ER membrane**

Several naturally occurring and synthetic small molecules (including CADA) have been shown to inhibit cotranslational translocation with varying degrees of specificity and have been recently reviewed by Van Puyenbroeck and Vermeire.\textsuperscript{19} Interestingly, these molecules have been shown to inhibit cotranslational translocation at different stages of the process, which provides a unique opportunity to modulate protein expression and advance our general understanding about a fundamental biological process. A few of the most significant inhibitors of cotranslational translocation will be discussed herein.
HUN-7293 (Figure 12), a naturally occurring cyclic heptadepsipeptide, was discovered to exhibit potent biological activity against several cell adhesion molecules (CAMs),\textsuperscript{28,29} a group of proteins responsible for maintaining cell-to-cell interactions and playing key roles in the inflammatory response.

A convergent total synthesis of HUN-7293 was achieved by Chen et al.,\textsuperscript{30} relying on a Mitsunobu esterification and macrolactamization to construct the cyclic peptide from linear precursors, which was versatile enough to generate a pharmacophore library of HUN-7293 analogs. A systematic investigation of structural features required for potent biologically activity included an alanine scan N-methyl substitutions, and a thorough investigation of the effects of substitution at each of the residues of HUN-7293. The results confirmed the importance of the N-methyl residues and identified the degree to which each residue could be substituted while maintaining biological activity.

Based on the cumulative structural investigation results reported by Chen et al.,\textsuperscript{30} a simplified analog of HUN-7293 named cotransin (Figure 13) was synthesized and evaluated for biological activity.\textsuperscript{30} Analogous
to HUN-7293, cotransin was found to inhibit only two proteins, VCAM-1 and P-selectin, likely occurring at a post-transcriptional or post-translational step.

Indeed, cotransin was found to have no effect on the targeting of the RNC-SRP complex to the translocon, apparently exerting its effect at a post-targeting stage. Using rabbit reticulocyte lysate in the presence or absence of rough microsomes (RM), the insertion of the VCAM-1 nascent chain into the Sec61 translocon was found to be inhibited by cotransin.\textsuperscript{30,31} In contrast, cotransin had no effect on the insertion and translocation of a pPrl nascent chain. Interestingly, translocation of chimeric constructs of VCAM-1 and pPrl signal sequences fused to an identical reporter were consistent with cotransins observed selectivity, resulting in significant inhibition for the VCAM-1 construct but not the pPrl construct.

Furthermore, targeting of the RNC-SRP to the SR receptor and subsequent transfer of the RNC to the Sec61 was found to be unaffected by cotransin. Instead, cotransin inhibits the insertion of VCAM-1 and subsequent pore opening in the Sec61 translocon (Figure 14).\textsuperscript{32} Indeed, an assay examining the effects of cotransin on VCAM-1 and pPrl nascent chains in the presence of purified Sec61 indicated that the insertion VCAM-1 nascent chain into Sec61 is selectively inhibited, as apparent from the resulting degradation by protease.

Figure 14. Cotransin inhibits the insertion of VCAM-1 and P-selectin into the Sec61 translocon.\textsuperscript{34}
To further investigate cotransin’s mechanism of action, an equipotent analog containing a photo-leucine and clickable propargyl for rhodium-azide reporting was synthesized (Figure 15). Subsequent photoaffinity labeling experiments identified the Sec61α as the primary target for cotransin.33

In turn, cotransin and equipotent analogs were found to inhibit a larger breadth of proteins, including ICAM-1, E-selectin, TNF-α, and others. Interestingly, a study of the interactions between TNF-α and the lateral gate in the presence of a cotransin analog CT8 has been proved useful in studying how TMD are transferred through the lateral gate of Sec61, which was previously poorly understood.34

Alternatively, the cyclic heptadepsipeptide apratoxin A (Figure 16) exhibits the biogenesis of a variety of secretory and TM proteins, inducing apoptosis in a variety of
cancer cell lines ($IC_{50} = 0.36-0.52$ nM). Several total syntheses of apratoxin A have been reported\textsuperscript{36,37} and a recent study by Paatero \textit{et al.} showed that apratoxin A binds to the Sec61\(\alpha\) (Figure 16).\textsuperscript{38} Interestingly, apratoxin A competes with cotransin for Sec61\(\alpha\) binding but, unlike cotransin, is not substrate-specific and instead blocks ER translocation of all Sec61 clients tested with comparable potency. Resistant cell cultures possessed mutations in the Sec61\(\alpha\) plug domain comparable to mutations previously observed for cotransin resistant cells, suggesting apratoxin A has a similar and perhaps overlapping binding site to that of cotransin.

![Figure 17. The chemical structure of decatransin, a cyclic decadepsipeptide that binds to the Sec61\(\alpha\) and inhibits cotranslational and post-translational translocation.](image)

More recently, a cyclic decadepsipeptide named decatransin (Figure 17) was found to inhibit both co- and post-translational translocation,\textsuperscript{39} which suggests that the site of inhibition is independent of the SRP pathway as well as the presence of an RNC docked to the translocon. Interestingly, resistant cell lines contained dominant mutations in Sec61\(\alpha\), as previously observed with cyclic depsipeptides targeting the Sec61.

Although further investigation of decatransin is warranted, it is interesting to note that decatransin, apratoxin A, and cotransin all appear to interact with the Sec61\(\alpha\) at a similar binding site. However, the apparent lack of specificity of decatransin and apratoxin A suggest that these small molecules stabilize a translocation intermediate that all proteins
of this pathway undergo, while cotransin seems to stabilize an intermediate that is specific for a smaller subset of translocated proteins.

Besides the described cyclic depsipeptides, a number of small molecules have been shown to modulate protein expression by inhibiting co-translational translocation, including eeyarestatin I (Figure 18), a small molecule that was shown to induce a rapid ER stress response in mammalian cells and showed potential as an anti-cancer agent. Like the cyclodepsipeptides, eeyarestatin I appears to target the Sec61 complex, but instead prevents the transfer of the nascent chain from the SRP-RNC to the translocon; demonstrating yet another component of the translocation pathway that may be potentially be targeted. Similar to apratoxin A and decatransin, eeyarestatin I does not discriminate between Sec61 clients and is subsequently highly cytotoxic, which may prove useful as an anti-cancer agent after more thorough investigation.

Finally, the polyketide-derived mycolactone (Figure 18) is the most recent example of a small molecule found to inhibit translocation across the ER membrane. Being the primary virulence factor for *Mycobacterium ulcerans*, mycolactone causes the severe ulcerative skin disease known as Buruli ulcer. Although mycolactone effectively

![Figure 18](image_url) Eeyarestatin I (top) and mycolactone (bottom), potent non-selective inhibitors of cotranslational translocation.
inhibits co-translational translocation, it also selectively inhibits post-translational translocation across the Sec61 based on signal sequence hydrophobicity as well as the length and folding propensity of the mature protein. Several total syntheses of mycolactone have been reported.\textsuperscript{42,43}

It seems that cotranslational translocation offers several opportunities for modulating protein expression with high specificity or broad inhibition. Cyclic depsidipeptides and small molecule inhibitors of cotranslational translocation have furthered our understanding of the intricacies of the process while providing unique opportunities for modulating protein expression. An overview of various inhibitors of cotranslational translocation is shown in Figure 19.\textsuperscript{19}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure19.png}
\caption{An overview of the stages of cotranslational translocation that are affected by various cyclic depsipeptides and small molecule inhibitors.\textsuperscript{19}}
\end{figure}
The signal peptide-specific small molecule cyclotriazadisulfonamide (CADA)

After CADA was found to exhibit biological activity against a number of HIV strains, a thorough investigation of structure-activity relationships and its mechanism of action was pursued in collaboration with Dr. Dominique Schols and Dr. Kurt Vermeire at the Rega Institute for Medical Research at the Katholieke Universiteit Leuven in Belgium. In 2002, Vermeire et al. reported that CADA’s anti-HIV activity was correlated with its ability to down modulate the expression of CD4 at the surface of MT-4 cells, SupT1 cells, and peripheral blood mononuclear cells (PBMCs). As previously mentioned, CD4 is the primary receptor that HIV uses to enter and infect immune cells.

Mechanism of action

In 2014, Vermeire et al. published an extensive study of CADA’s unique mechanism of action. As shown in Figure 20, CADA’s effect on CD4 expression is reversible and specific to CD4 expression, leaving the expression of a number of other glycoproteins unaffected. Furthermore, CADA was found to be selective for primate CD4, whereas the expression of murine CD4 expression was unaffected.

To analyze whether or not CADA was inhibiting de novo CD4 biogenesis, pulse-chase experiments with subsequent immunoprecipitation (IP) for CD4 revealed that cells treated with CADA exhibited a remarkable decrease in CD4 expression, yet overall protein biogenesis seemed unaffected. Additionally, CD4 mRNA levels were unaffected by treatment with CADA, suggesting that CADA was inhibiting CD4 expression at a post-transcriptional level during CD4 biogenesis.
As previously mentioned, CD4 consists of four extracellular domains, a TM domain, and a cytosolic domain. Single-domain mutants were generated to identify the domain(s) responsible for sensitivity to CADA. As shown in Figure 21, deletion of the CD4 cytosolic tail and the exchange the extracellular D3, D4, and TM domains of CD4 with a related protein (CD8, a similar type-I TM protein) were not affected by CADA. Interestingly, chimeric human/mouse constructs containing the hCD4 SP with murine D1 or D2 domains were incredibly sensitive to treatment with CADA, whereas murine CD4

Figure 20. A summary of CADA’s inhibitory effects on CD4 expression. (a) CADA down modulated CD4 expression in a dose-dependent manner various cell lines (b) CADA specifically down-modulates primate CD4 (c) CADA does not appear to affect the expression of similar glycoproteins (d) CADA’s inhibitory effects are reversible. 4
(mCD4) is unaffected by CADA. Furthermore, CD4 constructs containing the hCD4 SP with the TM removed was still susceptible to CADA. Therefore, the presence of the hCD4 SP and the first seven N-terminal residues of the mature hCD4 protein appeared to be necessary for sensitivity to CADA.\footnote{4}

In order to investigate exactly how and when CADA interacts with the hCD4 SP and first seven amino acids of the mature protein, \textit{in vitro} cell-free translation experiments were performed using rabbit reticulocyte lysate and rough microsomes (RMs). Rabbit reticulocyte lysate contains the molecular machinery for protein synthesis, while rough
microsomes accurately mimic the endoplasmic reticulum. Used together, the assay is useful for studying in vitro cotranslational translocation. When translation of hCD4 mRNA was carried out in the absence of RM and in the presence of CADA, CD4 expression was found to be unaffected by CADA. In contrast, translation of hCD4 mRNA in the presence of RM and CADA resulted in a significant reduction in the amount of hCD4 translocated across the membrane of the RM, which suggested that CADA was inhibiting the translocation of hCD4 across the ER membrane.4

As shown in Figure 22, CADA specifically reduces hCD4 translocation across the ER membrane, where pPL and a truncated mCD4 are unaffected. Furthermore, a construct containing the hCD4 SP fused to the mature pPL protein is translocated more efficiently than a hCD4-pPL construct which contains the first seven amino acids of the hCD4 mature protein.4

Figure 22. CADA inhibits the translocation of hCD4 across the ER membrane in a signal peptide-dependent manner. The first seven amino acids of the mature hCD4 protein appear to be important for maximum inhibition.4
protein. This observation suggested that sensitivity to CADA is dependent on the hCD4 signal peptide (25 amino acids) and the first seven residues of the hCD4 mature protein.\textsuperscript{4}

Vermeire \textit{et al.} also analyzed chimeric signal peptide constructs of the hCD4 SP in which one region (ex. the H-region) was substituted with that of the corresponding mCD4 SP (\textbf{Figure 23}). Exchanging the H-region of the hCD4 SP for that of mCD4 SP resulted in an almost complete loss of sensitivity to CADA, while the N-region and the C-region were less sensitive to substitution with the corresponding mCD4 sub-regions.\textsuperscript{4}

\textbf{Figure 23.} Chimeric constructs of the hCD4 SP revealed that the H-region is especially important for CADA sensitivity.\textsuperscript{4}
Finally, Vermeire et al. assayed a series of truncated hCD4 constructs containing a N-glycosylation site on the N-terminal in the presence and absence of CADA, providing snapshots of the translocation of the hCD4 SP through the translocon (Figure 23). Interestingly, SPs are thought to insert into the Sec61 translocon in a looped conformation with their N-terminus facing the cytosolic side of the ER membrane. However, some SPs may insert into the Sec61 translocon with the N-terminus initially facing the lumen of the ER membrane before the SP undergoes a flip-turn to reinitiate the N-terminus to face the cytosolic side. Since glycosylation takes place within the lumen of the ER, the N-glycosylation site introduced on the N-terminus of the hCD4 SP would be a diagnostic tool to determine the orientation of the hCD4 SP in the translocon.

![Diagram](image)

**Figure 24.** Truncated hCD4 SP constructs containing a 17 amino acid residue glycosylation tag were used to study the orientation of the SP through the Sec61 translocon. CADA was found to inhibit the reorientation of the SP within the translocon, preventing SP cleavage by signal peptidase on the luminal side of the ER membrane.

As shown in **Figure 24**, the translocation of constructs of shorter length (17+58mer) resulted in a high degree of glycosylation, which increased until the truncated hCD4 construct reached a length of 17+62mer before significantly decreasing. This
observation suggested that the hCD4 SP may initially be orientated with the N-terminus orientated towards the ER lumen before reorienting at later stages of translation. Upon treatment with CADA, N-glycosylation is reduced at longer chain lengths (17+62mer and higher) compared to the control, suggesting that the looped conformation of the hCD4 SP that occurs at higher chain lengths following reorientation within the translocon is required for potency. Furthermore, treatment with CADA markedly inhibits SP cleavage compared to the control, suggesting CADA is preventing the hCD4 SP from reaching the lumen of the ER membrane.

Taken together, the data presented by Vermeire et al. in 2014 showed that CADA inhibits the expression of hCD4 on the surface of immune cells through a highly specific interaction with the hCD4 SP within the Sec61 translocon of the ER membrane, preventing the successful cotranslational translocation of hCD4 and instead resulting in its ejection out of the channel into the cytosol where it is degraded (Figure 25). Importantly, CADA is the first example of a small molecule to inhibit protein expression through a specific interaction with the respective signal peptide, which ultimately begs an important question: are signal peptides a viable target for modulating protein expression? Since signal peptides are unique to each protein undergoing cotranslational translocation, they may be a
potentially invaluable target for small molecules to modulate protein expression with high specificity and broad therapeutic potential.

**Figure 25.** The proposed mechanism of action for the down modulation of hCD4 by CADA, which is thought to stabilize a folded conformation of the hCD4 SP within the Sec61 translocon, preventing SP inversion and subsequent cleavage by signal peptidase. (Bell, T.W.)

**Discovery of sortilin as a secondary substrate of CADA**

In 2017, a proteomic survey by Van Puyenbroeck et al. found that the expression of the type I glycoprotein sortilin was affected by CADA in a dose-dependent manner (Figure 26). Appropriately named, sortilin is a sorting receptor that aids in the transport of proteins through the secretory pathway. Sortilin is primarily expressed in the central nervous system and is involved in a number of biological processes, such as lipid metabolism and apoptosis. More recently, the role of sortilin in a variety of diseases such
as cancer and Alzheimer’s disease is becoming increasingly apparent. Thus, a small molecule that modulates sortilin expression may be of therapeutic value in the treatment of a number of different diseases.

Analogous to hCD4, sortilin is synthesized through cotranslational translocation before entering the secretory pathway, which suggested that CADA may be interacting with the sortilin SP in a similar manner to that of the hCD4 SP. The structures of the hCD4 SP and sortilin SP are shown in Figure 26, both of which are sensitive to CADA, resulting in a decrease in translocated protein when subjected to a cell free in vitro translation assay. Interestingly, CADA down-modulates sortilin expression to a lesser degree than hCD4 expression, suggesting the binding interactions between CADA and the sortilin SP is weaker than those of CADA and the hCD4 SP. A closer examination at the primary structure of each signal peptide show some notable similarities, such as the glutamine in...
the hydrophobic H-region, but other structural differences may explain the relative observed potencies.

**Structure-activity relationship studies between CADA analogs and hCD4 down modulation**

An expansive library of CADA analogs have been synthesized since CADA was first discovered to have activity against HIV. Even before CADA’s mechanism of action was fully understood, quantitative structure-activity relationship (QSAR) experiments correlating the chemical and physical features of CADA analogs and their corresponding effect on hCD4 expression enabled the design of increasingly more potent analogs. In general, CADA analogs are described in terms of the functional groups located on four major loci on the macrocycle: the head group, the side arms, and the tail (Figure 27).

Initial QSAR experiments focused on the importance of the tail group and side-arms. After years of research focused on synthesizing CADA analogs with various tail groups, QSAR experiments have revealed that a bulky, hydrophobic functional group correlates with favorable potency (Figure 28). Replacement of the benzyl group with an alkyl chain decreases potency, with branched alkanes typically being more potent than
straight chain alkanes (QJ038 vs. QJ033). CADA analogs containing small, polar groups tend to be inactive; along with the observed biological activity of the bulky sulfonamide tail group in AS112, diminishing the basicity of the tail group nitrogen may result in decreased biological activity. Several heteroaromatic tail groups tested (QJ027 AND QJ030) displayed moderate biological activity. However, the cyclohexylmethyl tail analog QJ028 was found to be more potent than CADA, suggesting the increased hydrophobicity of the cyclohexylmethyl group was responsible for enhanced potency. All else being equal,

\[
\begin{align*}
IC_{50} &= 0.5 \pm 0.046 \text{ µM} \\
IC_{50} &= 0.67 \pm 0.09 \text{ µM} \\
IC_{50} &= 4.1 \pm 0.8 \text{ µM} \\
IC_{50} &= 0.5 \pm 0.2 \text{ µM} \\
IC_{50} &= 0.6 \pm 0.3 \text{ µM} \\
IC_{50} &= 0.34 \pm 0.06 \text{ µM} \\
IC_{50} &= 5.5 \pm 2.1 \text{ µM} \\
IC_{50} &= 7.9 \pm 2.6 \text{ µM} \\
IC_{50} &= > 50 \text{ µM}
\end{align*}
\]

**Figure 28.** CADA analogs with various tail group functionalities. In general, a bulky, hydrophobic tail group results in the most potent biological activity in hCD4 down modulation.\textsuperscript{44}
CADA analogs exhibiting cyclohexylmethyl tail groups are more potent than their benzyl tail counterparts.

Dr. Liz Lumangtad recently synthesized a number of CADA analogs bearing fused pyridine tail groups that were demonstrated to considerably down-modulate hCD4 expression. Several of these analogs and their potencies are shown in Figure 29.47

![CADA analog structures](image)

\[ IC_{50} = 1.72 \pm 0.20 \, \mu M \quad IC_{50} = 0.94 \pm 0.14 \, \mu M \quad IC_{50} = 0.720 \pm 0.042 \, \mu M \quad IC_{50} = 0.677 \pm 0.057 \, \mu M \quad IC_{50} = 0.64 \pm 0.07 \, \mu M \quad IC_{50} = 0.416 \pm 0.20 \, \mu M \]

**Figure 29.** CADA analogs with functionalized pyridine tail groups and their biological activity against hCD4.47

QSAR experiments focused on the role of CADA side-arms and their corresponding hCD4 down modulation tendencies has been conducted more extensively than either the tail group or the head group.44-46 CADA analogs are described as either symmetrical or unsymmetrical, depending on whether the sulfonamide side arms are...
identical or not. In general, two aryl sulfonamide side-arms are necessary for favorable potency, as demonstrated by potencies of the bis-mesylate CADA analog MFS-105, as well as the analogs VGD040 and CK075 (Figure 30), which only possess one sulfonamide side-arm and are commonly referred to as the so-called “one-armed bandits.”

![Chemical structures of MFS-105, CK075, and VGD040](image)

**Figure 30.** QSAR experiments have shown that two aryl sulfonamide side-arms required for hCD4 potency.\(^{44-46}\)

A majority of CADA analogs currently being synthesized are unsymmetrical in respect to their side-arms. As shown in Figure 31, CADA analogs with identical side-arms (KKD023) are not as potent as analogs with two different side-arms. VGD020 is one of the most potent analogs synthesized to date and is reasoned to be more potent than VGD045 due to the increased hydrophobicity of the cyclohexylmethyl tail group.

![Chemical structures of KKD023, VGD045, and VGD020](image)

**Figure 31.** CADA analogs with identical side arms are less potent than CADA analogs with different side-arms.\(^{44-46}\)
Recent reports by Demillo et al. in 2011\textsuperscript{45} and Chawla et al. in 2016\textsuperscript{46} examined the hCD4 down modulation potencies of an expansive pharmacophore library of unsymmetrical CADA analogs with either a benzyl or cyclohexylmethyl tail, which led to the identification of key chemical features associated with potent biological activity. A few of these analogs and their hCD4 down modulation potencies are shown in Figure 32.

\begin{itemize}
  \item \textbf{CADA} \hspace{1cm} IC\textsubscript{50} = 0.56 \pm 0.0046 \mu M
  \item \textbf{CK023} \hspace{1cm} IC\textsubscript{50} = 5.25 \pm 1.10 \mu M
  \item \textbf{CK027} \hspace{1cm} IC\textsubscript{50} = 0.79 \pm 0.10 \mu M
  \item \textbf{CK137} \hspace{1cm} IC\textsubscript{50} = 1.10 \pm 0.15 \mu M
  \item \textbf{NCP001} \hspace{1cm} IC\textsubscript{50} = 1.14 \pm 0.02 \mu M
  \item \textbf{CK147} \hspace{1cm} IC\textsubscript{50} = 0.063 \pm 0.005 \mu M
  \item \textbf{VGD023} \hspace{1cm} IC\textsubscript{50} = 0.77 \pm 0.033 \mu M
  \item \textbf{VGD039} \hspace{1cm} IC\textsubscript{50} = 0.22 \pm 0.024 \mu M
  \item \textbf{CK148} \hspace{1cm} IC\textsubscript{50} = 1.10 \pm 0.15 \mu M
\end{itemize}

\textbf{Figure 32.} Various unsymmetrical CADA analogs and their observed hCD4 potencies.\textsuperscript{46}
In general, benzenesulfonamide side-arm containing an electron-donating group (EDG) in the para position are the more potent than having an electron-withdrawing group (EWG) in the para position, as demonstrated by the potencies of CK147 and VGD023. Furthermore, a hydrogen-bond donor in the para position of the benzenesulfonamide side-arm greatly decreases potency, as shown by the different biological activity of VGD020 and NCP001. Interestingly, EWGs in the ortho position seem to yield better potency than EWGs in the para position, as shown by the differential potencies of VGD023 and VGD039.

With the knowledge that CADA is interacting with the folded hCD4 SP, it is proposed that EDG without hydrogen-bond donating abilities improves the interaction between the CADA analog and the hCD4 SP by increasing the dipole moment of the benzenesulfonamide aromatic ring through a push-pull resonance phenomenon. As shown in Figure 32, the sulfonamide group is an inherent EWG in the absence of any functionality on the benzene ring. If an EDG is added in the para position, this greatly enhances the dipole moment, which may explain the potencies of CK147 and VGD020. This would also explain the difference in potency between VGD023 and VGD039; an EWG in the para position would create a dipole in the opposite direction, diminishing the dipole induced by the sulfonamide group. However, an EWG in the ortho position would create a net dipole moment in the direction shown in Figure 33.
Finally, a handful of QSAR experiments focused on the head group have shown that a relatively small, non-polar functionality is crucial for biological activity. Derived from VGD020, the CADA analog RA005 did not display favorable biological activity, while RA016 is actually more potent than CADA (Figure 34). In general, an isobutylene head group is typically installed on novel CADA analogs and usually results in favorable biological activity.

Figure 33. The dipole moment of the non-tosyl benzenesulfonamide side-arm is hypothesized to be important in potency. a) The sulfonamide is a natural EWG, creating a dipole in the aromatic ring. b) An EDG greatly enhances the dipole moment of the aromatic ring. c) An EWG in the para position creates a dipole opposite of the induced dipole of the sulfonamide. d) An EWG in the ortho position creates a dipole in conjunction with the sulfonamide group, which results in a more potent analog than if the EWG is in the para position.

Figure 34. VGD020 analogs with modified head groups and resulting hCD4 down-modulation potencies.
In summary, QSAR experiments have revealed that CADA analogs with one tosyl sulfonamide side-arm, one benzenesulfonamide side-arm containing an electron-donating group without hydrogen-bond donating abilities (ex. –OCH₃ or -N(CH₃)₂), an isobutylene head group, and a cyclohexylmethyl tail group correlate with potent hCD4 down-modulation. The dipole moment of the non-tosyl benzenesulfonamide side-arm is thought to be involved in binding to the hCD4 SP, of which electron-donating groups greatly enhance.

**General strategy for synthesizing CADA analogs**

The ability to access a large number of CADA analogs for understanding QSARs between CADA and the hCD4 signal peptide can largely be attributed to the development of a notably flexible synthetic strategy to access a variety of novel CADA analogs over the last 15 years. Since QSAR studies have shown that unsymmetrical CADA analogs containing cyclohexamethyl tails and isobutylene head groups tend to be the most potent, a brief discussion of the general synthetic pathway will be discussed herein.

The synthesis of unsymmetrical CADA analogs begins with the tosylation of 1,3-diaminopropane (Scheme 1).\textsuperscript{45} The mono-tosylated diamine is obtained after recrystallization from H₂O/CH₃OH in moderate to good yields (53-72%), with dichloromethane typically resulting in higher yields.

\begin{center}
\includegraphics[width=0.5\textwidth]{Scheme1.png}
\end{center}

**Scheme 1.** The monotosylation of 1,3-diaminopropane.
The mono-tosylated diamine is subjected to reductive amination to install the tail group. Reaction with cyclohexanecarboxaldehyde with magnesium sulfate in dichloromethane yields the Schiff base shown in Scheme 2, which is subsequently reduced with sodium borohydride in ethanol to install the cyclohexamethyl tail. Notably, substituting benzaldehyde for cyclohexanecarboxaldehyde followed by reduction of the Schiff base with sodium borohydride installs a benzyl tail. The yields of the addition of both tail groups are essentially quantitative over two steps.

As shown in Scheme 3, chain elongation with a protected primary amine is achieved in a two-step, one-pot sequence consisting of a Finkelstein reaction between N-3-bromopropylphthalimide and lithium iodide with subsequent reaction with refluxing intermediate 3 under basic conditions. Deprotection of intermediate 4 with hydrazine monohydrate in refluxing ethanol yields the primary amine, which is subjected to reaction with a desired sulfonyl chloride under basic conditions to yield an open-chain disulfonamide. The numerous amounts of sulfonyl chlorides available, commercially and

![Scheme 2](image)

**Scheme 2.** Installation of the cyclohexylmethyl tail group utilizing a reduction amination reaction with cyclohexanecarboxaldehyde and sodium borohydride.

![Scheme 3](image)

**Scheme 3.** Chain elongation utilizing N-3-bromopropylphthalimide to install a protected amine, which is subsequently deprotected with hydrazine monohydrate to reveal the primary amine.
otherwise, has and continues to enable the synthesis of a large number of CADA analogs containing side-arms with differing structural and electronic properties.

Finally, macrocyclization of the open-chain disulfonamide is achieved using Tsuji-Trost macrocyclization, as described in a recent report by Ali et al. The catalytic cycle for this transformation is given in Figure 35, which begins with the formation of an allylpalladium complex that undergoes an oxidative addition with 2-methylene-1,3-propanebis(tert-butylcarboxate), which displaces carbon dioxide and tert-butoxide. The allylpalladium complex then undergoes a nucleophilic addition reaction by the conjugate

*Figure 35*. Catalytic cycle for the macrocyclization of open-chain disulfonamide CADA analogs utilizing Tsuji-Trost macrocyclization conditions.
base of an aryl sulfonamide, which is presumably deprotonated by the \textit{tert}-butoxide generated in the previous step. The palladium dissociates and the cycle repeats to afford the macrocylized product.

In general, CADA and symmetrical CADA analogs can be obtained in five linear steps with \( \sim 30\% \) yield. However, some CADA analogs contain synthetic handles that enable further modification after macrocyclization, and most of the synthetic targets discussed in this research will be CADA analogs derived from post-macrocyclization modifications of unsymmetrical CADA analogs bearing an isobutylene head group and cyclohexylmethyl tail. This synthetic strategy certainly has advantages and disadvantages; a large number of CADA analogs can be synthesized in 5 linear steps with generally excellent yields, except for the macrocyclization step. However, post-macrocyclization modifications require a large amount of macrocyclic product, which can be costly, time consuming, and difficult to purify. The majority of CADA analogs discussed in this research (both novel and otherwise) will be the result of multiple late-stage functionalizations of CADA analogs post-macrocyclization.

\textbf{Summary & outlook}

CADA is the first and only small molecule that’s been shown to modulate protein expression through a specific interaction with the signal peptide, an unprecedented target in medicinal chemistry. A serendipitous realization of CADA’s anti-HIV activity was the result of its ability to down-modulate hCD4 expression, which ultimately led to the discovery of its unique mechanism of action. CADA is hypothesized to bind to the hCD4 SP in the Sec61 translocon during the translocation of nascent hCD4 across the ER.
membrane, stabilizing a folded conformation that prevents the inversion of the SP and preventing the nascent protein to cross the ER membrane. Unsuccessful cotranslational translocation of hCD4 results in a significant reduction in hCD4 expression in immune cells, which inhibits HIV infection by acting as an entry inhibitor. The expression of a secondary substrate, sortilin, was recently identified to be affected by CADA in a signal peptide-specific manner.

The therapeutic potential of CADA analogs is certainly appreciable and could eventually be realized in the treatment of HIV and other common diseases. However, further investigation of the feasibility of targeting signal peptides to modulate protein expression is certainly an important and exciting endeavor in the development of biologically active small molecules to treat disease and the advancement of our understanding of signal peptides and cotranslational translocation. In time, CADA may be remembered as the first example of a widely used method of modulating protein expression to treat a variety of diseases and ailments, improving the human condition while learning more about a fundamental process of protein expression.
CHAPTER 2

CADA analogs for photoaffinity labeling experiments with the signal peptides
Photoaffinity labeling: a technique for determining drug-substrate interactions

Photoaffinity labeling (PAL) is a biochemical technique for studying various binding site interactions via the formation of a covalent bond between a ligand and its substrate. First introduced in 1962, Frank Westheimer and coworkers used a diazoacetyl group in a PAL experiment to inactivate chymotrypsin. Since then, PAL has found extensive utility in determining ligand-receptor interactions, the molecular location of enzyme inhibitors, and the identification of unknown molecular targets of biologically active molecules. To the medicinal chemist, PAL offers valuable insight into identifying the chemical interactions taking place between a small molecule and its molecular target. After all, designing effective and efficient small molecule drug candidates requires a thorough understanding of the mechanism of action and the chemical interactions responsible for potency; both of which PAL can be used to help elucidate.

**Figure 36.** A general overview of PAL experiments. A typical PAL experiment involves the incorporation of a photophore onto the natural ligand, irradiation of the photoreactive group within the binding pocket, formation of a covalent bond between the ligand and the binding pocket, and subsequent analysis of the newly formed covalent bond.

As shown in **Figure 36,** PAL experiments generally involve the incorporation of a photoreactive group (a photophore) onto a ligand, which is irradiated in the presence of its target substrate to generate a reactive intermediate that quickly forms a covalent bond to the nearby substrate. The covalently modified substrate is then analyzed using an ever-increasing amount of chromatographic and spectroscopic methods.
The design of small molecules for PAL experiments requires careful consideration, as the size, reactivity, and stability of the incorporated photophore collectively factors into the utility and accuracy of a PAL agent. As the chemical structure of any given molecule is likely correlated with the observed biological activity, the incorporation of a photophore onto a small molecule shouldn’t induce significant structural changes from that of the natural structure; a PAL probe with biological activity that significantly differs from that of the natural molecule is likely interacting with the target in a different manner, providing an inaccurate representation of the chemical interactions between the natural ligand and its substrate. Accordingly, small photophores are generally favored over bulkier alternatives.

Perhaps most importantly, the reactive intermediate generated upon irradiation should be short-lived in order to avoid non-specific labeling. Long-lived reactive intermediates can potentially reorient their position within the binding pocket, again providing an inaccurate representation of the interactions between the natural ligand and the substrate. Furthermore, the photophore should be chemically stable to a wide variety of conditions and should ideally require a fairly long wavelength for photolysis as not to degrade biological macromolecules.

After photolysis, the covalently labeled substrate is analyzed through a variety of methods, such as mass spectroscopy. Alternatively, a terminal alkyne group may be incorporated onto the PAL probe. Subsequent click chemistry with a fluorophore containing an azide moiety can provide insight into the nature of interactions taking place in the binding pocket. This approach has been found appreciable value in PAL experiments, such as the identification of the cotransin binding site.
Photophores for photoaffinity labeling experiments

The most common photophores for PAL experiments are the benzophenones, aryl azides, and diazirines, which photochemically generate diradicals, nitrenes, and carbenes, respectively. An overview of the properties and advantages/disadvantages of common photophores for PAL experiments is given in Table 2, and each will be discussed in greater detail herein.

Table 2. A collective comparison of some of the most common photophores in PAL experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Benzophenone</th>
<th>Aryl azide</th>
<th>Diazirine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Benzophenone Structure" /></td>
<td><img src="image" alt="Aryl azide Structure" /></td>
<td><img src="image" alt="Diazirine Structure" /></td>
</tr>
<tr>
<td>Photolysis Product</td>
<td><img src="image" alt="Diradical" /></td>
<td><img src="image" alt="Nitrene" /></td>
<td><img src="image" alt="Carbene" /></td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>350-360</td>
<td>&lt; 280</td>
<td>350-380</td>
</tr>
<tr>
<td>Advantages</td>
<td>Long wavelength of irradiation, easy to synthesize</td>
<td>Small, easy to synthesize</td>
<td>Small, stable, long wavelength of irradiation, short-lived</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Steric bulk, long period of irradiation</td>
<td>Short wavelength of irradiation, nitrene is prone to rearrangement</td>
<td>Difficult to synthesize, can rearrange to diazo product</td>
</tr>
</tbody>
</table>
Benzophenones

Although typically not considered the most effective photophore, benzophenones have been shown to be useful in a number of PAL experiments. For example, Wu and coworkers utilized the benzophenone PAL probe shown in Figure 37 to study the mechanism of action for γ-secretase inhibition, a prominent target in the treatment of Alzheimer’s disease.54

Benzophenones are synthesized relatively easy compared to aryl nitrenes and aryl azides, which is certainly an attractive feature. A number of commercially available benzophenone precursors can be easily incorporated onto the PAL probe.50 However, incorporation of a benzophenone group may induce significant conformational changes to the molecule, which may decrease the PAL probe’s overall utility. As shown in Scheme 4, benzophenones undergo photolysis at a wavelength of 350-360 nm to yield a reactive carbonyl, which can undergo coupling reactions with other excited carbonyls, undergo hydrogen abstraction, or disproportionate.50 Unfortunately, the long period of irradiation

![Scheme 4. PAL experiment using benzophenone to generate a diradical, which can react with nearby bonds in the binding pocket. X = C, N, O, or S.](image)
required for the photolysis of benzophenones and the relatively weak reactivity of the excited carbonyl compared to nitrenes or carbenes may result in poor labeling efficiency.

**Aryl azides**

Owing to their small size and relatively quick and straightforward synthesis, aryl azides have found appreciable utility in PAL experiments. Upon irradiation, aryl azides expel molecular nitrogen (N$_2$) to generate an aryl nitrene, which is inherently more reactive than the excited carbonyl of an irradiated benzophenone. The nitrogen equivalent of a carbene, nitrenes are highly reactive electron deficient species with a single nitrogen atom containing six electrons in its valence shell. However, a high wavelength of irradiation (< 280 nm) is required to generate the aryl nitrene (Scheme 5), which may damage photosensitive biological macromolecules. Nonetheless, nitrenes are capable of C-H, N-H, O-H, and S-H bond insertion, which may provide superior labeling to that of the excited carbonyl of a benzophenone. The reactivity and rearrangements of photochemically derived aryl nitrenes via aryl azides have been extensively studied.$^{54-56}$ An aryl azide used in PAL experiments may forgo covalent modification of the binding site and instead undergo various rearrangements to produce several side products, reducing the labeling efficiency of the PAL probe.

In 1995, Platz and coworkers reported insightful research on the study of the irradiation of phenylnitrene, which is summarized in Scheme 6.$^{55}$ Upon irradiation, Platz observed the formation of azobenzene and generic azepine structures. Upon photolysis of
an aryl azide, the immediate intermediate formed is a singlet nitrene ($^1\text{N}$), containing two pairs of electrons and an empty, low-energy $p$-orbital. The singlet nitrene may undergo intersystem crossing (ISC) to the triplet nitrene ($^3\text{N}$), which contains an electron pair and a single electron in both of the remaining $p$-orbitals and may dimerize to form the observed azobenzene product. Alternatively, the singlet nitrene may rearrange to a highly strained benzazirine, which undergoes ring expansion to a ketamine that is susceptible to nucleophilic attack to yield an azepine. The triplet nitrene may also photochemically and thermally proceed along this pathway to produce an azepine product. Taken together, one

\[
\begin{align*}
\text{Aryl azide} & \xrightarrow{hv < 280 \text{ nm}} \text{Singlet nitrene} \xrightarrow{} \text{Benzazirine} \\
\text{Azobenzene} & \xrightarrow{\text{Dimerization}} \text{Triplet nitrene} \xrightarrow{hv, 77 \text{ K}} \text{Nucleophilic attack} \xrightarrow{} \text{Azepines}
\end{align*}
\]

\textbf{Scheme 6.} A summary of possible rearrangement products of aryl nitrenes generated from the photolysis of aryl azides.$^{55}$

...can see why aryl nitrenes may provide low labeling yields in PAL experiments.

Aryl azides can be synthesized utilizing a number of relatively straightforward and accommodative classical aromatic chemistry methods, a few of which are shown in

\textbf{Scheme 7.}$^{57}$ Aryl amines are easily converted to the diazonium salt, which can undergo a \textit{ipso}-substitution with sodium azide to afford the aryl azide. Nucleophilic aromatic substitution ($S_{NAr}$) and second order nucleophilic ($S_N2$) substitution reactions using aryl
and alkyl bromides, respectively, also yield aryl azides. Alternatively, aryl azides can be synthesized from boronic acids and hydrazines, or through a diazo transfer reaction.

Carbene mediated PAL: diazirines and diazo-compounds

Carbene-mediated PAL (cmPAL) via diazo and diazirine compounds have been shown to be the most successful avenues in PAL experiments.\textsuperscript{50,58,59} Both functionalities possess previously mentioned qualities that are favorable of an effective photophore for PAL experiments, owing to their small size, long wavelength of irradiation (350-380 nm), and the inherent reactivity of the carbene produced upon irradiation.
To understand the differences in reactivity (and therefore, the difference in utility for PAL experiments) of nitrenes and carbenes photochemically generated from aryl azides and diazirines/diazo compounds, respectively, one needs to consider the electronic differences between the two electron deficient species. As shown in Figure 38, the geometry a singlet carbene is thought to be planar with $sp^2$ hybridization. The lowest energy configuration is the $\sigma^2$ configuration, followed by the $\sigma\pi$, and finally the $\pi^2$ configuration.\textsuperscript{55}

Alternatively, singlet nitrenes are thought to be planar and $sp$ hybridized. The lone pair of electrons are situated in the $\sigma\pi$ orbital and the remaining $\sigma$ and $\pi$ or are thought to be approximately degenerate in energy. Unlike carbenes, the $\sigma\pi$ is the lowest singlet configuration for singlet nitrenes.\textsuperscript{55}

Furthermore, the difference in energy between the $\pi^2$ and the $\sigma^2$ of a nitrene is less than that of its carbene counterpart, which is a possible explanation for the higher tendency of aryl azides to rearrange to azepines compared to the analogous

![Figure 38: Possible triplet and singlet electronic configurations for carbenes and nitrenes. The $\sigma^2$ and the $\sigma\pi$ are the lowest energy configurations for carbenes and nitrenes, respectively. Although similar, minor differences result in different reactivity and labeling abilities between aryl azides and diazirines.\textsuperscript{55}](image)

![Figure 39: (a): Ring expansion generated by a carbene in the least favorable $\pi^2$ configuration. (b) Ring expansion of a nitrene in the $\pi^2$ configuration to form a highly strained ketimine.\textsuperscript{55}](image)
rearrangement in carbenes (Figure 39). These differences manifest themselves in a difference in reactivity and a difference in their likeliness to undergo insertion reactions with nearby C-C, C-H, O-H, and X-H (X=heteroatom) bonds. Concerted insertion reactions are widely thought to proceed through the coordination of an empty π orbital of a singlet carbene (or nitrene) with an electron pair of an σ or π bond.\textsuperscript{60} Carbenes are more likely to possess a favorable electronic configuration for insertion reactions to occur and may contribute to their superiority over nitrenes in photoaffinity labeling.

Diazo compounds have found appreciable use as a carbene source in many areas of organic synthesis and PAL experiments. Upon irradiation with UV light (~360 nm), a diazo compound expels molecular nitrogen (N\textsubscript{2}) and forms a carbene, which, in the case of the α-diazoketone shown in Scheme 8, can either insert into nearby X-H bonds (X = C, N, O, and S) or undergo a Wolff rearrangement to form a ketene that can react with various nucleophiles (Scheme 9).\textsuperscript{61}  

\begin{center}
\begin{tikzpicture}[thick, scale=0.6]

\node (a) at (0,0) {\textbf{Scheme 8. Reaction pathways of α-diazoketones. (a) The carbene may insert into nearby bonds (X = C, N, O, and S). (b) The carbene may also undergo a Wolff rearrangement to form a ketene, which readily reacts with nucleophiles.\textsuperscript{61,62}}}
\end{tikzpicture}
\end{center}
The Wolff rearrangement is undesirable for PAL experiments, as a change in structure likely changes the position of the PAL label in the binding pocket and can result in non-specific labeling. Chowdhry and coworkers incorporated an electron-withdrawing group α to the carbene in hopes of stabilizing the carbene so that it favors insertion reactions over the Wolff rearrangement. Indeed, the irradiation of diazotrifluoropropanoate in the presence of methanol overwhelmingly favored the bond insertion product over the Wolff rearrangement product, as shown in Scheme 9.

First synthesized in 1960, the diazirine (Figure 40) is a unique and beautiful functional group containing a three-membered ring with two nitrogen atoms and one carbon atom. Depending on the substitution of the R groups on a diazirine, they may be further classified into aliphatic and aryl diazirines, the latter of which are most successful in PAL experiments. Diazirines exhibit relatively high thermal and chemical stability; they are stable to a wide range of pH, to both oxidative and reductive conditions, temperatures of up to 70°C, and nucleophiles (such as thiols).
Diazirines decompose to expel molecular nitrogen and a carbene with exposure to UV light (~360 nm), which can subsequently undergo a series of reactions as shown in Scheme 10. A certain population of the highly reactive singlet carbene will rapidly undergo various insertion reactions with nearby C-H, N-H, O-H, and S-H bonds. As such, some singlet carbenes will simply react with water in the binding pocket. Intersystem crossing (ISC) may also occur to generate the triplet carbene, which can undergo an insertion reaction like the singlet carbene or reduce a nearby substrate through hydrogen abstraction. Notably, diazirines may isomerize (>30%) to the corresponding diazo compound.59

Scheme 10. Possible reactive species of a carbene photochemically generated from aryl trifluoromethyl phenyl diazirine.55

Alkyl and aromatic diazirines have found appreciable use in cmPAL experiments, although it was noticed early on that aromatic diazirines were generally more stable, photochemically generated a greater population of carbenes, and provided better labeling efficiency than their alkyl counterparts.63 Knowles and coworkers also noticed that
incorporating a trifluoromethyl moiety α to the aromatic diazirine introduced a new level of chemical stability and photochemical efficiency, as the electron-withdrawing nature of both the α-trifluoromethyl and the α-phenyl groups help to prevent rearrangements from occurring and to help stabilize the diazo isomer if formed. The electron-withdrawing nature of the resulting carbene greatly enhances its reactivity, thereby enhancing its labeling efficiency.63

Indeed, the 3-trifluoromethyl-3-phenyl diazirine moiety has been integrated onto many small molecules for cmPAL experiments and is arguably currently the move effective photophore for general PAL experiments.50 The major drawback of using diazirines over benzophenones and aryl azides lies in the difficulty of their synthesis, often requiring multiple linear steps to install the trifluoromethyl diazirine moiety in addition to synthesizing the small molecule itself. However, synthetic chemists have made strides in the development of reasonable synthetic routes to afford diazirines and commercial access is becoming increasingly available.

A summary of common diazirine syntheses is given in Scheme 11.58,59 The primary starting material in many diazirine syntheses is a (trifluoroacetyl)benzene derivative that is subjected to several transformations to afford the diazirine. (Trifluoroacetyl)benzene can be obtained via functional-group transformations involving aryl halide derivatives, utilizing Grignard chemistry or lithium-halogen exchange by treatment with n-BuLi and methyl trifluoroacetate. Aromatic aldehydes can also undergo conversion to the trifluoromethyl ketone via reaction with Dess-Martin periodinane and trifluoroacetic acid or reaction with trifluoromethyltrimethylsilane.
(Trifluoroacetyl)benzene is then converted to either an N-trimethylsilyl oxime with treatment of the ketone with lithium bis(trimethylsilyl)amide or to the oxime using hydroxylamine in acidic or basic conditions. The former is reduced to the imine, which is converted to the diaziridine using O-(2,4,6-trimethylbenzenesulfonyl)hydroxylamine and oxidized using a variety of methods to afford the diazirine. The latter is activated through a tosylation or mesylation reactions to yield the O-tosyl or O-mesyl oximes, respectively, which are converted to the diaziridine using liquid ammonia. Interestingly, Kumar and coworkers have recently shown that the diazirine could be synthesized directly from the O-tosyl oxime using liquid ammonia and sodium amide.

Despite the difficulty of their syntheses, aromatic diazirines have largely been the gold standard of PAL experiments due to their chemical and thermal stability, the labeling efficiency of the carbene produced upon photolysis at a relatively low wavelength of irradiation (which doesn’t damage biological systems), and their small size.
the realm of PAL experiments will almost certainly to continue to include diazirines, of which the ease of their synthesis and utility as a photophore for PAL experiments will surely increase in due time.

RA018: an aryl azide CADA analog for PAL experiments with the hCD4 SP

Around the same time that the mechanism of action for CADA was discovered, two CADA analogs containing aryl azides were synthesized by Dr. Rameez Ali and Dr. Reena Chawla (Figure 41).\textsuperscript{48,64} Notably, both analogs showed comparable potency to that of CADA in the down-modulation of CD4 and, consistent with previous SAR experiments, the unsymmetrical aryl azide with a cyclohexylmethyl tail analog RA018 was slightly more potent than the corresponding benzyl tail analog CK205. Thus, RA018 was envisioned to be a useful CADA analog for PAL experiments to study interactions of CADA analogs with the CD4 signal peptide.
The aryl azide RA018 was synthesized accordingly to previously described procedures for accessing unsymmetrical CADA analogs with cyclohexylmethyl tails.\textsuperscript{45} As shown in Scheme 12, the formation of the open-chain disulfonamide was achieved through the reaction of 5 with 4-nitrobenzenesulfonyl chloride to yield the open-chain disulfonamide VGD016, which undergoes palladium catalyzed macrocyclization to yield the macrocyclic disulfonamide VGD023. Reduction of the aryl nitro group on VGD023 utilizing a saturated solution of copper(II)acetate and sodium borohydride yields the aryl amine CK148 in quantitative yields, which was subjected to a classic Sandmeyer reaction using sodium nitrite and hydrochloric acid, followed by reaction with sodium azide to afford RA018.\textsuperscript{48}

\textbf{Scheme 12.} Synthesis of the unsymmetrical aryl azide RA018 via reduction of the unsymmetrical nitro CADA analog VGD023 followed by a Sandmeyer reaction with sodium azide to obtain the azide from the primary amine.
A variety of *ipso*-substitution methods exist for diazonium salts of aromatic compounds. Aromatic diazonium salts are obtained from the reaction of an aryl amine with hydrochloric acid and sodium nitrite, which forms nitrous acid (HONO) as shown in Scheme 13. Further treatment of HONO with acid results in the loss of water, forming a reactive intermediate that will undergo nucleophilic attack by an aryl amine. After series of protonation/deprotonation steps and the loss of water, the aryl diazonium salt is formed. The diazo group is an excellent leaving group and readily undergoes *ipso*-substitution with a variety of nucleophiles, such as CuCl, CuBr, CuI, CuCN (Sandmeyer reactions), H₂O, or sodium azide to afford the aryl chloride, aryl bromide, aryl iodide, aryl nitrile, phenol, and aryl azide, respectively.

**Exploring the photolysis of RA018**

The first photoaffinity probe to be used in studying the binding interactions between CADA and the hCD4 signal peptide was RA018. The overall experimental strategy aimed to introduce the compound to the signal peptide *in vitro*, irradiate the sample with UV light...
and isolate the signal peptide for spectroscopic analysis. Fortunately, the hCD4 SP consists of only 25 amino acid residues (although the first 7 residues of the mature protein have been shown to be important in the binding of CADA) and can be analyzed directly by mass spectrometry, avoiding the typical routine of trypsin digestion prior to analysis of the protein.

In order to confirm the utility of RA018 as a photoaffinity probe, the compounds were first tested for their biological activity in the down modulation of CD4 in MT-4 cells naturally expressing CD4 as well as transfected CHO.CD4-YFP cells. Three separate crops of RA018 were tested: RA018-1 was from a previously tested sample, RA018-2 was from the same crop but arrived several months after, and RA018-3 had been newly synthesized. The irradiated samples were also analyzed to determine whether or not a new species was being generated upon photolyzation. Table 3 shows the IC$_{50}$ values obtained for the aforementioned compounds in MT-4 cells.

On average, each sample of RA018 was shown to display comparable potency to CADA, suggesting that the compound will accurately reproduce the binding interactions occurring between CADA and the hCD4 SP. Furthermore, a significant loss in biological activity is observed when RA018 is irradiated with UV light (254 nm, 3 h), presumably due to chemical decomposition upon photolyzation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>0.78</td>
</tr>
<tr>
<td>RA018-1</td>
<td>0.53</td>
</tr>
<tr>
<td>RA018-2</td>
<td>1.23</td>
</tr>
<tr>
<td>RA018-3</td>
<td>1.05</td>
</tr>
<tr>
<td>RA018-2 (UV)</td>
<td>7.99</td>
</tr>
<tr>
<td>RA018-3 (UV)</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

Table 3. IC$_{50}$ values for the RA018 samples to be used in photoaffinity labeling, both prior and after exposure to UV irradiation (254nm) for 1 h.
Although the cellular assays strongly suggested that a chemical change was taking place upon irradiation, the details of the photolysis of RA018 were unknown. The wavelength of light as well as the length of time required for sufficient photolyzation was explored through a series of experiments prior to any experiments involving the hCD4 SP.

The first experiment attempted to examine changes in the UV-vis spectrum upon the samples exposure to the instruments internal UV radiation. A solution of RA018 for UV-Vis analysis was prepared in accordance to the Beer-Lambert equation:

\[
A = \varepsilon \cdot c \cdot l
\]

A: absorbance
\[\varepsilon: \text{molar absorptivity (L/mol} \cdot \text{cm)}\]
\[c: \text{concentration (mol/L)}\]
\[l: \text{pathlength (cm)}\]

Assuming \(\varepsilon = 10^4, l = 1 \text{ cm},\) and \(A = 0.3,\) 1.84 mg of RA018 was dissolved in 100 mL of HPLC grade methanol for UV-visible analysis. Several UV spectra were obtained in the range of 300-800 nm, scanning in 5 nm steps. The \(\lambda_{\text{max}}\) for each sample was approximately 300 nm, with a very broad peak appearing at 505 nm in one case. However, 300 nm was the lower limit of detection for the instrument, suggesting the true \(\lambda_{\text{max}}\) was likely at a lower wavelength and undetectable by the capabilities of the spectrometer. Indeed, later UV experiments with CADA analogs with a spectrometer capable of scanning below 300 nm would reveal that UV absorption of CADA analogs usually takes place at a wavelength less than 250 nm.
Instead of seeking a more sensitive instrument, a series of thin-layer chromatography (TLC) experiments were conducted to assess whether or not photolysis was occurring. In general, a minimal amount compound was dissolved in 1 mL of ethyl acetate and split into two glass vials, one of which was irradiated with a handheld UV lamp (254 nm) while the other was kept in the dark. The samples were spotted on alumina plates and placed in a TLC chamber containing 30% ethyl acetate in n-hexane.

The first set of TLC experiments compared irradiated and non-irradiated samples of RA018 over time to determine the length of time required for photolysis. Observing both samples by TLC every 20 minutes yielded no observable changes in Rf values after 1 hour of irradiation.

Two 100 μL aliquots of both the irradiated and non-irradiated samples were placed in separate wells of a 96 well polystyrene plate. The UV lamp (254 nm) was placed directly on top of the wells containing the samples. After 20 minutes of irradiation, both samples had turned into a viscous yellow residue. Although it is uncertain as to what the irradiated product(s) were, it was clear that RA018 was chemically modified after exposure to the UV lamp. This observation suggested that perhaps the glass vials used in the previous TLC experiment had been absorbing the UV radiation and preventing it from ever reaching the sample, or the sample was simply too far away from the lamp.

With this possibility in mind, the sample that had yet to be irradiated from the initial TLC experiment was split into two glass vials. One was kept in the dark while the other was exposed to the handheld UV lamp (254 nm) directly above the vial, without the lid, for 15 minutes; a mere fraction of what the previous samples in the capped glass vials had
been subjected to. The product was compared to the non-irradiated sample by TLC, which showed no RA018 present in the irradiated sample.

It now seemed likely that the photolysis of RA018 required direct exposure to UV irradiation. However, a series of control experiments were necessary to ensure that the UV exposure was the singular cause of the dissociation of RA018 as seen by TLC.

A similar experiment was conducted using CADA as a control (Figure 42). Four samples were prepared, each containing 500 μL of either RA018 (Lane A), CADA (Lane B), equal amounts of both compounds (Lane C), and ethyl acetate (Lane D). Each solution was split into two glass vials, one of which would be irradiated while the other was kept in the dark. TLC provided a great visual of the compounds prior to irradiation, as seen in Figure 41. The dissociation of RA018 is suggested by the TLC results in both samples in lane A and lane C after 30 minutes of direct UV exposure at 254 nm. Alternatively, CADA appears to be unchanged in both samples in lane B and lane C, indicating that RA018 is indeed photolyzed due to UV irradiation at 254 nm.
PAL between RA018 & hCD4 signal peptide in free solution

Photoaffinity labeling experiments between RA018 and the hCD4 SP were conducted in free solution to investigate amino acid residues that may have a high affinity for CADA compounds. A series of solutions were prepared from 10 mM CADA (DMSO), 10 mM RA018 (DMSO), and 0.5 mM hCD4 SP (PBS) stock solutions. Solutions of both CADA & hCD4 SP and RA018 & hCD4 SP were either kept in the dark or irradiated with UV radiation (254 nm) for 1 hour. In order to investigate whether wells would be sufficient for future testing, the experiments were run in both glass vials as well as polystyrene wells. The details are described in Table 4.

The samples were submitted for analysis by mass spectrometry. The interpretation of the results from this approach warrants caution, as the conformational relationship between the two molecules does not necessarily reflect what has been shown to exist in the translocon of the endoplasmic reticulum. It does, however, provide some insight into how to conduct the photolysis experiments and subsequently analyze them with mass spectrometry.

Cell free in vitro protein synthesis of hCD4 with RA018

The mRNA for CD4 needed to be synthesized before translation could be initiated. This process is shown in Figure 42 and begins with a polymerase chain reaction (PCR) of the CD4 gene with CD4-specific primers, after which the product was subjected to a second PCR with primers containing a tag that can later be identified to ensure successful PCR of CD4 had taken place. Finally, RNA polymerase is introduced to transcribe CD4 DNA into mRNA.
<table>
<thead>
<tr>
<th>I.D.</th>
<th>Contents</th>
<th>Concentration</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>CADA</td>
<td>10 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>02</td>
<td><strong>RA018</strong></td>
<td>10 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>03</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td>04</td>
<td>CADA (UV)</td>
<td>10 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>05</td>
<td><strong>RA018 (UV)</strong></td>
<td>10 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>06</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>CADA</td>
<td>1.0 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>07</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td><strong>RA018</strong></td>
<td>1.0 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>08</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>CADA (UV)</td>
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</tr>
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<td></td>
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</tr>
<tr>
<td>10</td>
<td>hCD4 SP</td>
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<td>PBS</td>
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<tr>
<td></td>
<td>CADA</td>
<td>1.0 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>11</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td><strong>RA018</strong></td>
<td>1.0 mM</td>
<td>DMSO</td>
</tr>
<tr>
<td>12</td>
<td>hCD4 SP</td>
<td>0.5 mM</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>CADA (UV)</td>
<td>1.0 mM</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

**Table 4.** Various solutions of CADA and **RA018** were prepared in a 2:1 concentration in DMSO and PBS. Solutions 1-3 were stock solutions in which all other solutions were comprised of.
A practicable method of studying translational processes *in vitro* is through the use of rabbit reticulocyte lysate and microsomes, which simulates cellular environment to that of *in vivo* studies without presenting many problems brought on by working with living cell cultures. *In vitro* translation studies of the hCD4 SP and RA018 would therefore be more representative of the actual conditions present when CADA and CADA analogs down modulate CD4 compared to the signal peptide and compounds in free solution.

The first *in vitro* experiment used a construct containing the hCD4 SP, the first seven residues of the mature protein, and pre-prolactin. Samples containing either DMSO, CADA, or RA018 were irradiated with UV light for 0.5 hours and compared to their non-irradiated counterparts. The analysis of the targeting of the RNC to the translocon, the translation of the nascent peptide chain, and the translocation of CD4 with the listed compounds are shown in Figure 43. Lanes 2-4 contain non-irradiated samples of DMSO, CADA, and RA018, respectively (red), in the presence of rough microsomes, while lanes 5-7 contain the same solutions with UV-irradiation for 0.5 hours (blue). All cases contain the intact tRNA-RNC as indicated by the black arrow, indicating targeting of the RNC to the translocon is successful.

The peptidyl-tRNA bond is broken with the addition of puromycin in lanes 8-13 (same conditions as lanes 2-7) and is apparent by the absence of the corresponding band seen in lanes 2-7. Treatment with only DMSO (lanes 8 and 11) shows the presence of the signal peptide, indicating that signal peptidase has cleaved the signal peptide and translation and translocation have successfully occurred. Alternatively, the addition of CADA and, to a lesser extent, RA018 result in a significant loss in the amount of signal
peptide present in the sample. This is true of both the irradiated and irradiated samples and indicates the compounds are disrupting the translation and translocation of hCD4.

This experiment showed that RA018 is binding analogously to CADA and disrupting the translation and translocation of CD4. Therefore, irradiating the samples containing RA018 could possibly result in a covalently modified nascent chain of CD4. However, no information from this gel could actually show that. The signal peptide would have to be labeled during *in vitro* translation studies and subsequently isolated and analyzed by mass spectrometry.

The experiment was repeated using a different construct that contained the hCD4 SP, the first seven residues of the mature CD4 protein, a V5 epitope, and a 70mer of the mature CD4 protein. The reasoning behind this experiment was to attempt to use the V5
tag to isolate the protein after exposure to UV-irradiation. The results from the second \textit{in vitro} translation experiments are comparable to that of the previous experiment and are shown in Figure 44. As observed with the pPL construct, lanes 9, 10, 12, and 13 show the absence of the signal peptide when treated with CADA or RA018 and puromycin, both with and without UV treatment.

Although it is still uncertain whether or not the signal peptide is being labeled by RA018, the V5 tag will now potentially provide an opportunity to isolate the signal peptide and determine whether labeling occurred. Through the use of immunoprecipitation and western blots, the proteins containing the V5 tag could potentially be isolated and cleaved with signal peptidase to yield a labeled or non-labeled hCD4 signal peptide.

**A summary of RA018 for PAL experiments with the hCD4 SP**

To date, the aryl azide RA018 has not provided insight on the nature of chemical interactions between the hCD4 SP and CADA. RA018 is synthesized with relative ease and appears to photolyze upon exposure to UV radiation. It was also found to down-
modulate hCD4 expression in cell free *in vitro* translation experiments at a comparable \( IC_{50} \) to that of CADA, suggesting it interacts with the hCD4 SP analogously to CADA. It seems that the primary barrier to RA018’s utility in PAL experiments with the hCD4 SP lies in the post-photolysis stage; RA018 may likely provide sufficient labeling but methods for determining the exact nature of the labeled SP have not been realized.

**Diazirine CADA analogs for PAL experiments**

Although the aryl azide RA018 has potential for PAL experiments with the hCD4 SP, a cmPAL CADA analog would likely be the most attractive route towards PAL experiments with the hCD4 SP. A CADA analog containing a diazirine (Figure 45) would, in theory, provide more accurate labeling of the hCD4 SP than the aryl azide RA018 due to the enhanced reactivity of the carbene compared to the nitrene generated upon photolysis. However, a synthetic route to a CADA analog with a diazirine had not been previously attempted, and the potency (and viability) of a diazirine CADA analog was unknown. Following literature precedence, the incorporation of a trifluoromethyl diazirine onto a CADA analog was envisioned to be ideal due to their reliable labeling track record.58,59

![Figure 45. Proposed diazirine CADA analogs for PAL with the hCD4 SP (R = -CH3, -CF3, -H)](image)

As shown in Scheme 14, the trifluoromethyl diazirine CADA analog was envisioned to be synthesized through a series of transformations of DJ001,67 the unsymmetrical disulfonamide containing a nitrile obtained from the reaction of...
intermediate 5 with 4-cyanobenzenesulfonyl chloride, the latter of which was synthesized from 4-aminobenzonitrile (Scheme 15).  

Following palladium catalyzed macrocyclization to the nitrile DJ002, formation of the methyl ester using a catalytic amount of acid in refluxing methanol would yield the methyl ester 6. Reaction of 6 with fluoroform (HCF₃, pKₐ = 25-28), and a suitable base (such as t-BuOK or (Me₂Si)₂NK) installs the trifluoromethyl ketone 7. Alternatively,
trifluoromethylation can be affected by treatment of a methyl ester with (trifluoromethyl)trimethylsilane and tetrabutylammonium fluoride (TBAF). A series of subsequent reactions based on established methods in the literature would theoretically afford the trifluoromethyl diazirine $8$.\textsuperscript{67}

This synthesis was pursued as far as the macrocyclic nitrile $\text{DJ002}$ before abandoning this target due previous QSAR experiments that suggested the electronic structure of the trifluoromethyl ketone side arm would negatively affect potency; a previously synthesized CADA analog containing a 4-fluorobenzene sulfonamide side arm showed modest potency (Figure 46).\textsuperscript{46} Furthermore, this was a particularly lengthy synthetic route in which the bottleneck occurred early on (macrocyclization), so obtaining appreciable amounts of 7 for four additional reactions may have proved difficult.

![Figure 46. Previously synthesized fluorinated CADA analog C201 (IC$_{50}$ = of 0.92 ± 0.11 µM).\textsuperscript{45}](image)

Keeping these considerations in mind, another CADA analog containing a diazirine was hypothesized to be accessible through $\text{DJ005}$, an unsymmetrical CADA analog with a 4-acetylbenzenesulfonamide side arm that could be modified through a series of reactions to afford the diazirine, as shown in Scheme 16.\textsuperscript{67} $\text{DJ005}$ was hypothesized to be accessible through the sulfonamide formation between intermediate 4 and commercially available 4-acetylbenzenesulfonyl chloride. Palladium-catalyzed macrocyclization of the open-chain disulfonamide $\text{DJ004}$ yields the macrocyclic analog $\text{DJ005}$ in moderate yield (28%) compared to other unsymmetrical CADA analogs. An oximation reaction of $\text{DJ005}$ with
hydroxylamine hydrochloride in pyridine afforded the oxime DJ006 in excellent yield, which was activated using \( p \)-toluenesulfonyl chloride to obtain the \( O \)-tosyl oxime DJ007.

![Scheme 16. Proposed synthetic route to access the aryl methyl diazirine DJ009 from DJ005 following literature methods. Oxidation of the diaziridine DJ008 to the diazirine DJ009 proved problematic.](image)

The \( O \)-tosyl oxime DJ007 was reacted with liquid ammonia and THF in a pressure tube to afford the diaziridine DJ008, which then required a mild oxidation to the corresponding diazirine. Numerous attempts utilizing a variety of different methods\(^{68-71}\) to oxidize DJ008 proved unsuccessful, as shown in Figure 47. As a result, this synthesis was abandoned after several years of trying to oxidize the diaziridine to the diazirine.
Since initial attempts to oxidize the diaziridine to the diazirine proved unsuccessful, an alternative target (Figure 48) containing a phenyl diazirine side arm was proposed based on a synthetic procedure that did not include a diaziridine intermediate. Platz and coworkers have reported a one-pot synthetic procedure for obtaining diazirines from aldehydes with non-enolizable protons,\textsuperscript{72} which addresses the instability of diaziridines by instead forming a diaziridine in which one of the hydrogens is substituted with a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temperature (°C)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I\textsubscript{2}, TEA, EtOH</td>
<td>rt</td>
<td>0%</td>
</tr>
<tr>
<td>I\textsubscript{2}, TEA, EtOH</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Ag\textsubscript{2}O, MeOH</td>
<td>rt</td>
<td>0%</td>
</tr>
<tr>
<td>Mn\textsubscript{2}O, MeOH</td>
<td>rt</td>
<td>0%</td>
</tr>
<tr>
<td>Swern</td>
<td>-78-rt</td>
<td>0%</td>
</tr>
</tbody>
</table>

Figure 47. An overview of the various conditions explored for the oxidation of the diaziridine DJ008 to DJ009, all of which were unsuccessful.\textsuperscript{68-71} (rt: room temperature)

Figure 48. The unsymmetrical CADA analog DJ022, containing a phenyl diazirine side-arm.
trimethylsilyl (TMS) group, as shown in Scheme 17.

![Scheme 17. Synthesis of phenyl diazirines from aldehydes with non-enolizable protons.](image)

The synthesis of an unsymmetrical CADA analog containing a phenyl diazirine side arm was hypothesized to be obtainable through the reduction of the previously described unsymmetrical nitrile analog DJ002 to the aldehyde DJ003 (Scheme 18).

![Scheme 18. Synthesis of the phenyl diazirine CADA analog DJ009 via DJ002.](image)

The reduction of the nitrile moiety of DJ002 to the aldehyde DJ003 was shown to occur in moderate yields using 5 equiv. of DIBAL-H. Notably, using 1.5 equiv. of DIBAL-H did not yield any reduced product. Unfortunately, the conversion of the aldehyde DJ003 to DJ022 proved unsuccessful using the proposed method; only a small amount of starting material was recovered.
CD4 down modulation of CADA analogs for PAL experiments

The hCD4 down-modulation potencies of several DJ analogs synthesized in route to the diazirines and are summarized in Table 5. Consistent with previous QSAR experiments, the open-chain disulfonamide DJ004 is not active and the 4-acetyl and 4-cyano analogs DJ005 and DJ002 are not exceptionally potent, presumably due to the electron-withdrawing nature of each functional group.

<table>
<thead>
<tr>
<th>ID</th>
<th>$IC_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ002</td>
<td>1.19 ± 0.084</td>
</tr>
<tr>
<td>DJ004</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>DJ005</td>
<td>1.79 ± 0.040</td>
</tr>
</tbody>
</table>

Table 5. CD4 down-modulation potencies of several DJ CADA analogs

Summary

CADA is hypothesized to interact with the hCD4 and sortilin signal peptides in the Sec61 translocon, preventing successful cotranslational translocation to occur and resulting in the down-modulation of both proteins. The hCD4 signal peptide is 25 amino acids long, of which the hydrophobic H-region has been found to be involved in binding to CADA. However, the exact amino acid residues involved in this binding interaction are unknown.

Photoaffinity labeling (PAL) is a useful method of examining ligand-binding site interactions. The synthesis and utility of several CADA analogs for PAL experiments with the hCD4 signal peptide were investigated. The aryl azide RA018 is synthetically available
through the reduction of the 4-nitro CADA analog VGD023 followed by a Sandmeyer reaction using sodium azide with CK148. RA018 appears to photolyze upon exposure to a handheld UV lamp commonly found in chemistry laboratories. RA018 down modulates hCD4 expression analogously to CADA in cell free in vitro translation experiments, but techniques to isolate any labeled signal peptide have not been realized.

The synthesis of several diazirine CADA analogs was also explored, albeit with no success. The diazirine DJ009 was envisioned to be obtained from several functional group transformations of the 4-acetyl CADA analog DJ005, but conversion of the diaziridine DJ008 to DJ009 proved unsuccessful utilizing several oxidation conditions reported in the literature. Another diazirine CADA analog DJ022 was pursued through the reduction of DJ002 to DJ003. Ultimately, DJ022 was also found to be unobtainable from DJ003. Synthetic access to diazirines is notoriously difficult, but alternate methods in the literature may eventually enable the synthesis of CADA analogs with diazirine moieties.

**Experimental**

All reactions were carried out under an atmosphere of dry nitrogen using reagents and solvents were purchased from Aldrich Chemical Company, Acros Organics, Fisher Scientific, or AK Scientific and were of ACS reagent grade or better. All of the equipment used for macrocyclization reactions, such as spatulas, magnetic stir bars, and round-bottomed flasks were dried overnight at 100-110 °C. Anhydrous acetonitrile for macrocyclization, PEGylation, and mPEGylation reactions was distilled from CaH2. Organic solutions were dried with anhydrous Na2SO4. “Overnight” periods are ca. 16 h. HCl salts were synthesized using methanolic HCl (2 N) in methanol/water, which was
prepared from 84 mL of conc. aq. HCl (12.1 N) and 420 mL of anhydrous methanol. Trituration of HCl salts were performed in triplicate by sonicking the sample in anhydrous ethyl ether (30 mL, unless otherwise noted) for 15 min with subsequent filtration. Samples were dried in vacuo at 0.1 mm at room temperature. Samples for elemental analysis were dried in a drying pistol for 48-72 h at 78 °C (0.1 mm) and submitted to NuMega Resonance Labs, Inc. Samples for biological testing were greater than 95% pure, as shown by combustion microanalysis. Column chromatography was performed with Sorbent Technologies neutral alumina (50-200 μm) or standard grade silica (32-63 μm). Melting points were measured on a Mel-Temp apparatus and are uncorrected. $^1$H-NMR (400 MHz or 500 MHz) and $^{13}$C NMR (100 MHz or 125 MHz) spectra were acquired on a Varian 400 or a Varian Unity+ 500 spectrometer. All chemical shifts (δ) are reported in ppm units relative to the following solvent resonances: $^1$H, CDCl$_3$/TMS = 0.00, DMSO-$d_6$ = 2.50, CD$_3$OD = 3.31; $^{13}$C, CDCl$_3$ = 77.16. Low-resolution mass spectra (MS) were obtained using a Waters Micromass ZQ electrospray ionization quadrupole mass spectrometer using positive ion detection (capillary voltage = 3.5 kV). Infrared spectra (IR) spectra were obtained using a Nicolet 6700 FTIR spectrometer.

$N$-(3-Aminopropyl)-$p$-toluenesulfonamide (1):$^{46}$ Into a 500-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet, a 500-mL addition funnel, and a magnetic stir bar, 65 mL of 1,3-diaminopropane (58 g, 0.78 mol) in 180 mL of toluene was added.
A filtered solution of 50.5 g (26 mol) of p-toluenesulfonyl chloride in 180 mL of toluene was added to the addition funnel and added dropwise over 6 h to the vigorously stirred solution under nitrogen. The milky white reaction mixture was allowed to stir overnight under nitrogen, after which it was filtered using vacuum filtration. The white residue was washed with 30 mL of cold toluene (0 °C) and dried in vacuo to yield 77.8 g of a white powder. A solution of the crude product in 1 L of 1:1 (v/v) H₂O:MeOH and was stirred vigorously for 1 h. The cloudy white solution was vacuum filtered and the filtrate was concentrated by boiling to a volume of approximately 350 mL and was then allowed to cool to room temperature. The solution was placed in the freezer for 0.5 h, after which a pale white precipitate was formed, which was vacuum filtered. The residue was dried in vacuo to yield 25.8 g (43%) of mono-tosylated product as a white powder. The filtrate was further reduced to a volume of 150 mL and placed in the freezer for 0.5 h, after which it was vacuum filtered a second time to yield a second crop of product. The combined crops yielded a total of 30.9 g (51%) of 1 as a fine white powder. ¹H NMR (400 MHz, CD₃OD) δ 7.71 (d, 8 Hz, 2 H, o-Ts), 7.36 (d, 8 Hz, 2 H, m-Ts), 2.86 (t, 7 Hz, 2 H, CH₂NH), 2.62 (t, 7 Hz, 2 H, CH₂NH₂), 2.40 (s, 3 H, CH₃), 1.56 (quint., 7 Hz, 2 H, CCH₂C).

N-(3-p-Toluenesulfonamidopropyl)cyclohexylmethylamine (2):⁴⁶ Into a 250-mL, one-necked, round-bottomed flask equipped with a magnetic stir bar and a nitrogen inlet, 9.04
g of (40.0 mmol) N-(3-aminopropyl)-p-toluenesulfonamide (I), 14.6 g (122 mmol) MgSO$_4$, 6.15 mL cyclohexanecarboxaldehyde, and 110 mL of CH$_2$Cl$_2$ were added. The reaction mixture was stirred vigorously for 24 h under nitrogen. The resulting milky white mixture was filtered using a medium sintered glass funnel and the residue was washed with 30 mL of CH$_2$Cl$_2$. The filtrate was concentrated via rotary evaporation to yield a yellow oil, which was further dried in vacuo. A solution of the oil in 45 mL of absolute ethanol was stirred for 1 h at room temperature, then 1.91 g (51.4 mmol) of NaBH$_4$ was added in portions over the course of 0.5 h. The resulting yellow mixture turned to a pale, foamy mixture, which was allowed to stir overnight at room temperature. The mixture was filtered through a medium sintered glass funnel and the residue was washed with 20 mL of absolute ethanol (x3). The combined filtrates were concentrated via rotary evaporation to yield a pale white solid. A solution of this solid in 90 mL H$_2$O was stirred for 0.5 h at room temperature. The mixture was extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic extracts were dried (NaSO$_4$), filtered, concentrated via rotary evaporation, and the residue was dried in vacuo to yield 13.0 g (100%) of 2 as a light yellow oil. $^1$H NMR (400 MHz, CDCl$_3$/TMS) $\delta$ 7.73 (d, 8 Hz, 2 H, o-Ts), 7.30 (d, 8 Hz, 2 H, m-Ts), 3.06 (t, 6 Hz, 2 H, CH$_2$NH), 2.36 (t, 6 Hz, 2 H, CH$_2$NH-Cy), 2.43 (s, 3 H, CH$_3$), 2.35 (d, 7 Hz, 2 H, CH$_2$Cy), 1.70 (m, 4 H, CCH$_2$C, Cy), 1.59 (quintet, 2 H, Cy), 1.39 (m, 1 H, Cy), 1.20 (m, 2 H, Cy), 0.91 (m, 4 H, Cy).
A mixture of 25.9 g (50.7 mmol) of N-(3-p-toluenesulfonamidopropyl)cyclohexylmethylamine (2), 2.6 g of LiI (19.4 mmol), 8.8 g of (84 mmol) Na₂CO₃, and 300 mL of MeCN were added to a 500 mL, two-necked, round-bottomed flask equipped with a condenser, a magnetic stir bar, a rubber septum, and a nitrogen inlet. The pale mixture was boiled under reflux under nitrogen. A solution of 52.0 g (192 mmol) of N-(3-bromopropyl)phthalimide was dissolved in 100 mL of MeCN, which was slowly added to the refluxing solution via a syringe. The resulting solution was boiled at reflux under nitrogen for 24 h, then cooled to room temperature. The solution was filtered through a medium sintered glass funnel and the residue was washed with 30 mL of MeCN (x3). The combined yellow filtrates were concentrated by rotary evaporation. The crude product was purified using alumina filtration chromatography, as follows. A slurry of 800 g of alumina in 500 mL of 5:2 (v/v) hexanes/ethyl acetate was added to a 2 L medium sintered glass funnel. A solution of the crude product in 40 mL of ethyl acetate was uniformly loaded onto the column before the slurry could dry, then 3 L of of 5:2 (v/v) hexanes/ethyl acetate used to elute the column, which was then eluted with 1.5 L of ethanol. The desired ethanol fractions were combined, concentrated via rotary evaporation, and the residue was dried in vacuo to yield 24.9 g (96%) of pure product (3) as a clear yellow oil.

[^1H NMR (400 MHz, CDCl₃/TMS) δ 7.86 (m, 2 H, Phth), 7.74 (m, 4 H, o-Ts, Phth), 7.26](#)
(d, 8 Hz, 2 H, m-Ts), 6.32 (bs, 1 H, NH), 3.62 (t, 8 Hz, 2 H, CH$_2$NPhth), 3.06 (t, 6 Hz, 2 H, CH$_2$NHTs), 2.39 (m, 4 H, CH$_2$CH$_2$N), 2.34 (s, 3 H, CH$_3$), 2.09 (d, 7 Hz, 2 H, CH$_2$Cy), 1.69 (m, 10 H, CCH$_2$C, Cy), 1.38 (m, 1 H, Cy), 1.17 (m, 2 H, Cy), 0.84 (m, 2 H, Cy).

$N$-(3-Aminopropyl)-$N$-(3-(p-toluenesulfonamido)propyl)-benzylamine (4):$^{46}$ Into a 250-mL, one-necked, round-bottomed flask equipped with a magnetic stir bar, a condenser, and a nitrogen inlet, 2.20 g (4.17 mmol) of $N$-(3-(N'-phthalimido)propyl)-$N$-(3-(p-toluenesulfonamido)-propyl)benzylamine, 35 mL of hydrazine monohydrate, and 35 mL of ethanol were added. The solution was boiled under reflux under nitrogen for 3 h and then allowed to cool to room temperature, resulting in the formation of a white precipitate. The solvent was removed via rotary evaporation to yield a pale white solid. A mixture of this solid and 40 mL of 2 N HCl was stirred at room temperature (open container) for 2 h, until no white fumes were apparently visible. The milky white solution was made basic (pH=10) using a solution of 2 N aq. NaOH solution, then extracted with CH$_2$Cl$_2$ (3 x 25 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated via rotary evaporation. The resulting yellow oil was dried in vacuo to yield 1.52 g (95%) of 4 as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$/TMS) $\delta$ 7.78 (d, 8 Hz, 2 H, o-Ts), 7.29 (d, 8 Hz, 2 H, m-Ts), 5.76 (bs, 3 H, NH), 3.03 (t, 6 Hz, 2 H, CH$_2$NHTs),
2.96 (t, 6 Hz, 2 H, CH$_2$NH$_2$), 2.49 (t, 6 Hz, 2 H, CH$_2$NCH$_2$Cy), 2.45 (t, 7 Hz, 2 H, CH$_2$N), 2.41 (s, 3 H, CH$_3$), 2.12 (d, 7 Hz, 2 H, CH$_2$Cy), 1.80 (m, 2 H, CCH$_2$C), 1.66 (m, 7 H, CCH$_2$C, Cy), 1.40 (m, 1 H, Cy), 1.17 (m, 3 H, Cy), 0.82 (m, 2 H, Cy).

\[ \text{p-Cyanobenzenesulfonyl chloride:} \]

In a 2-L, 3-necked round-bottomed flask equipped with a magnetic stir bar, a nitrogen inlet, a thermometer, and a 100-mL addition funnel, 570 mL (32 mol) of H$_2$O was stirred at 0 °C (ice bath) as 79 mL (1.1 mol) of thionyl chloride was added over 2 h at 0 °C under nitrogen, then the reaction mixture was allowed to warm to room temperature. Next, 0.29 g (2.9 mmol) CuCl was added in one portion and the solution was stirred at 0 °C for 4 h under nitrogen, during which time the solution turns from light green to light yellow.

In a 500-mL, 3-necked round-bottomed flask equipped with a magnetic stir bar, a nitrogen inlet, a thermometer, and a 250-mL addition funnel was added 30 g (0.25 mol) of 4-aminobenzonitrile was stirred at 0°C using an ice bath under nitrogen, as 260 mL (8.3 mmol) of concentrated aq. HCl was added dropwise over 5 h, producing a thick, milky white mixture. A solution of 19 g (275 mmol) of sodium nitrite in 72 mL H$_2$O was added dropwise to the milky white mixture over 1.5 h at 0 °C, then the reaction mixture was stirred for 1 h at 0 °C. The resulting diazonium salt slurry was added dropwise to the sulfur dioxide solution at 0 °C over 2 h. The foamy reaction mixture was allowed to warm to room temperature and stirred under nitrogen overnight.
The reaction mixture was filtered by vacuum filtration and dried in vacuo, yielding 46 g (90%) of a beige solid. The crude product was dissolved in a minimal amount (ca. 60 mL) of boiling CHCl₃ (ca. 61 °C). Insoluble material was removed using vacuum filtration and hot hexane was added to the orange filtrate until the solution became turbid, and then cooled to room temperature before placing in the freezer overnight (-13 °C). The bright-orange, needle-like crystals were collected via vacuum filtration and dried in vacuo to yield 32 g (64%) of pure product, mp 106.5-108.2 °C (lit. 68.107-110 °C). 

¹H NMR (400 MHz, CDCl₃/TMS) δ 8.20 (d, 8.8 Hz, 2 H, o-ArSO₂), 7.96 (d, 8.8 Hz, 2 H, m-ArSO₂).

N’-(p-Cyanobenzenesulfonyl)-N’-(p-toluenesulfonyl)[N,N-bis(3-aminopropyl)cyclohexylmethylamine (DJ001): A mixture of 3.0 g (7.9 mmol) of N-(3-aminopropyl)-N-(3-p-toluenesulfonylimidopropyl)cyclohexylmethylamine, 1.6 g (7.9 mmol) of p-cyanobenzenesulfonyl chloride, 51 mL of saturated aq. Na₂CO₃ solution, 51 mL of saturated aq. NaCl solution, and 51 mL of CH₂Cl₂ were added and stirred at room temperature for 24 h, then the layers were separated. The aqueous layer was extracted with CH₂Cl₂ (3 x 30 mL). The combined organic solutions were dried (Na₂SO₄), filtered, and concentrated to dryness by rotary evaporation to give a light orange oil. A solution of the crude product in 103 mL of a 2 N solution of HCl in aq. MeOH was stirred at room
temperature for 4 h, then the solvent was removed via rotary evaporation. The residue was
dried in vacuo, triturated with anhydrous diethyl ether (3 x 20 mL) and dried again in vacuo
to give 1.26 g (28 %) of DJ001•HCl. The HCl salt was converted back to the free base by
stirring with a mixture of 138 mL of 2 N aq. NaOH solution, 138 mL of saturated aq. NaCl
solution, and 138 mL of CH2Cl2 for 6 h, then the layers were separated. The aqueous layer
was extracted with CH2Cl2 (2 x 50 mL). The combined organic solutions were dried
(Na2SO4), filtered, and concentrated by rotary evaporation to give a light orange oil, which
was dried overnight in vacuo to yield 1.09 g (25 %) of pure DJ001 as a light orange oil. 1H
NMR (400 MHz, CDCl3) δ 7.97 (d, 8 Hz, 2 H, o-ArSO2), 7.78 (d, 8 Hz, 2 H, o-Ts), 7.70
(d, 8 Hz, 2 H, m-ArSO2), 7.29 (d, 8 Hz, 2 H, m-Ts), 3.04 (t, 6 Hz, 2 H, CH2NH2Ar),
2.96 (t, 6 Hz, 2 H, CH2NHTs), 2.41 (s, 3 H, CH3), 2.38 (d, 7 Hz, 4 H, CH2NCH2Cy), 2.04
(d, 7 Hz, 2 H, CH2Cy), 1.64 (m, 10 H, CCH2C, Cy), 1.36 (m, 1 H, Cy), 1.12 (m, 2 H, Cy),
0.82 (m, 2 H, Cy). 13C NMR (125 MHz, CDCl3) δ 144.6, 143.3, 136.8, 132.8, 129.7, 128.9,
127.7, 127.0, 117.4, 67.0, 62.1, 53.1, 42.7, 42.6, 35.6, 31.9, 26.5, 26.0, 21.5.
MS (ESI+) m/z 547 (MH+).

2-Methylene-1,3-propanebis(tert-butylcarbonate) [11]: Into a 1-L, one-necked,
round bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 122.65 g (562
mmol) of di-tert-butyl dicarbonate, 21.85 g (248 mmol) of 2-methylene-1,3-propanediol,
and 600 mL of anhydrous diethyl ether were added. The solution was thoroughly mixed before adding 9.04 g (74.0 mmol) of 4-dimethylaminopyridine (DMAP) was added. The reaction mixture was stirred at room temperature for 24 h, after which the organic layer was separated and washed with sat. \textit{aq.} CuSO$_4$ (3 x 45 mL), sat. \textit{aq.} NaHCO$_3$ (3 x 45 mL), and sat. \textit{aq.} NaCl (3 x 45 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation. The clear viscous residue was dried \textit{in vacuo} to yield 68.18 g (95%) of pure product as a clear oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.32 (s, 2 H, C=CH$_2$), 4.60 (s, 4 H, CH$_2$C=C), 1.49 (s, 6 H, CH$_3$).

\[ \text{9-Cyclohexylmethyl-1-(p-cyanophenylsulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ002):}^{49} \]

Into a 125-mL, one-necked, round-bottomed flask equipped with a condenser, a nitrogen inlet, and a magnetic stir bar, 0.92 g (1.93 mmol) of \textit{N}-(4-cyanobenzenesulfonyl)-\textit{N}'-(p-toluenesulfonyl)bis(3-aminopropyl)cyclohexylmethylamine (DJ001), 2.6 g (9.2 mmol) of 2-methylene-1,3-propanebis(tert-butylcarbonate), 0.10 g (0.23 mmol) of 1,4-bis(diphenylphosphino)butane (dppy), 0.11 g (0.11 mmol) of tris(dibenzyldieneacetone)dipalladium (0) (Pd$_2$(dba)$_3$), 0.031 g (0.33 mmol) of Na$_2$CO$_3$, and 20 mL of anhydrous acetonitrile was added. The dark mixture was boiled under reflux under nitrogen for 24 h, then allowed to cool to room
temperature. The solvent was removed via rotary evaporation and the dark residue was dissolved in 50 mL of CH$_2$Cl$_2$. The solution was washed with sat. aq. NaHCO$_3$ (3 x 50 mL) and sat. aq. NaCl (50 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 3.3 g of crude product as an amber oil. A solution of the crude product in a minimal amount of ethyl acetate was loaded onto an alumina column, which was eluted with 3:7 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated via rotary evaporation to yield 0.42 g (36%) of product as a light orange oil. $^1$H NMR (400 MHz, CDCl$_3$/TMS) $\delta$ 7.92 (d, 8.2 Hz, 2 H, o-ArSO$_2$), 7.81 (d, 8.7 Hz, 2 H, o-Ts), 7.63 (d, 8.2 Hz, 2 H, m-ArSO$_2$), 7.32 (d, 8.0 Hz, 2 H, m-Ts), 5.14 (s, 2 H, C=CH$_2$), 3.96 (s, 2 H, H2), 3.67 (s, 2 H, H4), 3.36 (m, 2 H, H12), 2.99 (t, 6.3 Hz, 2 H, H6), 2.43 (s, 3 H, CH$_3$), 2.33 (m, 4 H, H8/H10), 1.96 (d, 6.9 Hz, 2 H, CH$_2$Cy), 1.60 (m, 10 H, H7/H11, Cy) 1.13 (m, 3 H, Cy), 0.73 (m, 2 H, Cy). $^{13}$C NMR (125 MHz, CDCl$_3$/TMS) $\delta$ 144.0, 143.6, 137.6, 134.4, 133.0, 129.8, 127.7, 127.3, 117.2, 62.1, 53.0, 50.6, 49.6, 45.3, 43.3, 35.8, 31.9, 27.7, 26.7, 26.0, 25.5, 23.8, 21.5. MS (ESI+) m/z 601 (MH$^+$). Anal. calcd. for C$_{31}$H$_{42}$N$_4$O$_4$S$_2$·HCl·CH$_3$OH·0.5H$_2$O: C, 56.83; H, 7.15; N, 8.28. Found: C, 56.64; H, 6.82; N, 8.01.
9-Cyclohexylmethyl-1-(\(p\)-formylbenzenesulfonyl)-3-methylene-5-(\(p\)-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ003).\(^7\) Into a 50-mL, two-necked, round bottomed flask equipped with a magnetic stir bar, thermometer, and a nitrogen inlet was added a solution of 0.18 g (0.30 mmol) of 9-cyclohexylmethyl-1-(\(p\)-cyanophenylsulfonyl)-3-methylene-5-(\(p\)-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ002) in 1.00 mL of anhydrous CH\(_2\)Cl\(_2\), which was cooled to 0 °C (ice bath) under nitrogen. Next, 1.25 mL of a 1.2 M solution of DIBAL-H in toluene (1.50 mmol) was added slowly to the solution via a syringe. The ice bath was removed and the solution was stirred at room temperature for 3 h under nitrogen, a mixture of 6.0 mL of 6 N HCl and 3.1 g of crushed ice was added slowly (vigorous effervescence occurs). The mixture was stirred at room temperature for 1.5 h, then 10 mL of a 5% \(aq\) solution of NaHCO\(_3\) was added slowly. The mixture was extracted with CH\(_2\)Cl\(_2\) (3 x 10 mL) and the organic extracts were combined, dried (Na\(_2\)SO\(_4\)), and concentrated by rotary evaporation to yield 0.23 g of a yellow oil. A solution of the crude product in a minimal amount of CH\(_2\)Cl\(_2\) and loaded onto an alumina column chromatography, which was eluted with 4:6 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated by rotary evaporation to yield 0.082 (48%) of pure product as a light yellow oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)/TMS) \(\delta\) 10.10 (s, 1 H, CHO) 8.03 (d, 8.1 Hz, 2 H, \(o\)-ArSO\(_2\)), 7.97 (d, 8.5 Hz, 2 H, \(m\)-ArSO\(_2\)), 7.64 (d, \(J = 8.3\) Hz, 2 H, \(o\)-PhSO\(_2\)), 7.31 (d, 8.5
Hz, 2 H, m-PhSO₂), 5.15 (s, 2 H, C=CH₂), 3.94 (s, 2 H, H2), 3.69 (s, 2 H, H4), 3.33 (t, 7.2 Hz, 2 H, H6), 3.02 (t, 6.1 Hz, 2 H, H12), 2.42 (s, 3 H, TsCH₃), 2.30 (m, 4 H, H8,H10), 1.95 (d, 6.9 Hz, 2 H, CH₂Cy), 1.62 (m, 10 H, H7/H11, Cy), 1.14 (m, 3 H, Cy), 0.68 (m, 2 H, Cy). ¹³C NMR (101 MHz, CDCl₃) δ 190.7, 130.2, 129.8, 127.8, 127.5, 127.3, 127.2, 52.0, 36.0, 35.9, 31.8, 29.7, 26.1, 21.5. MS (ESI⁺) m/z 602 (MH⁺).

N'-(p-Acetylbenzenesulfonyl)-N''-(p-toluenesulfonyl)[N,N-bis(3-aminopropyl)cyclohexylmethylamine] (DJ004): A mixture of 4.0 g (10.5 mmol) of N-(3-aminopropyl)-N-(3-p-toluenesulfonamidopropyl)cyclohexylmethylamine, 2.33 g (10.7 mmol) of p-acetylbenzenesulfonyl chloride, 69 mL of saturated aq. Na₂SO₄ solution, 69 mL of saturated aq. NaCl solution, and 69 mL of CH₂Cl₂ was stirred at room temperature for 24 h under nitrogen, then the layers were separated the aqueous layer was extracted with CH₂Cl₂ (3 x 40 mL). The combined organic extracts were dried (Na₂CO₃), filtered, and concentrated by rotary evaporation to yield a light orange oil. A solution of the crude product in 140 mL of 2 N HCl in MeOH was stirred at room temperature for 4 h then concentrated to dryness by rotary evaporation. The residue was dried in vacuo, triturated with anhydrous diethyl ether (3 x 30 mL) and again dried in vacuo to yield 3.24 g (87%) of DJ004•HCl. The HCl salt was converted back to the free base by stirring with 185 mL of 2 N aq. NaOH solution, 185 mL of saturated aq. NaCl solution, and 185 mL of DCM
for 5 h. The layers were separated and the aqueous phase was extracted with DCM (2 x 50 mL). The combined organic solutions were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to a light-yellow oil, which was dried \textit{in vacuo} to give 2.64 g (78\%) of pure DJ004. \textsuperscript{1}H NMR (400 MHz, CDCl$_3$) $\delta$ 8.07 (d, 9 Hz, 2 H, o-ArSO$_2$), 7.95 (d, 9 Hz, 2 H, m-ArSO$_2$), 7.72 (d, 8 Hz, 2 H, o-Ts), 7.30 (d, 8 Hz, 2 H, m-Ts), 3.04 (t, 6 Hz, 2 H, CH$_2$NHSO$_2$Ar), 2.97 (t, 6 Hz, 2 H, CH$_2$NHTs), 2.65 (s, 3 H, Ac), 2.43 (s, 3 H, CH$_3$), 2.38 (t, 7 Hz, 4 H, CH$_2$NCH$_2$Cy), 2.05 (d, 7 Hz, 2 H, CH$_2$Cy), 1.65 (m, 10 H, CCH$_2$C, Cy), 1.35 (m, 1 H, Cy) 1.14 (m, 2 H, Cy), 0.78 (m, Hz, 2 H, Cy). \textsuperscript{3}C NMR (101 MHz, CDCl$_3$) $\delta$ 196.9, 143.7, 143.3, 139.8, 136.5, 129.7, 128.9, 127.4, 127.1, 61.0, 52.3, 41.1, 34.2, 31.6, 26.9, 26.1, 25.6, 21.5. MS (ESI$^+$) $m/z$ 564 (MH$^+$). Anal. Calcd. for C$_{28}$H$_{42}$N$_3$O$_5$S$_2$·HCl·0.2 H$_2$O: C, 55.70; H, 7.08; N, 6.96. Found: C, 55.64; H, 6.89; N, 6.89.

9-Cyclohexylmethyl-1-(p-acetylbenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ005):\textsuperscript{48} Into a 250-mL, one-necked round bottomed flask equipped with a magnetic stir bar, a condenser, and a nitrogen inlet, 1.31 g (2.33 mmol) of N-(p-acetylbenzenesulfonyl)-N’-(p-toluenesulfonyl)bis(3-aminopropyl)cyclohexylmethylamine (DJ004), 1.68 g (5.84 mmol) of 2-methylene-1,3-propanebis(tert-butylcarbonate), 0.056 g (0.13 mmol) of 1,4-bis(diphenylphosphinobutane (dppb), 0.064 g (0.070 mmol) of tris(dibenzylideneacetone)dipalladium (0) (Pd$_2$(dba)$_3$),
0.13 g of Na₂CO₃ (1.21 mmol), and 120 mL of anhydrous acetonitrile were added. The dark mixture was boiled under reflux under nitrogen for 24 h and then allowed to cool to room temperature. The solvent was removed by rotary evaporation to yield an amber oil. A solution of this oil in 25 mL of CH₂Cl₂ was washed with saturated aq. NaHCO₃ solution (2 x 25 mL) and saturated aq. NaCl solution (25 mL). The combined organic extracts were and dried (Na₂SO₄), filtered, concentrated by rotary evaporation, and dried in vacuo to yield 2.55 g of crude product as a dark orange oil. A solution of the crude product in a minimal amount of CH₂Cl₂ and carefully loaded onto the alumina column, which was eluted with 3:7 (v/v) ethyl acetate/hexane to yield 0.41 g (28%) of pure product as a fluffy, pale-yellow solid (Rf = 0.4). ¹H NMR (400 MHz, CDCl₃/TMS) δ 8.08 (d, 8.7 Hz, 2 H, o-ArSO₂), 7.89 (d, 8.7 Hz, 2 H, m-ArSO₂), 7.64 (d, J = 8.3 Hz, 2 H, o-Ts), 7.32 (d, 9.1 Hz, 2 H, m-Ts), 5.15 (s, 2 H, C=CH₂), 3.91 (s, 2 H, H₂), 3.71 (s, 2 H, H₄), 3.28 (t, 7.2 Hz, 2 H, H₁₂), 3.04 (t, 6.6 Hz, 2 H, H₁₆), 2.65 (s, 3 H, Ac), 2.43 (s, 3 H, CH₃), 2.25 (m, 4 H, H₈,H₁₀), 1.95 (d, 6.9 Hz, 2 H, CH₂Cy), 1.65 (m, 10 H, H₇/H₁₁, Cy), 1.13 (m, 3 H, Cy), 0.73 (m, 2 H, Cy). ¹³C NMR (125 MHz, CDCl₃) δ 196.6, 143.4, 140.5, 139.9, 136.2, 129.7, 129.0, 127.4, 127.1, 61.4, 60.3, 51.9, 51.6, 47.8, 36.0, 31.8, 29.7, 27.7, 26.8, 26.1, 21.5. MS (ESI⁺) 616. Anal. Calcd. for C₃₂H₄₅N₃O₅S₂·HCl·0.2 H₂O: C, 57.52 H, 7.22 N, 6.27. Found: C, 57.52, H, 7.51, N, 6.12.
9-Cyclohexylmethyl-1-(p-acetyloximebenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ006): 

Into a 5-mL, one-necked, round bottomed flask equipped with a magnetic stir bar, a condenser, and a nitrogen inlet, 0.098 g (0.18 mmol) of 9-cyclohexylmethyl-1-(p-acetyloximebenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ005), 0.035 g (0.51 mmol) of hydroxylamine hydrochloride, and 1 mL of pyridine were added. The reaction mixture was boiled under reflux under nitrogen for 2 h and then allowed to cool to room temperature. The solvent was removed in vacuo to yield a pale-yellow residue, which was triturated with 5 mL of a 0.2 M aq. solution of citric acid and then extracted with CH₂Cl₂ (3 x 5 mL). The combined organic extracts were dried (Na₂SO₄), filtered, and concentrated by rotary evaporation to yield 0.10 g (100%) of crude product as a fluffy, light yellow solid. A solution of the crude product in a minimal amount of CH₂Cl₂ was absorbed on an alumina column, which was eluted with 3:7 (v/v) ethyl acetate/hexanes. The desired fractions were concentrated by rotary evaporation and dried in vacuo to yield 0.95 g (94%) of pure product as a light yellow glassy solid (Rf = 0.2). ¹H NMR (400 MHz, CDCl₃/TMS) δ 8.12 (bs, 1 H, C=NOH), 7.78 (m, 4 H, o-ArSO₂, p-ArSO₂), 7.65 (d, 8.1 Hz, 2 H, o-Ts), 7.31 (d, 8.0 Hz, 2 H, p-Ts), 5.16 (s, 2 H, C=CH₂), 3.84 (s, 2 H, H4/2), 3.75 (s, 2 H, H2/H4), 3.19 (t, 6.9 Hz, 2 H, H6/12), 3.10 (t, 6.6 Hz, 2 H, H6/12), 2.42 (s, 3 H, CH₃), 2.30 (s, 3 H, C(N=OH)CH₃),
2.25 (m, 4 H, H8, H10), 1.95 (d, 6.8 Hz, 2 H, CH$_2$Cy), 1.60 (m, 10 H, H11, H7, Cy), 1.12 (m, 2H, Cy), 0.67 (m, 2 H, Cy). $^{13}$C NMR (125 MHz, CDCl$_3$/TMS) $\delta$ 154.6, 143.5, 140.6, 139.1, 137.8, 135.2, 129.7, 127.3, 126.6, 62.1, 51.4, 50.3, 50.1, 44.4, 44.0, 35.8, 31.9, 26.7, 26.0, 24.6, 24.2, 21.5, 14.2, 11.9. MS (ESI$^+$) $m/z$ 631 (MH$^+$).

9-Cyclohexylmethyl-1-(p-O-acetyltosyloximebenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ007): Into a 25-mL, one-necked round bottomed flask equipped with a magnetic stir bar, an addition funnel, and a nitrogen inlet, 0.86 g (1.49 mmol) of 9-cyclohexylmethyl-1-(4-acetyloximebenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ006), 3.2 mg (0.026 mmol) of 4-dimethylaminopyridine, 0.30 mL (2.21 mmol) of triethylamine, and 1.5 mL of CH$_2$Cl$_2$ were added. The mixture was stirred under nitrogen at room temperature as a solution of 0.28 g (1.48 mmol) of p-toluenesulfonyl chloride in 2.15 mL of CH$_2$Cl$_2$ was added dropwise over 10 min. The resulting yellow mixture was stirred at room temperature for 1 h and then 5 mL of a 0.2 M $a$q. citric acid solution was added. The mixture was extracted with CH$_2$Cl$_2$ (3 x 5 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 1.09 g of a light yellow solid.
solution of the crude product in a minimal amount of CH₂Cl₂ was absorbed on an alumina column, which was eluted with 1:9 (v/v) ethyl acetate/hexanes. The desired fractions were concentrated and dried *in vacuo* to yield 0.77 g (71%) of pure product as a fluffy, pale-yellow solid. ¹H NMR (500 MHz, CDCl₃/TMS) δ 7.95 (d, 8.3 Hz, 2 H, o-ArSO₂), 7.82 (d, 8.6 Hz, 2 H, m-ArSO₂), 7.74 (d, 8.7 Hz, 2 H, o-OTs), 7.67 (d, 8.3 Hz, 2 H, o-NTs), 7.39 (d, 8.6 Hz, 2 H, m-NTs/m-OTs), 7.34 (d, 8.4 Hz, 2 H, m-OTs/m-NTs), 5.18 (s, 2 H, C=CH₂), 3.91 (s, 2 H, H2/H4), 3.74 (s, 2 H, H4/H2), 3.27 (t, 7.1 Hz, 2 H, H6/H12), 3.07 (t, 6.5 Hz, 2 H, H12/H6), 2.47 (s, 3 H, OTsCH₃), 2.45 (s, 3 H, TsCH₃), 2.40 (s, 3 H, CH₃C=NOTs), 2.28 (m, 4 H, H8, H10), 1.98 (d, 6.9 Hz, 2 H, CH₂Cy), 1.62 (m, 10 H, H7, H11, Cy), 1.16 (m, 2 H, Cy), 0.70 (q, 11.5 Hz, 2 H, Cy). ¹³C NMR (125 MHz, CDCl₃/TMS) δ 161.8, 145.4, 143.6, 141.4, 137.7, 134.9, 132.4, 129.7, 129.0, 127.6, 127.3, 116.6, 62.1, 60.4, 52.2, 50.6, 49.9, 44.8, 43.6, 35.9, 31.9, 26.8, 26.0, 25.1, 24.0, 21.5, 14.2. MS (MS⁺) m/z 785 (MH⁺).

9-Cyclohexylmethyl-1-(p-(3-methyldiaziridin-3-yl)benzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ008):⁶⁷ Into a thoroughly dried pressure tube containing a magnetic stir bar, a solution of 0.11 g (0.14 mmol) of crude 9-cyclohexylmethyl-1-(p-O-acetyltoyoxyloxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ007) in 2 mL of tetrahydrofuran was added. A pressure tube was placed in a dewar containing a dry/ice ethanol bath and
connected to a cold finger containing a dry ice/ethanol bath and an ammonia inlet. Approximately 10 mL of liquid ammonia was allowed to collect in the pressure tube before the tube was tightly sealed, then removed from the dry ice/ethanol. The reaction vessel was allowed to warm to room temperature and was stirred for 24 h, after which it was placed back into a dry ice/ethanol bath for 1 h. The cap was carefully removed and the tube was warmed to room temperature. The solution was added to a separatory funnel and extracted with CH$_2$Cl$_2$ (3 x 20 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ was absorbed on an alumina column, which was eluted with 3:7 (v/v) ethyl acetate/hexanes. The desired fractions were concentrated by rotary evaporation and dried in vacuo to yield 0.05 g (56%) of product as a light yellow solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.96 (d, 8.6 Hz, 2 H, o-ArSO$_2$), 7.84 (d, 8.6 Hz, 2 H, m-ArSO$_2$), 7.65 (d, 8.3 Hz, 2 H, o-Ts), 7.40 (d, 8.0 Hz, 2 H, m-Ts), 5.01 (d, 16.3 Hz, 2 H, C=CH$_2$), 4.10 (s, 2 H, HN-NH), 3.73 (m, 4 H, H2/H4), 3.11 (m, 4 H, H12/H6), 2.36 (s, 3 H, CH$_3$), 2.17 (m, 4 H, H8/H10), 1.92 (d, 2 H, CH$_2$Cy), 1.54 (m, 10 H, H7, H11, Cy), 1.14 (m, 2 H, Cy). MS (MS$^+$) m/z 632 (MH$^+$).

![Chemical Structure](image)

$N$-(p-Nitrobenzenesulfonyl)-$N'$-(p-toluenesulfonyl)bis(3aminopropyl) cyclohexylmethylamine (VGD016)

Into a 250-mL, one-necked, round-bottomed flask
equipped with a nitrogen inlet and a magnetic stir bar, 2.30 g (6.05 mmol) of \( N-(3\text{-aminopropyl})-N-(3\text{-p-toluenesulfonamidopropyl})\text{cyclohexylmethylamine (4)} \), 1.39 g (6.27 mmol) of \( p\text{-nitrobenzenesulfonyl chloride} \), 17 mL of saturated \( aq. \) \( Na_2CO_3 \) solution, 17 mL of saturated \( aq. \) \( NaCl \), and 17 mL of \( CH_2Cl_2 \) were added. The mixture was stirred at room temperature under nitrogen for 24 h and transferred to a separatory funnel. The aqueous layer was extracted with \( CH_2Cl_2 \) (3 x 20 mL). The combined organic extracts were dried (\( Na_2SO_4 \)), filtered, and concentrated by rotary evaporation to yield 3.26 g of crude product as an amber oil. The crude was purified by conversion to the HCl salt followed by trituration with diethyl ether. The crude oil was dissolved in 40 mL of 2 N \( HCl \) in MeOH and stirred at room temperature for 4 hours. The solvent was removed via rotary evaporation and dried \textit{in vacuo} before being triturated with anhydrous diethyl ether (3 x 30 mL). The residue was dried \textit{in vacuo} to yield 3.41 g (98\%) of VGD016·HCl. The HCl salt was converted back to the free base by stirring VGD016·HCl with 40 mL of 2 N \( aq. \) \( NaOH \), 40 mL of \( aq. \) sat. \( NaCl \), and 40 mL of \( CH_2Cl_2 \) for 5 hours. The mixture was transferred to a separatory funnel and the aqueous phase was extracted with \( CH_2Cl_2 \) (2 x 30 mL). The combined organic extracts were dried (\( Na_2SO_4 \)), filtered, and concentrated by rotary evaporation to yield a dark amber oil, which was dried \textit{in vacuo} to yield 2.50 g (78\%) of pure product as an amber oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)/TMS) \( \delta \) 8.34 (d, 9 Hz, 2 H, \( m\text{-ArSO}_2 \)), 8.05 (d, 9 Hz, 2 H, \( o\text{-ArSO}_2 \)), 7.72 (d, 8 Hz, 2 H, \( o\text{-Ts} \)), 7.31 (d, 8 Hz, 2 H, \( m\text{-Ts} \)), 6.30 (bs, 2 H, NH), 3.07 (t, 6 Hz, 2 H, H6/H12), 2.98 (t, 6 Hz, 2 H, H12/H6), 2.43 (s, 3 H, CH\(_3\)), 2.40 (m, 4 H, H8, H10), 2.06 (d, 7 Hz, 2 H, CH\(_2\)Cy), 1.65 (m, 10 H, H7, H11, Cy), 1.35 (m, 1 H, Cy), 1.13 (m, 2 H, Cy), 0.77 (quintet, 11 Hz, 2 H, Cy).
9-Cyclohexylmethyl-1-(4-nitrobenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (VGD023):13 Into a 100-mL, one-necked, round bottomed flask equipped with a condenser, a nitrogen inlet, and a magnetic stir bar, 1.50 g (2.28 mmol) of \( \text{N-(4-nitrobenzenesulfonyl)-N'}-(p\text{-toluenesulfonyl)bis(3aminopropyl)cyclohexylmethylamine} \) (VGD016), 3.65 g (12.7 mmol) of 2-methylene-1,3-propanebis(\textit{tert}-butylcarbonate), 0.13 g (0.16 mmol) of 1,4-bis(diphenylphosphino)butane (dppb), 0.15 g (0.15 mmol) of tris(dibenzyldieneacetone)dipalladium (0) (Pd\textsubscript{2}(dba)\textsubscript{3}), 0.36 g (3.42 mmol) of Na\textsubscript{2}CO\textsubscript{3}, and 100 mL of anhydrous acetonitrile were added. The resulting dark mixture was boiled under reflux under nitrogen for 24 h, then allowed to cool to room temperature. The solvent was removed by rotary evaporation and a solution of the resulting dark residue 50 mL of CH\textsubscript{2}Cl\textsubscript{2} was washed with a saturated \textit{aq.} NaHCO\textsubscript{3} solution (3 x 50 mL) and a saturated \textit{aq.} NaCl solution (50 mL). The combined organic extracts were dried (Na\textsubscript{2}SO\textsubscript{4}), filtered, and concentrated by rotary evaporation. A solution of the crude product in a minimal amount of ethyl acetate and loaded onto an alumina column, which was eluted with a gradient of 1:4 (v/v) ethyl acetate/hexanes, 2:3 (v/v) ethyl acetate/hexanes, and finally 3:2 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated by rotary evaporation to yield 0.46 g (33%) of product as a light yellow oil. \(^1\text{H NMR} \text{ (500 MHz,}
CDCl₃/TMS) δ 8.38 (d, 9 Hz, 2 H, m-ArSO₂), 8.02 (d, 9 Hz, 2 H, o-ArSO₂), 7.65 (d, 8 Hz, 2 H, o-Ts), 7.34 (d, 8 Hz, 2 H, m-Ts), 5.17 (s, 1 H, C=CH₂), 5.15 (s, 1 H, C=CH₂), 4.01 (s, 2 H, H2/H4), 3.69 (s, 2 H, H4/H2), 3.38 (t, 8 Hz, 2 H, H6/H12), 3.01 (t, 6 Hz, 2 H, H12/H6), 2.45 (s, 3 H, CH₃), 2.33 (t, 6 Hz, 2 H, H8/H10), 2.25 (t, 6 Hz, 2 H, H10/H8), 1.98 (d, 7 Hz, 2 H, CH₂Cy), 1.73 (m, 2 H, H7/H11), 1.63 (m, 6 H, Cy), 1.53 (m, 2 H, H11/H7), 1.15 (m, 3 H, Cy), 0.70 (m, 2 H, Cy).

9-Cyclohexylmethyl-1-(p-aminophenylsulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (CK148): Into a 500-mL, one-necked, round bottomed flask containing a nitrogen inlet and a magnetic stir bar, 1.0 g (1.64 mmol) of 9-cyclohexylmethyl-1-(p-nitrobenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (VGD023), 35 mL of sat. Cu(OAc)₂ and 140 mL of absolute ethanol were added. The reaction mixture was stirred at room temperature under nitrogen for 1 h and then the solution was cooled to 0°C (ice bath) before 2.31 g (61.02 mmol) of NaBH₄ was added in small portions over 0.5 h. The resulting dark mixture was allowed to warm to room temperature and was stirred overnight under nitrogen. The mixture was filtered through a medium sintered glass funnel and the black residue was washed with 20 mL of
absolute ethanol. The pale white filtrate was concentrated by rotary evaporation and dried in vacuo. A solution of the resulting residue in 60 mL of CH$_2$Cl$_2$ was washed with a saturated $aq$. NaHCO$_3$ solution (3 x 60 mL). The combined organic extract was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 1.36 g of crude product as a pale oil. A solution of the crude product in a minimal amount of ethyl acetate was absorbed on an alumina column, then eluted with 3:7 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated by rotary evaporation to yield 0.61 g (64%) of pure product as a light yellow oil. $^1$H NMR (400 MHz, CDCl$_3$/TMS) $\delta$ 7.67 (d, 8.3 Hz, 2 H, o-Ts), 7.54 (d, 7.8 Hz, 2 H, o-ArSO$_2$), 7.32 (d, 8.2 Hz, 2 H, m-Ts), 6.69 (d, 9.2 Hz, 2 H, m-ArSO$_2$), 5.18 (s, 2 H, C=CH$_2$), 4.13 (s, 2 H, ArNH$_2$), 3.82 (s, 2 H, H2/H4), 3.73 (s, 2 H, H2/H4), 3.18 (t, 7.4 Hz, 2 H, H6/H12), 3.06 (t, 7.4 Hz, 2 H, H6/H12), 2.44 (s, 3 H, CH$_3$), 2.26 (m, 4 H, H8, H10), 1.96 (d, 7.3 Hz, 2 H, CH$_2$Cy), 1.62 (m, 10 H, CCH$_2$C, Cy), 1.12 (m, 3 H, Cy), 0.70 (m, 2 H, Cy). $^{13}$C NMR (500 MHz, CDCl$_3$/TMS) $\delta$ 150.4, 143.3, 138.1, 136.0, 129.7, 129.4, 127.2, 116.4, 114.1, 62.1, 50.5, 50.2, 44.4 43.8, 35.9, 31.9, 26.8, 26.0, 24.7, 24.0, 21.5.

![Chemical Structure](attachment:image.png)

9-Cyclohexylmethyl-1-(p-azidophenylsulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (RA018):\textsuperscript{48} Into a 50 mL, one-necked, round-bottomed flask
equipped with a nitrogen inlet and a magnetic stir bar, 0.29 g (1.05 mmol) of 9-cyclohexylmethyl-1-(p-aminophenylsulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (CK148) in 5 mL of ethyl acetate was added. The solution was cooled to 0°C (ice bath) and 0.1 mL of concentrated hydrochloric acid was added, followed by the addition of a solution of 0.44 g (6.37 mmol) of NaNO₂ in 1 mL of H₂O. The reaction mixture was stirred for 1 h at 0°C under nitrogen. Next, a solution of 0.035 g (0.54 mmol) of NaN₃ in 6 mL of H₂O was slowly added. The dark orange mixture was removed from the ice bath and stirred overnight under nitrogen. A 1 N aq. NaOH solution was added dropwise to the reaction mixture until the pH of the mixture was 11, then the mixture was extracted with of ethyl acetate (3 x 30 mL). The organic extracts were combined, dried (Na₂SO₄), filtered, and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 0.24 g of crude product. A solution of the crude product in a minimal amount of CH₂Cl₂ was absorbed on an alumina column, which was eluted using 1:1 (v/v) ethyl acetate/hexanes. The desired fractions were concentrated by rotary evaporation and dried in vacuo to yield 0.15 g (48%) of pure product as a clear yellow oil. ¹H NMR (400 MHz, CDCl₃/TMS) δ 7.77 (d, 8.5 Hz, 2 H, o-ArSO₂), 7.67 (d, 8.2 Hz, 2 H, o-Ts), 7.32 (d, 9.2 Hz, 2 H, m-Ts), 7.13 (d, 8.9 Hz, 2 H, m-ArSO₂), 5.16 (s, 2 H, C=CH₂), 3.84 (s, 2 H, H4), 3.74 (s, 2 H, H2), 3.22 (t, 7.1 Hz, 2 H, H6), 3.08 (t, 6.7 Hz, 2 H, H12), 2.44 (s, 3 H, CH₃), 2.25 (m, 4 H, H8, H10), 1.96 (d, 6.9 Hz, 2 H, CH₂Cy), 1.62-1.54 (m, 5 H, H7, H11, CHCy), 1.24 (m, 4 H, Cy), 0.85 (m, 2 H, Cy). ¹³C NMR (500 MHz, CDCl₃/TMS) δ 144.7, 143.5, 137.8, 135.2, 129.8, 129.1, 127.3, 119.5, 62.2, 51.7, 50.5, 50.1, 44.6, 43.8, 35.9, 32.0, 29.7, 29.7, 29.4, 26.8, 26.0, 24.9, 22.7, 14.2.
CHAPTER 3

PEGylated and mPEGylated CADA analogs
Challenges in drug design and development

The medicinal chemist seeks to design and synthesize drugs with the purpose of improving human health. Utilizing a blend of synthetic chemistry and biology, scientists in and around medicinal chemistry have produced small molecules that have irreversibly changed people’s lives for (mostly) the better, providing cures and treatments for life-threatening diseases and ailments. However, the road to a successful drug is often far from trivial, and almost always more troublesome than most people realize.

Indeed, the modern drug discovery process time consuming, expensive, and risky. The drug development process differs between drugs and companies, but can generally be split into five main categories:  

(1) Drug discovery & development  
(2) Preclinical research  
(3) Clinical research  
(4) FDA drug review  
(5) FDA post-marketing safety monitoring

Each of these phases is elaborated on in Figure 49. The drug discovery and development phase involves the identification of a molecular target to achieve a desired biological response. Generally, a high-throughput method of screening an enormous number of small molecules will be employed to identify a small subset of potent small molecules, known as “hits.” Research will focus on the identification of the mechanism of action (MoA) and structure-activity relationship (SAR) experiments, which will produce a
handful of synthetic small molecules with optimized biological activity, known as lead compounds.\textsuperscript{75}

Lead compounds are then moved into the development phase, in which a variety of characteristics not necessarily related to the observed biological activity will be explored.

\textbf{Figure 49}. An overview of the drug development process.\textsuperscript{74}
Collectively, these characteristics are generally belong to one of two classes: (i) pharmacodynamics, the study of the effects of the drug on the body, and (ii) pharmacokinetics, which involves the effects the body has on the drug. The former involves the biochemical and physiological effects of the drug and its mechanism of action, while the latter explores drug metabolism, absorption, metabolism, toxicity, solubility, and many other characteristics. Both are crucial in the development of a successful drug candidate.

If a lead compound displays potent biological activity and favorable pharmacodynamics and pharmacokinetic properties, it may advance to clinical trials. Phase I clinical trials aim to analyze the safety of the drug, in which a small number of healthy individuals (20-100) are paid to take the drug so researchers can study the drugs absorption, metabolism, excretion, and potential side effects.

Phase II clinical trials are designed to study the efficacy of the drug, in which several hundred individuals participate in a double blind study (half of the participants receive the drug and half of the participants receive a placebo, but neither the participants nor the researchers know which they are distributing or receiving, respectively). Over the course of 1-2 years, researchers analyze the effects of the administered drug compared to the effects of the control group receiving the placebo to assess the effectiveness and safety of the drug. Approximately one-third of lead compounds make it through both phase I and phase II clinical trials.

Phase III clinical trials aim to analyze the effectiveness and safety of the drug on a much larger scale than phase II clinical trials, often utilizing thousands of individuals over
the course of several years to strengthen the understanding of the drug’s efficacy and potential side effects.\textsuperscript{74,75} Once phase III clinical trials are complete, an application for FDA approval can be submitted.

Finally, an FDA approved drug on the market remains in phase IV clinical trials, also known as post-market surveillance trials, in which researchers assess the efficacy, safety, and cost-effectiveness of a drug compared to similar drugs on the market.

When all is said and done, the journey of a drug from the laboratory to the market is long, arduous, and expensive. A recent study reported by Wong \textit{et al.} analyzed approximately 186,000 clinical trials of 21,000 clinical compounds from 2000-2015 found that a mere 13.8\% of lead compounds make it from phase I clinical trials to FDA approval.\textsuperscript{76} Furthermore, approval rates differ significantly depending on the type of drug entering clinical trials. Cancer drugs have the lowest approval rate (3.4\%), while vaccines for infectious diseases have the highest approval rate (33.4\%).

\textbf{PEGylation and mPEGylation as means of improving drug-like properties}

Common setbacks encountered by medicinal chemists in the drug development process are the result of poor pharmacokinetic properties of lead compounds. Collectively referred to as drug-like properties, various properties such as a drugs’ aqueous solubility, cell permeability, lipophilicity, and metabolic stability will be exhaustively studied utilizing a variety of assays to establish a pharmacokinetic profile of a clinical drug candidate. Unfortunately, the structural features associated with potent biological activity will not necessarily be correlated with favorable drug-like properties, requiring scientists
to find novel methods of improving drug-like properties while maintaining biological activity.

One method that has found appreciable use in improving drug-like properties is the covalent incorporation of poly(ethylene glycol) (PEG) onto the chemical structure of biologically active small molecules; a process referred to as PEGylation.\(^{78,79}\) PEG (Figure 50) is an inert, linear, and water-soluble polyether comprised of ethylene glycol monomers. Various PEG polymers can be found in many household items, such as shampoo, conditioner, and toothpaste. Conveniently, PEG is commercially available in a wide range of molecular weights, which is highly beneficial given the inherently expensive nature of the drug development process. In medicinal chemistry, PEGylated drugs have been demonstrated to exhibit enhanced metabolic stability and aqueous solubility compared to their non-PEGylated counterparts, providing a safe and effective method of improving the pharmacokinetics of a lead compound with inherently poor drug-like properties. Alternatively, PEG polymers capped with a methoxy group at one end (mPEG) have also found appreciable use for improving drug-like properties (Figure 51).\(^{78,79}\)

In the late 1960s, a professor of biochemistry at Rutgers University named Frank Davis became interested in developing methods of utilizing bioactive proteins for various

![Figure 50](image1.png)

**Figure 50.** Poly(ethyleneglycol), a water-soluble polyether found in many common household products.

![Figure 51](image2.png)

**Figure 51.** The structure of methoxy-polyethylene glycol (mPEG).
therapeutic purposes; at the time, relatively few proteins of non-human origin had any measure of therapeutic value due to the resulting adverse immunological responses if injected into humans.\textsuperscript{80} Davis hypothesized that attaching a hydrophilic polymer might make the foreign protein less immunogenic. However, it was ultimately a report on the infusion of a copolymer of PEG and polypropylene glycol into the blood of patients who had undergone major blood vessel surgery in order to prevent undesirable post-surgical side effects. In an interesting and inspiring account of the origin of PEGylation in medicinal chemistry, Davis recalls that it was this report that caused him to realize that PEG likely had utility beyond traditional industrial and commercial ventures.\textsuperscript{80}

Davis would later publish two back-to-back reports in June of 1977 confirming PEGs ability to increase the immunogenicity of bovine serum albumin and bovine liver catalase, respectively. In each case, mPEGs of 1.9-5 kDa were covalently attached to the protein of interest, and the mPEGylated bovine proteins were introduced intravenously to rabbits.\textsuperscript{80} Remarkably, the mPEGylated proteins went largely unnoticed by the rabbit immune system, with blood circulation life time comparable to the non-mPEGylated bovine serum without the development of antibodies against the protein. Furthermore, the mPEGylated proteins were found to retain over 90\% of their catalytic activity and no toxicity was observed. Davis also noted altered physical properties, such as solubility and electrophoretic mobility. Thus, the pharmacokinetic value of PEGylated molecules for therapeutic purposes in biological systems was clearly demonstrated, paving the way for decades of research into the utility and consequences of PEGylation in medicinal chemistry.
PEG chains are readily hydrated in an aqueous environment, which each ethylene oxide unit of the PEG polymer coordinating 2-3 water molecules through the hydrogen bond acceptor (HBA) ability of the ether oxygens. Depending on the size of the PEGs attached to the structure, they may also provide a masking effect from endogenous proteases. For example, PEGylated uricase (Figure 52) shows increased solubility, increased serum half-life, and reduced immunogenicity compared to uricase; all without decreasing activity.81

There are currently 16 FDA approved PEGylated drugs on the market, in which an mPEG moiety is present in every case. An overview of these drugs is given in Table 6.82,83 In every instance but one, mPEGylation has been used to increase the pharmacokinetic properties of biological macromolecules, such as enzymes, antibodies, hormones, and liposomes to treat a variety of disorders. Naloxegol (Figure 53) is currently the only example of a PEGylated small molecule approved by the FDA. An mPEGylated derivative of naloxol (which, itself is close
<table>
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<th>Generic Name</th>
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<th>PEG Size (kDa)</th>
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<td>20</td>
<td>1</td>
<td>Neutropenia</td>
</tr>
<tr>
<td>Mircera</td>
<td>CERA, PEG-EPO</td>
<td>60</td>
<td>30</td>
<td>1</td>
<td>Anemia, chronic renal failure</td>
</tr>
<tr>
<td>Macugen</td>
<td>Pegaptanib</td>
<td>50</td>
<td>40</td>
<td>1</td>
<td>Macular degeneration</td>
</tr>
<tr>
<td>PEGASYS</td>
<td>Peg-interferon α-2a</td>
<td>60</td>
<td>40</td>
<td>1</td>
<td>Chronic hepatitis B, C</td>
</tr>
<tr>
<td>Cimzia</td>
<td>Certolizumab b pegol</td>
<td>40</td>
<td>1</td>
<td></td>
<td>Rheumatoid arthritis, Crohn’s disease, and psoriatic arthritis</td>
</tr>
<tr>
<td>Plegridy</td>
<td>Peg-interferon β-1</td>
<td>44</td>
<td>20</td>
<td>1</td>
<td>Multiple sclerosis</td>
</tr>
</tbody>
</table>

Table 6. An overview of FDA-approved PEGylated drugs for a variety of diseases.
derivative of naloxone (trade name: Narcan), a common and life-saving opioid used to combat opioid overdoses) which is used to treat opioid-induced constipation.\textsuperscript{83}

**Improving the pharmacokinetic properties of CADA analogs**

After almost a decade of syntheses devoted to developing an understanding of structural features of CADA analogs associated with potent biological activity,\textsuperscript{11-13} a focus on designing potent analogs which could feasibly be used in a biological setting has come into focus. Given the poor drug-like properties of CADA and CADA analogs, the synthesis of potent CADA analogs with enhanced aqueous solubility, metabolic stability, and various other pharmacokinetic properties is necessary for advancing our understanding of CADA’s mechanism of action. Furthermore, potent CADA analogs with favorable drug-like properties is necessary in order to study CADA analogs in biological systems and, perhaps, even moving CADA into clinical trials.

The previously synthesized unsymmetrical CADA analog **VGDO20** (Figure 54), containing a cyclohexylmethyl tail and a 4-methoxybenzenesulfonamide side-arm is one of the most potent CADA analogs found to date (\(IC_{50} = 0.15 \pm 0.01~\mu M\)). QSAR

![Figure 54. Structures of CADA analogs VGD020 and CK165.\textsuperscript{45}](image)
experiments reported by Chawla et al. found that the dipole moment of the side-arm of an unsymmetrical CADA analog is crucial for potency.\textsuperscript{46} Furthermore, the ethoxy analog CK165 (Figure 54) was also potent ($IC_{50} = 0.19 \pm 0.01 \mu\text{M}$), suggesting potency may be retained as the hydrocarbon chain extends beyond the benzene ring.\textsuperscript{45}

**(m)PEGylated CADA analogs**

Combining SAR experiments and known methods of increasing drug-like properties of small molecules, it was hypothesized that a CADA analog of the general structure shown in Figure 55 would be a potent CADA analog with enhanced pharmacokinetic properties. Therefore, a series of PEGylated CADA analogs of different chain lengths ($n = 1-4$) and terminal groups (-OH and –OCH$\textsubscript{3}$) were synthesized with the following aims: (i) to investigate the biological activity of PEGylated CADA analogs with various chain lengths, (ii) to investigate the biological activities of PEGylated CADA analogs with either a hydrogen bond donating (HBD) or a hydrogen bond accepting (HBA) at the terminus of the PEG group, and (iii) develop a pharmacokinetic profile of PEGylated CADA analogs.

![Figure 55. The general structure of PEGylated and mPEGylated CADA analogs with varying chain lengths.](image_url)
Utilizing **NCP001** as a parent compound, a series of PEGylated CADA analogs of various chain length and terminal functionalities were synthesized according to previous literature precedence for the addition of activated PEG and mPEG polymers to phenolic substrates under basic conditions, as shown in **Scheme 19**.\(^{84}\)

![Scheme 19](image.png)

**Scheme 19.** Proposed synthetic route towards of a series of CADA analogs with varying PEG (R = -OH) and mPEG (R = -CH\(_3\)) chain lengths (n = 1-4) via the phenolic CADA analog NCP001.\(^{84}\)

**Tosylation of PEG and mPEG polymers**

Activated PEGs and mPEG polymers were synthesized in excellent yields utilizing previously reported literature methods,\(^{85-87}\) as summarized in **Figure 56**. In the case of the PEG substrates, the mono-tosylated glycols were synthesized in moderate to excellent yields, and each PEG polymer (n = 1-4) was cheap and commercially available. The synthesis of the tosylated PEG polymers was achieved by adding a solution of p-toluenesulfonyl chloride in THF dropwise to a solution of the PEG polymer in aqueous sodium hydroxide and THF over 1-2 hours. The solution was stirred at 0°C for an additional 2 hours to afford the monotosylated product in excellent yields following purification using
The n = 1-3 mPEG polymers were also commercially available and relatively cheap, and tosylation was achieved in excellent yields by adding a solution of p-toluenesulfonyl chloride in THF dropwise to a solution of the mPEG polymer in sodium hydroxide at 0°C, after which it was allowed to warm to room temperature and was stirred for an additional 16 h. As shown in Figure 56, the tosylation of the mPEG polymers was achieved in excellent yields.

![Figure 56. Synthesis and yields of various tosylated PEG and mPEG polymers for the synthesis of PEGylated CADA analogs.](image_url)
However, tetraethylene glycol monomethyl ether is more expensive than the \( n = 1 \)-3 mPEGs, and was synthesized from tetraethylene glycol in a straightforward reaction shown in Scheme 20.\(^{87}\) Sodium hydride was carefully added to a solution of tetraethylene glycol in THF at 0\(^\circ\)C. After stirring vigorously for 10 minutes, iodomethane was added dropwise at 0\(^\circ\)C over 0.5 h. The solution was removed from an ice bath and warmed to room temperature, then refluxed for 16 h to afford the monomethylated product in excellent yields. Notably, refluxing this reaction for 16 h rather than 24 h as reported in the literature provided an improved yield.

\[
\text{HO(\(\text{O})_4\text{H} 1. \text{NaH, THF, 0}^\circ\text{C} 2. \text{CH}_3\text{I, 0}^\circ\text{C - reflux, 16 h} \rightarrow \text{O(\(\text{O})_4\text{H}} 99\%
\]

**Scheme 20.** Methylation of tetraethylene glycol utilizing sodium hydride and iodomethane.\(^{87}\)

**Synthesis of NCP001**

The synthesis of the unsymmetrical CADA analog **NCP001** was previously developed by Nicholas Plfug, who derived the phenolic compound via the demethylation of **VGD020**, the macrocyclic product of the open chain disulfonamide **VGD013** (Scheme 21).\(^{46}\) Following the general synthetic route to unsymmetrical CADA analogs bearing a cyclohexylmethyl tail, the phenolic **NCP001** is synthesized in seven linear steps in a 17\%
yield from 1,3-diaminopropane. The advanced intermediate 5 is reacted with 4-methoxybenzenesulfonyl chloride to yield the open-chain disulfonamide VGD013, which undergoes a Tsuji-Trost macrocyclization reaction as described by Ali et al. to yield VGD020. Notably, yields for the macrocyclization of VGD013 to VGD020 were relatively higher than the analogous conversion for various other CADA analogs, which enhanced the practicality of obtaining a significant amount of starting material for the synthesis of eight novel CADA analogs.

**BBBr3-mediated cleavage of ethers**

A normally unreactive group, aryl and alkyl ethers are commonly cleaved using boron tribromide (BBBr3), which yields an alcohol and a brominated compound. In the case of aryl methyl ethers, treatment with BBBr3 at -78°C produces a phenol and bromomethane.
Surprisingly, the mechanism of BBr$_3$-mediated ether cleavage has not been extensively studied, experimentally or computationally, and the exact mechanistic details are somewhat contested. The traditional understanding of the mechanism of BBr$_3$-mediated cleavage of ethers is shown in Scheme 22, which involves the ether oxygen undergoing nucleophilic attack of the Lewis acidic boron center.$^{88}$ As a result, a bromide anion is displaced, which undergoes intra- or intermolecular attack of the methyl group attached to the ether oxygen to produce methyl bromide and a neutral dibromo borate species. Similar to the hydroboration of alkenes, the resulting dibromo borate could potentially undergo nucleophilic attack by 2 additional ether oxygens. Finally, oxidative cleavage using ammonium hydroxide yields the phenol product.

Scheme 22. The traditional proposed mechanism for the BBr$_3$-mediated cleavage of aryl ethers.

However, recent computational studies by Sousa et al. in 2013 and Kosak et al. in 2015 have challenged the traditional understanding of the mechanism of BBr$_3$-mediated demethylation.$^{89,90}$ Sousa et al. found that the formation of the ether-BBr$_3$ adduct shown in Scheme 22 readily forms in a variety of alkyl and aryl ethers. However, their computations suggested that, although ethers of secondary and tertiary alkyl groups likely proceed through the traditional mechanism as shown in Scheme 22, ethers of primary alkyl or aryl groups seem to proceed by a bimolecular mechanism involving two ether-BBr$_3$
adducts, as shown in Figure 57.

**Figure 57.** Proposed unimolecular and biomolecular mechanisms for the BBr$_3$-mediated cleavage of phenyl ethers (Kosak et al.$^{89,90}$)

Given the phenol and tertiary amine functionalities of NCP001, it was hypothesized that the structure may exist as a zwitterion that could readily react with a tosylated glycol to afford the desired PEGylated product (Scheme 23). Accordingly, this transformation was first attempted by combining NCP001 and ethylene glycol (monotosylate) in room temperature to refluxing acetonitrile. After several attempts, this synthetic strategy was found to yield exclusively starting material and was therefore unsuitable for obtaining PEGylated CADA analogs.

**Scheme 23.** The phenolic NCP001 was initially hypothesized to be a zwitterion that could react with a tosylated PEG or mPEG without a base. Ultimately, this transformation was not achievable under these conditions.
However, a variety of methods for adding PEG groups to phenols have been reported in the literature using several common bases in aprotic solvents. The entire series of PEGylated and mPEGylated CADA analogs were accessible by heating **NCP001** with the respective tosylated PEG or mPEG polymer with potassium carbonate ($K_2CO_3$) in either dimethylformamide (DMF) at 90°C or refluxing acetonitrile. Ultimately, it was realized that acetonitrile was a more viable solvent for this transformation due to the ease of its removal in the purification process compared to DMF in the preparation of analytically pure compounds. Furthermore, DMF is toxic and may impede biological studies. The yields for a series of PEGylated CADA analogs are shown in **Figure 58**, most of which were obtained in moderate to excellent yields.

![Figure 58](image)

<table>
<thead>
<tr>
<th>ID</th>
<th>n</th>
<th>R</th>
<th>Yield</th>
</tr>
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<tbody>
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<td>1</td>
<td>-OH</td>
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<tr>
<td>DJ011</td>
<td>1</td>
<td>-OCH₃</td>
<td>68%</td>
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<tr>
<td>DJ012</td>
<td>2</td>
<td>-OH</td>
<td>83%</td>
</tr>
<tr>
<td>DJ013</td>
<td>2</td>
<td>-OCH₃</td>
<td>51%</td>
</tr>
<tr>
<td>DJ014</td>
<td>3</td>
<td>-OH</td>
<td>54%</td>
</tr>
<tr>
<td>DJ015</td>
<td>3</td>
<td>-OCH₃</td>
<td>61%</td>
</tr>
<tr>
<td>DJ016</td>
<td>4</td>
<td>-OH</td>
<td>45%</td>
</tr>
<tr>
<td>DJ017</td>
<td>4</td>
<td>-OCH₃</td>
<td>47%</td>
</tr>
</tbody>
</table>

**Figure 58.** An overview of the synthesis of various PEGylated and mPEGylated CADA analogs.
Biological activities of PEGylated and mPEGylated CADA analogs

PEGylated and mPEGylated CADA analogs were hypothesized to exhibit potent CD4 down modulation due to previous QSAR experiments while continuing to develop our understanding of CADAs interactions with the hCD4 SP and sortilin SP. Indeed, a majority of PEGylated and mPEGylated CADA analogs exhibited moderate potency in the down modulation of sortilin and hCD4 (Table 7).

<table>
<thead>
<tr>
<th>ID</th>
<th>IC$_{50}$ hCD4 (µM)</th>
<th>IC$_{50}$ SORT (µM)</th>
</tr>
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<tbody>
<tr>
<td>DJ010</td>
<td>1.96 ± 0.52</td>
<td>5.28</td>
</tr>
<tr>
<td>DJ011</td>
<td>0.29 ± 0.077</td>
<td>1.61</td>
</tr>
<tr>
<td>DJ012</td>
<td>0.404 ± 0.404</td>
<td>n/d</td>
</tr>
<tr>
<td>DJ013</td>
<td>1.062 ± 0.073</td>
<td>n/d</td>
</tr>
<tr>
<td>DJ014</td>
<td>0.912 ± 0.03</td>
<td>n/d</td>
</tr>
<tr>
<td>DJ015</td>
<td>-</td>
<td>n/d</td>
</tr>
<tr>
<td>DJ016</td>
<td>0.702 ± 0.057</td>
<td>n/d</td>
</tr>
</tbody>
</table>

Table 7. Biological activity of PEGylated and mPEGylated CADA analogs

Besides obtaining potent CADA analogs with enhanced drug-like properties (ex. water solubility, cell permeability, metabolic stability, etc.), one of the specific aims of this project was to investigate the effects that PEG or mPEG chain length have on potency. Furthermore, previous SAR experiments suggested that a side-arm containing a HBD and HBA group in the para position is generally less potent than an analog having a side arm with only a HBA group in the para position; for example, the methyl ether VGD020 is
significantly more potent than the phenolic NCP001. Thus, the synthesis of a series of PEGylated and mPEGylated CADA analogs provided an avenue to explore the role of HBD and HBA groups on the aryl sulfonamide.

In regards to chain length, the n = 1 and 2 PEG and mPEG analogs were the most potent CD4 down modulators in the series. In particular, DJ011 was found to have an IC$_{50}$ of 0.29 µM, which may not be surprising given its structural and electronic similarity to VGD020. The n = 2-4 analogs were slightly less potent than DJ010 and DJ011, but still relatively potent among previously synthesized CADA analogs. Notably, the higher chain analogs are some of the largest CADA analogs synthesized to date, with molecular weights topping out at 780. Such a large structure suggests that there is ample room in the binding pocket of CADA and the hCD4 SP, as previously hypothesized. PEG and mPEG have been shown to be exceptionally flexible, often wrapping around the structure in which they are attached to. It seems these higher chain PEGylated and mPEGylated CADA analogs are able to arrange themselves in the binding pocket fairly well despite their size. The synthesis of mPEGylated and PEGylated CADA analogs of even higher chain lengths may be relevant in the future, although small molecules of high molecular weight (>500) violate Lipinski’s rules and may be unsuccessful drug candidates.

Interestingly, the relative potencies between DJ010 and DJ011 were opposite of DJ012 and DJ013; in the n = 1 analogs, a terminal methyl ether was more potent than a terminal hydroxyl group, as predicted based on previous SAR experiments. However, the opposite is true of the n = 2 analogs, albeit with a slightly smaller difference than the n = 1 analogs. This observation suggests that the effects on potency observed for analogs with
HBD/HBA and HBA groups in the para position of the aryl sulfonamide may not apply as the structure elongates away from the aryl ring.

**Summary and outlook**

A series of PEGylated and mPEGylated CADA analogs have been synthesized and analyzed for their CD4 and sortilin down modulation activities. These analogs were obtained in moderate yields utilizing the unsymmetrical phenolic CADA analog NCP001 as a parent substrate, which is obtained in 7 linear steps in a 17% yield from 1,3-diaminopropane. PEG and mPEG polymers (n = 1-4) were activated using p-toluenesulfonyl chloride in moderate to excellent yields and reacted with NCP001 under basic conditions to yield 8 novel CADA analogs.

The rationale for synthesizing DJ010-DJ017 had several aims: (i) to synthesize biologically active CADA analogs with improved drug-like properties compared to previously synthesized analogs utilizing PEGylation and mPEGylation, a well-known method of increasing solubility and metabolic stability of small molecules; (ii) to investigate the relationship between PEG/mPEG chain length and biological activity; and (iii): to investigate the relationship between having a hydrogen-bond acceptor or a hydrogen-bond donor at the chain terminus and biological activity.

mPEGylated and PEGylated CADA analogs exhibit moderate potency in hCD4 down modulation. Consistent with previous QSAR experiments, the mPEG DJ011 is more potent than PEG DJ010. However, the opposite is true for DJ012 and DJ013, suggesting that the presence of a hydrogen-bond acceptor may not have a negative effect on potency.
as the group extends farther from the aromatic ring. Indeed, it will be interesting to assess the relative potencies of the higher chain mPEG and PEG CADA analogs.

The drug-like properties of mPEG- and PEGylated CADA analogs were also investigated and will be discussed in the next chapter. Pharmacokinetic properties of mPEG- and PEGylated CADA analogs, such as solubility and lipophilicity, are significantly better than those found for CADA. Ultimately, this series of biologically active CADA analogs with enhanced drug-like properties may prove useful in studying hCD4 and sortilin down-modulation using in vivo models.

**Experimental**

\[ N-(p\text{-}Methoxybenzenesulfonyl)\text{-}N'-(p\text{-}toluenesulfonfyl)bis(3-aminopropyl)cyclohexylmethylamine (VGD013):^{45} \]

Into a 500-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 4.03 g (10.5 mmol) of \( N-(3\text{-}aminopropyl)\text{-}N-(3-p\text{-}toluenesulfonamidopropyl)cyclohexylmethylamine (4) \), 2.2 g (10.6 mmol) of 4-methoxybenzenesulfonyl chloride, 69 mL of a saturated \( aq. \) Na\(_2\)SO\(_4\) solution, 69 mL of a saturated \( aq. \) NaCl solution, and 69 mL of CH\(_2\)Cl\(_2\) were added. The mixture was stirred at room temperature under nitrogen for 24 h and then the aqueous layer
was extracted with CH$_2$Cl$_2$ (3 x 40 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 4.87 g (84%) of crude product as a light yellow oil. The crude was purified by conversion to the HCl salt followed by trituration with diethyl ether. The crude oil was dissolved in 138 mL of 2 N HCl in MeOH and stirred at room temperature under nitrogen for 4 hours. The solvent was removed by rotary evaporation and the resulting residue was dried in vacuo before being triturated with anhydrous diethyl ether (3 x 30 mL). The residue was again dried in vacuo to yield 3.65 g (90%) of VGD013-HCl. The HCl salt was converted back to the free base by stirring VGD013-HCl with 183 mL of a 2 N aq. NaOH solution, 183 mL of a saturated aq. NaCl solution, and 183 mL of CH$_2$Cl$_2$ for 5 hours. The mixture was transferred to a separatory funnel and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 x 50 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, concentrated via rotary evaporation, and dried in vacuo to yield 3.27 g (95%) of pure product as a light yellow oil.

$^1$H NMR (500 MHz, CDCl$_3$/TMS) $\delta$ 7.79 (d, 8 Hz, 2 H, o-ArSO$_2$), 7.73 (d, 8 Hz, 2 H, o-Ts), 7.30 (d, 8 Hz, 2 H, m-Ts), 6.98 (d, 9 Hz, 2 H, m-ArSO$_2$), 5.88 (bs, 2 H, NH), 3.87 (s, 3 H, OCH$_3$), 2.97 (m, 4 H, H$_6$/H$_{12}$), 2.42 (s, 3 H, CH$_3$), 2.36 (t, 6 Hz, 4 H, H$_8$/H$_{10}$), 2.04 (d, 7 Hz, 2 H, CH$_2$Cy), 1.63 (m, 10 H, H$_7$/H$_{11}$, Cy), 1.34 (m, 1 H, Cy), 1.15 (m, 2 H, Cy), 0.78 (q, 12 Hz, 2 H, Cy).
9-Cyclohexylmethyl-1-(p-methoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclodecane (VGD020):\textsuperscript{49} Into a 250-mL, one-necked, round-bottomed flask equipped with a condenser, a nitrogen inlet, and a magnetic stir bar, 4.10 g (7.45 mmol) of $N$-(p-methoxybenzenesulfonyl)-$N'$-(p-toluenesulfonyl)bis(3-aminopropyl)cyclohexylmethylamine (VGD013), 10.6 g (36.43 mmol) of 2-methylene-1,3-propanebis(tert-butylocarbonate), 0.33 g (0.91 mmol) of 1,4-bis(diphenylphosphino)butane (dppb), 0.11 g (0.41 mmol) of tris(dibenzylideneacetone)dipalladium (0) (Pd$_2$(dba)$_3$), 0.37 g (3.73 mmol) of Na$_2$CO$_3$, and 60 mL of anhydrous acetonitrile were added. The resulting dark mixture was boiled under reflux under nitrogen, then allowed to cool to room temperature. The solvent was removed by rotary evaporation and a solution of the resulting dark residue in 70 mL of CH$_2$Cl$_2$ was washed with a saturated $aq$. NaHCO$_3$ solution (3 x 70 mL) and a saturated $aq$. NaCl solution (70 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 5.3 g of crude product. A solution of the crude product in a minimal amount of ethyl acetate was absorbed on an alumina column, which was eluted with 250 mL of hexanes, 250 mL of 1:4 (v/v) ethyl acetate/hexanes, 250 mL of 2:3 (v/v) ethyl acetate/hexanes, and finally 3:2 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated by rotary
evaporation to yield 2.29 g (51%) of product as a light yellow oil. $^1$H NMR (500 MHz, CDCl$_3$/TMS) $\delta$ 7.72 (d, 9 Hz, 2 H, o-ArSO$_2$), 7.67 (d, 7 Hz, 2 H, o-Ts), 7.32 (d, 8 Hz, 2 H, m-Ts), 6.99 (d, 7 Hz, 2 H, m-ArSO$_2$), 5.18 (s, 2 H, C=CH$_2$), 3.88 (s, 3 H, OCH$_3$), 3.79 (m, 4 H, H2/H4), 3.15 (m, 4 H, H6/H12), 2.44 (s, 3 H, CH$_3$), 2.27 (m, 4 H, H8/H10), 1.96 (d, 7 Hz, 2 H, CH$_2$Cy), 1.62 (m, 10 H, H7, H11, Cy), 1.15 (m, 3 H, Cy), 0.69 (m, 2 H, Cy).

9-Cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001):$^{46}$ Into a 250-mL, three-necked, round-bottomed flask equipped with a nitrogen inlet, a 50-mL addition funnel, a rubber septum, and a magnetic stir bar, 0.62 g (1.3 mmol) of 9-cyclohexylmethyl-1-(p-methoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (VGD020) in 52 mL of CH$_2$Cl$_2$ was added. The solution was stirred vigorously as 13 mL of a 1 M solution of BBr$_3$ in CH$_2$Cl$_2$ (13 mmol) was carefully added dropwise to the solution using a syringe. The resulting brown mixture was stirred for 1.5 h and then cooled to 0 °C (ice bath) before 49 mL of an aq. solution of NH$_4$OH (30% by weight) was slowly added using an addition funnel. A thick, milky-white vapor is formed upon addition of NH$_4$OH and the mixture slowly turned to a light yellow color. The mixture was warmed to room temperature and then stirred overnight. The mixture was transferred to a separatory
funnel and the organic layer was washed with H$_2$O (3 x 30 mL). The organic extracts was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation, yielding 0.49 g (81%) of pure product as a flaky yellow solid. $^1$H NMR (500 MHz, CDCl$_3$/TMS) δ 7.76 (d, 8 Hz, 2 H, o-Ts), 7.66 (d, 8 Hz, 2 H, o-ArSO$_2$), 7.33 (d, 8 Hz, 2 H, m-Ts), 6.98 (d, 5 Hz, 2 H, m-ArSO$_2$), 5.19 (s, 2 H, C=CH$_2$), 3.79 (t, 6 Hz, 2 H, H4), 3.76 (t, 5.8 Hz, 2 H, H2), 3.17 (m, 4 H, H12/H6), 2.44 (s, 3 H, CH$_3$), 2.40 (m, 4 H, H8/H10), 2.07 (m, 2 H, CH$_2$Cy), 1.65 (m, 10 H, H7, H11, Cy), 1.16 (m, 2 H, Cy), 0.75 (q, 12 Hz, 2 H, Cy).

2-Hydroxyethyl $p$-toluenesulfonate.\textsuperscript{85} Into a 50-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 3.3 g (52 mmol, 10 equiv.) of ethylene glycol and 6 mL of CH$_2$Cl$_2$ were added. The solution was cooled to 0°C and then 0.80 g (7.8 mmol, 1.5 equiv.) of triethylamine was added. The solution was allowed to stir for 0.5 h at 0°C before 1.00 g (5.24 mmol) of $p$-toluenesulfonyl chloride was slowly added in small portions. After complete addition, the reaction mixture was allowed to warm to room temperature and was then stirred for 18 h. The milky white mixture was poured into 5 mL of H$_2$O and the organic layer was separated and transferred to an addition funnel. The pale solution was washed with 5% aq. solution of citric acid (3 x 5 mL), dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield a crude white solid. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ was absorbed on a silica column, then eluted with 50:1 (v/v) CH$_2$Cl$_2$/MeOH). The desired fractions were combined and concentrated via rotary evaporation to yield 0.75 g (65%) as a bright yellow oil. $^1$H NMR
(300 MHz, CDCl₃/TMS) δ 7.81 (d, 8.0 Hz, 2 H, o-Ts), 7.36 (d, 8.2 Hz, 2 H, m-Ts), 4.14 (t, 7.0 Hz, 2 H, TsOCH₂), 3.81 (t, 6.9 Hz, 2 H, HOCH₂), 2.45 (s, 3 H, ArCH₃), 2.24 (s, 1 H, OH).

2-Methoxyethyl p-toluenesulfonate:⁸⁶ Into a 100-mL, one-necked round bottomed flask equipped with a magnetic stir bar and a nitrogen inlet was added 2.0 g (26 mmol) of 2-methoxyethanol and 15 mL of anhydrous THF. Next, a solution of 1.58 g (39.51 mmol) of a solution of NaOH in 7.50 mL of H₂O was added and the solution was cooled to 0 °C (ice bath) as 5.01 g of p-toluenesulfonyl chloride was added in portions over 30 min., which resulted in the formation of a milky white mixture. The reaction mixture was slowly warmed to room temperature and stirred vigorously overnight. The mixture was then extracted with CH₂Cl₂ (3 x 30 mL). The combined organic extracts were dried (Na₂SO₄), filtered, concentrated by rotary evaporation, and dried in vacuo to yield 5.12 g (85%) of product as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, 8.4 Hz, 2 H, o-Ts), 7.30 (d, 7.9 Hz, 2 H, m-Ts), 4.11 (m, 2 H, TsOCH₂), 3.53 (m, 2 H, CH₂OCH₃), 3.25 (s, 3 H, OCH₃), 2.39 (s, 2 H, ArCH₃).

2-(2-Hydroxyethoxy)ethoxyethyl p-toluenesulfonate:⁸⁵ Into a 50-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 5.56 g (52.4 mmol, 10 equiv.) of diethylene glycol and 6 mL of CH₂Cl₂ were added. The solution was
cooled to 0°C (ice bath) and then 0.8 g (7.8 mmol, 1.5 equiv.) of triethylamine was added. The solution stirred for 0.5 h at 0°C and then 1.00 g (5.24 mmol) of p-toluenesulfonyl chloride was slowly added in small portions over 30 min, then the reaction mixture was allowed to warm to room temperature and stirred overnight. The milky white mixture was poured into 5 mL of H2O and the organic layer was separated and transferred to an addition funnel. The pale solution was washed with 5% _aq._ solution of citric acid (3 x 5 mL), dried (Na2SO4), filtered, and concentrated by rotary evaporation to yield a crude white solid. A solution of the crude product in a minimal amount of CH2Cl2 was absorbed on a silica column, then eluted with 50:1 (v/v) CH2Cl2/MeOH. The desired fractions were combined and concentrated by rotary evaporation to yield 0.72 g (53%) as a bright yellow oil. 1H NMR (400 MHz, CDCl3) δ 7.79 (d, 8.1 Hz, 2 H, o-Ts), 7.32 (d, 8.6 Hz, 2 H, m-Ts), 4.16 (m, 2 H, CH2OCH3), 3.68 (m, 2 H, TsOCH2), 3.56 (m, 2 H, CH2CH2OCH3), 3.46 (m, 2 H, TsOCH2CH2O), 3.34 (s, 3 H, OCH3), 2.43 (s, 3 H, ArCH3).

2-(2-Methoxyethoxy)ethyl p-toluenesulfonate: 86 Into a 100-mL, one-necked round bottomed flask equipped with a magnetic stir bar and a nitrogen inlet was added 3.12 g (26.0 mmol) of diethylene glycol methyl ether and 15 mL of anhydrous THF. Next, a solution of 1.58 g (39.51 mmol) of NaOH in 7.50 mL of H2O was added and the solution was cooled to 0°C (ice bath) before 5.01 g of p-toluenesulfonyl chloride was added in portions over approximately 0.5 h. The mixture was slowly warmed to room temperature and stirred vigorously overnight. The mixture was then extracted with CH2Cl2 (3 x 30 mL). The combined organic extracts were dried (Na2SO4), filtered, concentrated via rotary
evaporation, and dried in vacuo to yield 6.23 g (87%) of pure product as a colorless oil. 1H NMR (400 MHz, CDCl3) δ 7.79 (d, 8.1 Hz, 2 H, o-Ts), 7.32 (d, 8.6 Hz, 2 H, m-Ts), 4.16 (m, 2 H, TsOCH2), 3.68 (m, 2 H, CH2OCH3), 3.57 (m, 2 H, TsOCH2CH2), 3.47 (m, 2 H, CH2CH2OCH3), 3.34 (s, 3 H, OCH3), 2.43 (s, 3 H, ArCH3).

2-(2-(2-Hydroxyethoxy)ethoxy)ethoxyethyl p-toluenesulfonate: Into a 50-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 7.9 g (52 mmol, 10 equiv.) of triethylene glycol and 6 mL of CH2Cl2 were added. The solution was cooled to 0℃ (ice bath) and then 0.80 g (7.8 mmol, 1.5 equiv.) of triethylamine was added. The solution was stirred for 30 min at 0℃ before 1.0 g (5.2 mmol) of p-toluenesulfonyl chloride was slowly added in small portions. The resulting reaction mixture was allowed to warm to room temperature and was then overnight. The milky white mixture was poured into 5 mL of H2O and the organic layer was separated and transferred to an addition funnel. The pale solution was washed with 5% aq. solution of citric acid (3 x 5 mL), dried (Na2SO4), filtered, and concentrated by rotary evaporation to yield a crude white solid. A solution of the crude product in a minimal amount of CH2Cl2 was absorbed on a silica column, then eluted with 50:1 (v/v) CH2Cl2/MeOH. The desired fractions were combined and concentrated via rotary evaporation to yield 1.10 g (60%) as a bright yellow oil. 1H NMR (300 MHz, CDCl3/TMS) δ 7.80 (d, 8.0 Hz, 2 H, o-Ts), 7.35 (d, 8.2 Hz, 2 H, m-Ts), 4.19 (m, 2 H, TsOCH2), 3.70 (m, 4 H, TsOCH2CH2OCH2), 3.58 (m, 6 H, CH2OCH2CH2OH), 2.44 (s, 3 H, ArCH3), 2.30 (bs, 1 H, OH).
2-[2-(2-Methoxyethoxy)ethoxy]ethyl p-toluenesulfonate:86 Into a 100-mL, one-necked round bottomed flask equipped with a magnetic stir bar and a nitrogen inlet was added 4.0 g (24.4 mmol) of triethylene glycol monomethyl ether and 38 mL of THF. A solution of 3.95 g (101.3 mmol) of NaOH in 18.75 mL H₂O was added and the solution was cooled to 0°C via an ice bath before 12.5 g (65.9 mmol) p-toluenesulfonyl chloride was added in small portions over 30 min., then the mixture was slowly warmed to room temperature and stirred overnight. The mixture was extracted with CH₂Cl₂ (30 mL x 3) and the combined organic extracts were dried (Na₂SO₄), filtered, and concentrated by rotary evaporation to yield 8.02 g (98%) of pure product as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.79 (d, 8.5 Hz, 2 H, o-Ts), 7.32 (d, 8.0 Hz, 2 H, m-Ts), 4.15 (m, 2 H, TsOCH₂), 3.67 (m, 2 H, CH₂OCH₃), 3.59 (m, 6 H, OCH₂CH₂O(CH₂)₂O), 3.52 (d, 2 H, CH₂CH₂OCH₃), 3.36 (s, 3 H, OCH₃), 2.43 (s, 3 H, ArCH₃).

2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]ethoxy]ethyl p-toluenesulfonate:85 Into a 50-mL, one-necked, round-bottomed flask equipped with a nitrogen inlet and a magnetic stir bar, 10.0 g (52.4 mmol, 10 equiv.) of tetraethylene glycol and 6 mL of CH₂Cl₂ were added. The solution was cooled to 0°C (ice bath) and then 0.80 g (7.8 mmol, 1.5 equiv.) of triethylamine was added. The solution was stirred for 0.5 h at 0°C before 1.00 g (5.24
mmol) of p-toluenesulfonyl chloride was slowly added in small portions. The reaction mixture was allowed to warm to room temperature and was then stirred under nitrogen overnight. The milky white mixture was poured into 5 mL of H₂O and the organic layer was separated and transferred to an addition funnel. The pale solution was washed with 5% aq. solution of citric acid (3 x 5 mL), dried (Na₂SO₄), filtered, and concentrated by rotary evaporation to yield a crude white solid. A solution of the crude product in a minimal amount of CH₂Cl₂ was absorbed on a silica column, then eluted with 50:1 (v/v) CH₂Cl₂/MeOH. The desired fractions were combined and concentrated via rotary evaporation to yield 1.11 g (66%) of pure product as a bright yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 7.80 (d, 8.2 Hz, 2 H, o-Ts), 7.34 (d, 8.1 Hz, 2 H, m-Ts), 4.17 (m, 2 H, TsOCH₂), 3.65 (m, 14 H, CH₂CH₂), 2.45 (s, 3 H, CH₃).

2-(2-[2-(2-Methoxyethoxy)ethoxy]ethoxy)ethanol:⁸⁶,⁸⁷ Into a 250-mL, one-necked, round bottomed flask equipped with a nitrogen inlet, a magnetic stir bar, and a 25-mL addition funnel was added 4.08 g (21.00 mmol) of tetraethylene glycol in 100 mL of anhydrous THF and cooled to 0°C using an ice bath. Meanwhile, 1.00 g (41.68 mmol) of sodium hydride was washed with anhydrous hexane (30 mL x 3) before being carefully added to the cooled solution in small portions, causing the solution to effervescence. Next, 8.00 g (56.4 mmol, 3.51 mL) of iodomethane was added dropwise over 0.5 h. The solution was then warmed to room temperature and then refluxed for 24 h, after which it was concentrated by rotary evaporation to yield 4.44 g (100%) of crude product as an orange
oil. The crude product was loaded into a 100-mL, one-necked round bottomed flask equipped with a magnetic stir bar and a nitrogen inlet with 10 mL of anhydrous THF. Next, a solution of 0.95 g (31.5 mmol) of NaOH in 5.50 mL of H₂O was added and the solution was cooled to 0°C (ice bath) before 8.0 g (41.1 mmol) of p-toluenesulfonyl chloride was added in portions over approximately 0.5 h. The mixture was slowly warmed to room temperature and stirred vigorously overnight. The mixture was then extracted with CH₂Cl₂ (3 x 30 mL). The combined organic extracts were dried (Na₂SO₄), filtered, concentrated via rotary evaporation, and dried in vacuo to yield 7.88 g (99%) of pure product as a colorless oil. H-NMR (400 MHz, CDCl₃) δ 7.78 (d, 8.4 Hz, 2 H, o-Ts), 7.33 (d, 8.5 Hz, 2 H, m-Ts), 4.16 (m, 2 H, CH₂OH), 3.59 (m, 14 H, CH₂CH₂), 3.36 (s, 3 H, OCH₃), 2.43 (s, 3 H, ArCH₃).

9-Cyclohexylmethyl-1-(p-[2-hydroxy]ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ010): Into a 250-mL, one-necked, round-bottomed flask equipped with a reflux condenser, a nitrogen inlet, and a magnetic stir bar, 0.24 g (0.41 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.392 g (0.666 mmol) of 2-hydroxyethyl p-toluenesulfonate, 1.26 g (9.13 mmol) of K₂CO₃, and 48 mL of

![Chemical Structure Image]
anhydrous acetonitrile were added. The mixture was boiled under reflux under nitrogen for 24 h and then allowed to cool to room temperature. The mixture was concentrated by rotary evaporation to yield an amber oil. A solution of this oil in 25 mL of CH$_2$Cl$_2$ was washed with a saturated aq. NaHCO$_3$ solution (2 x 25 mL) and a saturated solution of aq. NaCl (25 mL). The organic extract was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield an orange oily residue. A solution of the crude product was dissolved in a minimal amount of CH$_2$Cl$_2$ and carefully absorbed on an alumina column, which was eluted with 1:99 (v/v) methanol/ethyl acetate. The desired fractions were combined and concentrated via rotary evaporation. The resulting residue was dried in vacuo to yield 0.32 (77%) of pure product as a yellow glassy solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.73 (d, 9.0 Hz, 2 H, o-Ts), 7.66 (d, 8.4 Hz, 2 H, o-ArSO$_2$), 7.33 (d, 7.8 Hz, 2 H, m-Ts), 7.03 (d, 9.0 Hz, 2 H, m-ArSO$_2$), 5.22 (s, 2 H, C=CH$_2$), 4.17 (d, 8.5 Hz, 2 H, SO$_2$ArOCH$_2$), 4.02 (d, 8.8 Hz, 2 H, SO$_2$ArOCH$_2$CH$_2$), 3.78 (d, 1.4 Hz, 4 H, H2/4), 3.15 (m, 4 H, H12/H6), 2.45 (s, 3 H, CH$_3$), 1.68 (m, 10 H H7, H11, Cy). 1.08 (m, 2 H, Cy), 0.69 (t, 11 Hz, 2 H, Cy). $^{13}$C-NMR (101 MHz, CDCl$_3$) $\delta$ 141.7, 130.7, 130.6, 130.0, 129.7, 127.5, 119.3, 69.7, 61.1, 52.7, 48.6, 47.1, 33.7, 31.6, 29.7, 25.6, 25.5, 21.5. MS (MS$^+$) $m/z$ 634. (MH$^+$) Anal. Calcd. for C$_{32}$H$_{47}$N$_3$O$_6$S$_2$ · HCl · 0.25 H$_2$O: C: 56.96, H: 7.24, N: 6.23. Found: C: 57.34, H: 7.22, N: 6.27.
9-Cyclohexylmethyl-1-(p-[2-methoxy]ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ011):²⁴ Into a 10-mL, one-necked round bottomed flask equipped with a magnetic stir bar, a condenser, and a nitrogen inlet was added 0.21 g (0.36 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.123 g (0.53 mmol) of 2-methoxyethyl p-toluenesulfonate, 0.101 g (0.73 mmol) of K₂CO₃, and 5 mL of anhydrous acetonitrile was added. The resulting mixture was boiled under reflux under nitrogen for 24 h. The reaction mixture was cooled to room temperature and concentrated by rotary evaporation to yield an amber oil. A solution of this oil in 10 mL of CH₂Cl₂ and washed with a saturated aq. NaHCO₃ solution (3 x 10 mL) and a saturated aq. NaCl solution (10 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated by rotary evaporation to yield a crude amber oil. A solution of the crude product in a minimal amount of CH₂Cl₂ was carefully absorbed on an alumina column, which was then eluted with 3:7 (v/v) ethyl acetate/hexane. The desired fractions were combined and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 0.15 g (68%) of pure product as a yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 7.71 (d, 8.8 Hz, 2 H, o-Ts), 7.67 (d, 8.1 Hz, 2 H, o-ArSO₂), 7.32 (d, 8.1 Hz, 2 H, m-Ts), 7.02 (d, 8.8 Hz, 2 H, m-ArSO₂), 5.17 (s, 2 H, C=CH₂), 4.19 (m, 2 H, SO₂ArOCH₂), 3.78 (m, 4 H, H₂/4), 3.46 (s, 3 H, OCH₃), 3.14 (m, 4 H, H6/H12), 2.44 (s, 3 H, TsCH₃), 2.26 (m, 4 H,
H8/H10), 1.96 (d, 6.8 Hz, 2 H, CH$_2$Cy), 1.58 (m, 10 H, H7, H11, Cy), 1.13 (m, 2 H, Cy), 0.74 (m, 2 H, Cy). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 162.1, 143.4, 138.0, 135.6, 130.9, 130.4, 129.7, 129.3, 128.8, 127.3, 116.5, 114.8, 70.7, 67.7, 62.1, 59.3, 50.9, 50.8, 50.3, 44.1, 35.8, 31.9, 29.7, 26.7, 26.0, 21.5. MS (ESI$^+$): m/z 648. Anal. Calcd. for C$_{32}$H$_{47}$N$_3$O$_6$S$_2$ $\cdot$ HCl $\cdot$ 0.25 H$_2$O: C: 57.54, H: 7.39, N: 6.10. Found: C: 56.17, H: 7.35, N: 5.90.

9-Cyclohexylmethyl-1-(p-[2-hydroxy]ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ012):$^{84}$ Into a 10-mL, one-necked round bottomed flask equipped with a nitrogen inlet, a condenser, and a magnetic stir bar was added 0.25 g (0.424 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.162 g (0.623 mmol) of 2-(2-hydroxyethoxy)ethoxy)ethyl p-toluenesulfonate, 0.117 g (0.847 mmol) of K$_2$CO$_3$, and 5 mL of anhydrous acetonitrile. The resulting mixture boiled under reflux under nitrogen for 24 h. The reaction mixture was cooled to room temperature and concentrated via rotary evaporation to yield an orange-amber oil. A solution of this oil in 10 mL of CH$_2$Cl$_2$ was washed with a saturated aq. NaHCO$_3$ solution (3 x 10 mL) and a saturated aq. NaCl solution (10 mL). The organic solution was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 0.23 g of crude as an amber oil. A solution
of the crude product in a minimal amount of CH₂Cl₂ was absorbed on an alumina column, which was eluted with 1:99 (v/v) methanol/ethyl acetate. The desired fractions were combined and concentrated via rotary evaporation. The resulting residue was dried *in vacuo* to yield 0.281 g (83%) of pure product as a yellow-orange oil. 

**¹H NMR (400 MHz, CDCl₃)** \( \delta \): 7.70 (d, 9.0 Hz, 2 H, \( \alpha \)-Ts), 7.65 (d, 8.3 Hz, 2 H, \( \alpha \)-ArSO₂), 7.30 (d, 8.3 Hz, 2 H, \( m \)-Ts), 7.00 (d, 8.9 Hz, 2 H, \( m \)-ArSO₂), 5.15 (s, 2 H, C=CH₂), 4.19 (m, 2 H, SO₂ArOCH₂), 3.89 (m, 2 H), 3.76-3.67 (m, 10 H, CH₂C=CH₂, OCH2CH2OCH2CH2OH), 3.13 (d, 4.8 Hz, 4 H, H6/H12), 2.42 (s, 3 H, TsCH₃), 2.25 (t, 5.8 Hz, 4 H, H8/H10), 1.94 (d, 6.9 Hz, 2 H, CH₂Cy), 1.61 (m, 10 H, H7, H11, Cy), 1.23 (m, 2 H, Cy), 0.67 (m, 2 H, Cy). 

**¹³C NMR (101 MHz, CDCl₃)** \( \delta \): 161.9, 143.4, 137.9, 135.5, 129.7, 129.3, 127.2, 116.5, 114.8, 72.6, 72.4, 70.8, 70.6, 70.3, 69.4, 69.3, 67.7, 62.1, 50.3, 35.9, 31.9, 26.7, 26.0, 21.5. 


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**9-Cyclohexylmethyl-1-(\( \rho \)-[2-(2-methoxyethoxy)ethoxy]methoxybenzenesulfonyl)-3-methylene-5-(\( \rho \)-toluenesulfonyl)-1,5,9-triazacyclododecane** (DJ013):\(^{84}\) Into a 10-mL, one-necked round bottomed flask equipped with a nitrogen inlet, a condenser, and a magnetic stir bar was added 0.22 g (0.338 mmol) of 9-cyclohexylmethyl-1-(\( \rho \)-hydroxybenzenesulfonyl)-3-methylene-5-(\( \rho \)-toluenesulfonyl)-1,5,9-triazacyclododecane.
(NCP001), 0.155 g (0.567 mmol) of 2-(2-methoxyethoxy)ethyl p-toluenesulfonate, 0.105 g (0.76 mmol) of K$_2$CO$_3$, and 5 mL of anhydrous acetonitrile. The resulting mixture was boiled under reflux under nitrogen for 24 h. The mixture was cooled to room temperature and then concentrated by rotary evaporation to yield an orange-amber oil. A solution of this oil in 10 mL of CH$_2$Cl$_2$ was washed with a saturated aq. NaHCO$_3$ solution (3 x 10 mL) and a saturated aq. NaCl solution (10 mL). The organic layer was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 0.19 g of crude product as a dark amber oil. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ was absorbed on an alumina column, which was then eluted with 3:7 (v/v) ethyl acetate/hexane. The desired fractions were combined and concentrated via rotary evaporation. The resulting residue was dried in vacuo to yield 0.13 g (51%) of pure product as a yellow oil.$^1$H NMR (400 MHz, CDCl$_3$) δ 7.69 (d, 8.9 Hz, 2 H, o-Ts), 7.65 (d, 8.1 Hz, 2 H, o-ArSO$_2$), 7.30 (d, 8.5 Hz, 2 H, m-Ts), 6.99 (d, 8.7 Hz, 2 H, m-ArSO$_2$), 5.15 (s, 2 H, C=CH$_2$), 4.19 (t, 2 H, SO$_2$ArOCH$_2$), 3.86 (m, 2 H, ArOCH$_2$CH$_2$OCH$_2$), 3.77 (d, 6.4 Hz, 4 H, H2/4), 3.70 (m, 2 H, ArOCH$_2$CH$_2$OCH$_2$CH$_2$), 3.56 (m, 2 H, ArOCH$_2$CH$_2$OCH$_2$CH$_2$), 3.37 (s, 3 H, OCH$_3$), 3.12 (m, 4 H, H6/H12), 2.42 (s, 3 H, TsCH$_3$), 2.25 (m, 4 H, H8/H10), 1.94 (d, 6.9 Hz, 2 H, CH$_2$Cy), 1.61 (m, 10 H, H7, H11, Cy), 1.13 (m, 2 H, Cy), 0.69 (m, 2 H, Cy). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 162.1, 143.4, 138.0, 135.6, 130.3, 129.7, 129.3, 127.2, 114.8, 71.9, 70.8, 69.5, 67.8, 62.1, 59.1, 51.0, 50.3, 44.1, 35.9, 31.9, 29.7, 26.7, 26.0, 24.4, 21.5. MS (ESI$^+$): m/z 692 (MH$^+$). Anal. Calcd. for C$_{36}$H$_5$N$_3$O$_9$S$_2$·HCl·0.25 H$_2$O: C: 57.01, H: 7.44, N: 5.54. Found: C: 56.88, H: 7.07, N: 5.58.
9-Cyclohexylmethyl-1-(p-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ014): Into a 10-mL, one-necked round bottomed flask equipped with a nitrogen inlet, a condenser, and a magnetic stir bar was added 0.43 g (0.73 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.33 g (1.1 mmol) of 2-(2-(2-hydroxyethoxy)ethoxy)ethoxyethyl p-toluenesulfonate, 0.204 g (1.4 mmol) of K$_2$CO$_3$, and 5 mL of anhydrous acetonitrile. The resulting mixture was boiled under reflux under nitrogen for 24 h, then cooled to room temperature and concentrated by rotary evaporation to yield an orange-amber oil. A solution of this oil 15 mL of CH$_2$Cl$_2$ was washed with a saturated aq. NaHCO$_3$ solution (3 x 15 mL) and a saturated aq. NaCl solution (15 mL). The organic layer was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 0.60 g of crude product as a dark amber oil. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ was absorbed on an alumina column, which was eluted with 1:99 (v/v) methanol/ethyl acetate. The desired fractions were combined and concentrated via rotary evaporation. The resulting residue was dried in vacuo to yield 0.18 g (54%) of pure product as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.71 (d, 8.6
Hz, 2 H, o-Ts), 7.66 (d, 7.7 Hz, 2 H, o-ArSO₂), 7.30 (d, 7.8 Hz, 2 H, m-Ts), 6.98 (d, 8.7 Hz, 2 H, m-ArSO₂), 5.16 (s, 2 H, C=CH₂), 3.87 (s, 2H), 3.77 (d, J = 6.0 Hz, 3H), 3.65 (s, 1H), 3.13 (q, J = 6.9 Hz, 4H), 2.42 (s, 3H), 2.29 – 2.21 (m, 5H), 1.95 (d, J = 6.8 Hz, 2H), 1.61 (d, J = 11.5 Hz, 16H), 1.24 (s, 4H), 0.91 – 0.83 (m, 3H), 0.69 (t, 11.7 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 171.1, 162.7, 144.3, 141.7, 133.3, 129.7, 128.0, 127.6, 115.3, 60.4, 48.6, 33.4, 30.5, 25.7, 25.4, 21.5, 21.0, 14.2. MS (ESI⁺): m/z 722 (MH⁺). Analog. Calcd. for C₃₆H₅₅N₃O₉S₂ · HCl · 0.25 H₂O: C: 57.01, H: 7.44, N: 5.54. Found: C: 56.88, H: 7.07, N: 5.58.

9-Cyclohexylmethyl-1-(4-(2-(2-(2-methoxyethoxy)hydroxy)ethoxy)ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ015):

Into a 10-mL, one-necked round bottomed flask equipped with a nitrogen inlet, a condenser, and a magnetic stir bar was added 0.20 g (0.339 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.161 g (0.51 mmol) of 2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate, 0.093 g (0.67 mmol) of K₂CO₃, and 5 mL of anhydrous acetonitrile. was boiled under reflux under nitrogen for 24 h, then cooled to room
temperature and concentrated by rotary evaporation to yield an amber oil. A solution of this oil in 10 mL of CH$_2$Cl$_2$ was washed with saturated aq. NaHCO$_3$ solution (2 x 10 mL) and saturated aq. NaCl (10 mL). The organic layer was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 0.60 g of crude product as a dark amber oil. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ and was absorbed on an alumina column, which was eluted with 3:7 (v/v) ethyl acetate/hexanes. The desired fractions were combined and concentrated by rotary evaporation. The resulting residue was dried in vacuo to yield 0.12 g (61%) of pure product as a yellow oil. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.72 (d, 8.9 Hz, 2 H, O-Ts), 7.68 (d, 8.3 Hz, 2 H, O-ArSO$_2$), 7.33 (d, 8.0 Hz, 2 H, m-Ts), 7.02 (d, 8.9 Hz, 2 H, m-ArSO$_2$), 5.18 (s, 2 H, C=CH$_2$), 4.20 (m, 2 H, SO$_2$ArOCH$_2$), 3.90 (m, 2 H, ArOCH$_2$CH$_2$OCH$_2$), 3.80 (d, 9.0 Hz, 4 H, H2/4), 3.75 (d, 5.3 Hz, 2 H, ArOCH$_2$CH$_2$OCH$_2$CH$_2$), 3.70 (d, 5.4 Hz, 2 H, ArOCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$), 3.66 (d, 4.9 Hz, 2 H, ArOCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$), 3.58 (m, 3 H), 3.39 (s, 3H, OCH$_3$), 3.15 (m, 4 H, H6/H12), 2.45 (s, 3 H, CH$_3$), 2.27 (m, 4 H, H8/H10), 1.97 (d, 6.8 Hz, 2 H, CH$_2$Cy), 1.63 (d, 9.2 Hz, 10 H, H7, H11, Cy), 1.13 (m, 2 H, Cy), 0.69 (m, 2 H, Cy). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 162.1, 143.4, 129.7, 129.3, 127.3, 114.9, 114.2, 71.9, 70.9, 70.6, 70.6, 69.4, 67.8, 62.1, 59.0, 56.3, 31.9, 26.7, 26.0, 21.5, 20.0, 13.5. MS (ESI$^+$): m/z 738 (MH$^+$).
9-Cyclohexylmethyl-1-(2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy)ethoxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (DJ016): Into a 10-mL, one-necked round bottomed flask equipped with a nitrogen inlet, a condenser, and a magnetic stir bar was added 0.136 g (0.231 mmol) of 9-cyclohexylmethyl-1-(p-hydroxybenzenesulfonyl)-3-methylene-5-(p-toluenesulfonyl)-1,5,9-triazacyclododecane (NCP001), 0.121 g (0.347 mmol) of 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate, 0.063 g (0.670 mmol) of K$_2$CO$_3$, and 5 mL of anhydrous acetonitrile. The resulting mixture was boiled under reflux under nitrogen for 24 h, then cooled to room temperature and concentrated by rotary evaporation to yield an amber oil. A solution of this oil in 10 mL of CH$_2$Cl$_2$ was washed with a saturated aq. NaHCO$_3$ solution (3 x 10 mL) and a saturated aq. NaCl solution (2 x 10 mL). The organic extract was dried (Na$_2$SO$_4$), filtered, and concentrated by rotary evaporation to yield 0.28 g of crude product as an amber oil. A solution of the crude product in a minimal amount of CH$_2$Cl$_2$ and absorbed on an alumina column, then eluted using 1:99 (v/v) methanol/ethyl acetate. The desired fractions were combined and concentrated by rotary evaporation to yield a yellow oil, which was dried in vacuo to yield 0.084 g (45%) of pure product as a yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.67 (d, 8.1 Hz, 2 H, o-Ts), 7.64 (d, 8.6 Hz, 2 H, o-ArSO$_2$), 7.29 (d, 7.5 Hz, 2 H, m-Ts), 6.99 (d,
8.3 Hz, 2 H, m-ArSO₂, 5.15 (s, 2 H, C=CH₂), 4.18 (t, 4.7 Hz, 2 H, SO₂ArOCH₂), 3.87 (d, 4.4 Hz, 2 H, ArOCH₂CH₂OCH₂), 3.76 (d, 5.9 Hz, 4 H H2/4), 3.71 – 3.61 (m, 12 H), 3.59 (d, 6.3, 2.4 Hz, 2 H), 3.12 (q, 7.8 Hz, 4 H, H6/H12), 2.41 (s, 3 H, CH₃), 2.24 (d, 5.9 Hz, 4 H, H8/H10), 1.94 (d, 6.8 Hz, 2 H, CH₂Cy), 1.64 (m, 10 H, H7, H11, Cy), 1.08 (m, 2 H, Cy), 0.68 (t, 11.7 Hz, 2 H, Cy). ¹³C NMR (101 MHz, CDCl₃) δ 162.1, 143.4, 138.0, 135.6, 129.7, 129.3, 127.2, 116.5, 114.9, 72.4, 70.8, 70.6, 70.3, 69.4, 67.8, 62.1, 61.7, 50.3, 44.1, 35.9, 31.9, 26.7, 26.0, 21.5. MS (ESI⁺): m/z 778 (MH⁺). Anal. Calcd. for C₃₈H₆₁N₃O₉S₂ · HCl · 1.25 H₂O: C: 55.32, H: 7.64, N: 5.09. Found: C: 55.14, H: 7.24, N: 5.08.
CHAPTER 4

Pharmacokinetics of various biologically active CADA analogs
An overview of pharmacokinetics

It is perhaps an understatement to say that the journey of almost any given drug from the pharmacy to its target in the patient's body is anything but trivial. Regardless of the mechanism of action or mode of administration, there exists many physiological factors that the drug must accommodate in order to successfully reach its target. Drug development must not only consider the design of novel small molecules with remarkable biological activity, but must also consider the complexity of the environment in which the drug must exist and traverse through; the human body. The study of how a drug affects the human body, such as a drug’s mechanism of action, is known as pharmacodynamics.\textsuperscript{91-93} Conversely, the study of how the human body affects the drug is known as pharmacokinetics (PK), which is an area of major concern in the development of successful drugs.

Broadly speaking, pharmacokinetics involves the study of drug administration, distribution, metabolism, excretion, and toxicity, often abbreviated as ADMET and collectively referred to as drug-like properties.\textsuperscript{91-93} Understanding and enhancing the PK properties of a drug is absolutely crucial in its success as a useful drug but often presents a difficult problem to the medicinal chemist. After all, a novel drug that cures cancer is

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure59.png}
\caption{The crucial balance between the biological activity of a drug and its drug-like properties.}
\end{figure}
simply useless if the drug cannot be absorbed appropriately to reach its intended target, is metabolized at a rate that exceeds its ability to exert a biological effect(s), or, conversely, remains in the body with toxic consequences rather than being excreted in a timely manner. Thus, a balanced relationship between the biological activity and the pharmacokinetics of a successful drug (Figure 59).

Drug-like properties are often a direct result of the chemical and physical properties of the drug’s structure. Various functional groups and structural features present on a drug will influence the drug’s physical properties in a biological system, such as hydrophobicity, hydrophilicity, and stability towards metabolic enzymes. For example, many drugs may need to pass through a hydrophobic cell membrane to reach their target, yet they must traverse through an aqueous environment in order to get to the surface of the cell membrane. These seemingly contradictory requirements require a chemical structure that can accommodate both hydrophobic and hydrophilic environments while maintaining specificity for a molecular target.

In order to understand the difficulty of designing a successful drug, one must consider the complex journey of an orally administered drug to its target, which begins with the administration of the drug. Drugs may be, but are not limited to, administration through intravenous (IV) injection, insertion into the rectal cavity, nebulization and/or inhalation, intramuscular injection, or injection through the skin (topical or subcutaneous). However, the oral administration of a drug is by far the most convenient and preferential mode of drug administration and a vast amount of research has been dedicated to the development of biologically active orally administered drugs.
Although oral administration is the preferred method, it is unfortunately the mode of administration that meets the most scrutiny in terms of the physiological barriers encountered by the drug. In general, many successful orally administered drugs follow a set of guidelines known as Lipinski’s rule of five,\textsuperscript{94,95} which, in effect, states the following:

\textit{A viable drug for oral absorption should have a molecular weight of 500 or less, fewer than 5 hydrogen bond donor (HBD) functional groups, fewer than 10 hydrogen bond acceptor (HBA) functional groups, and a LogP (the partition coefficient, which will be explained in greater detail in the upcoming discussion) of less than 5.}

That being said, Lipinski’s rule of five is certainly a rule of thumb rather than law, as there have been many examples of successful drugs that do not abide by the rule of five.

\textbf{The journey of a drug through the gastrointestinal tract}

In general, an orally administered drug will move through the gastrointestinal tract (GI) tract, where it eventually moves into the bloodstream and travels throughout the body to reach its target.\textsuperscript{93} Collectively, the organs that comprise the GI tract are the mouth, throat, stomach, lower intestine, and large intestine.

\textbf{Figure 60}. An overview of the human gastrointestinal tract (GIT).
intestine (Figure 60), each of which pose variable chemical environments and pharmacokinetic barriers.

Assuming an orally administered drug spends little of its time in the mouth, the first major physiological environment the drug encounters is the stomach. Highly acidic in nature, the stomach contains hydrochloric acid and various digestive enzymes that operate at a pH of approximately 1-2 in a fasting state and 3-7 in fed state. The stomach also contains various digestive enzymes that assist in the degradation of proteins and other macromolecules into smaller chemical components, although the absorption of these molecules is relatively insignificant compared to absorption in later stages of the GI tract.

For an orally administered drug, the movement of stomach muscles and the acidic aqueous environment aid in the degradation of the capsule or pill containing the drug. The rate at which the solid drug dissolves into solution in the stomach is known as the dissolution rate and is an important factor to consider in designing an orally bioavailable drug. Depending on whether an individual is fasting, a drug may spend as long as one hour in the stomach before moving into the small intestine. Ultimately, an orally administered drug must be soluble and stable for an appreciable amount of time in the harsh, acidic environment of the stomach.

As an orally administered drug moves from the stomach to the small intestine, pancreatic fluid containing various hydrolytic enzymes is added, providing potential challenges to drugs containing functional groups prone to hydrolysis. Also, the gallbladder secretes bile acids that aid in the solubility of lipophilic molecules, which may ultimately increase the absorption of lipophilic drugs.
The small intestine consists of three sections: the duodenum, jejunum, and ileum. Each section of the small intestine presents different chemical environments, as summarized in Table 8. Notably, a pH gradient exists between the stomach and the progressing sections of the small intestine, which will have a direct impact on drug absorption. Analogous to the stomach, drug structure is correlated with drug solubility in the small intestine, which ultimately affects drug absorption. Drugs containing basic functional groups will mostly exist in protonated cationic salts in the stomach and the duodenum and will be more soluble than acidic or neutral drugs. However, the solubility of neutral and acidic drugs tend to increase with increasing pH as the drug moves from the duodenum to the jejunum and ileum.

<table>
<thead>
<tr>
<th>GIT region</th>
<th>pH in a fasting state</th>
<th>pH in a fed state</th>
<th>Transit time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1.4-2.1</td>
<td>3-7</td>
<td>0.5-1 h</td>
</tr>
<tr>
<td>Duodenum</td>
<td>4.4-6.6</td>
<td>5.2-6.2</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>4.4-6.6</td>
<td>5.2-6.2</td>
<td>2-4 h</td>
</tr>
<tr>
<td>Ileum</td>
<td>6.8-8</td>
<td>6.8-8</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. An overview of the chemical environments and the transit times in the major organs of the gastrointestinal tract (GIT).
The majority of drug absorption takes place in the small intestine,\textsuperscript{93} which exhibits an anatomy that greatly enhances the absorption of important biological molecules and nutrients. As shown in Figure 61, the human small intestine is a muscular, tubular organ with an average length of 6 meters (20 feet) long. To maximize absorption, the surface area of the inner surface of the small intestine (the lumen) is increased by a factor of almost 400 due to large folds that line the length of the small intestine, which are lined with villi (microscopic structures that extend approximately 1 mm into the lumen).\textsuperscript{93} The surface of villi contain a layer of epithelial cells, which is the primary barrier molecules must cross in order to enter the bloodstream. Remarkably, the individual epithelial cells contain microvilli on the luminal side, which further increase the surface area of the small intestine!

\textbf{Figure 61.} The structure of the small intestine, which contains several morphological features that significantly increase the surface area of the small intestine lumen in order to enhance the absorption of nutrients.\textsuperscript{91}
Transportation of drugs across the epithelial layer of cells in the small intestine can occur via several mechanisms; active transport, endocytosis, or even paracellular transport (if the drug is low molecular weight). However, passive diffusion is the most common mode of drug transport across a lipid bilayer. Phosphatidylcholine (Figure 62) is a representative structure of a class of molecules called phospholipids, which are amphiphilic molecules containing an anionic phosphate group attached to a number of fatty acid hydrocarbon chains. In an aqueous environment, phospholipids self-assemble into bilayers by orientating the hydrophobic fatty acid chains towards each other (and away from the water molecules) while exposing their polar head groups to the polar environment (Figure 62). The passive transport of a drug across this membrane requires the drug to interact with...
the polar head groups, displacing water molecules that are normally near the surface of the bilayer. The drug must then make its way across the tightly-packed, hydrophobic fatty acid chains, and finally through the tangential polar phosphate groups. Regardless of polarity, movement across biological membranes is simply fascinating, highlighting the paradoxical necessity of a drug to accommodate a host of different chemical and physical environments in order to be successful in exerting its biological effect.

**Methods of analyzing drug solubility: kinetic & thermodynamic solubility**

Drug solubility is perhaps one of the most important pharmacokinetic properties of an orally administered drug. After all, what good is a potent drug that is not soluble in a biological system like the human body? Drug solubility is defined as the maximum dissolved concentration of a drug in a particular solvent. The medicinal chemist seeks to maximize the biological solubility of a drug, as a drug with low solubility is highly undesirable to the patient, requiring frequent dosing as a consequence of low absorption.

Much to the dismay of a medicinal chemist, the structure of an active drug often contains chemical and physical moieties that do not necessarily correlate with solubility in a biological system. Though largely flexible depending on the chemical environment, solubility is mainly governed by chemical structure. Obtaining an orally administered and biologically active drug is certainly difficult, as the drug will encounter a variety of different chemical environments in route to its target. For example, the drug must be hydrophobic enough to pass through cell membranes and hydrophilic enough to remain soluble in the cytoplasm. A host of strategies for introducing chemical and physical
features correlated with solubility exist, such as incorporating ionic functionalities onto the drug structure, altering the polarity and molecular weight of the drug, and changing the number of hydrogen bonding groups on the drug.  

A number of assays to determine solubility of drug candidates have been developed, two of which have emerged as common and convenient methods of determining solubility. Kinetic solubility is measured by creating a concentrated solution of the drug in an organic solvent (DMSO) and creating serial dilutions in an aqueous buffer (PBS) at various concentrations. Presumably, the drug will be soluble at very low concentrations but will become increasingly insoluble at higher concentrations. Typically, a solution of the drug in dimethyl sulfoxide (DMSO) is introduced to an aqueous buffer (ex. PBS) in serial dilutions and incubated at 37 °C in a micro-well plate, after which each well is analyzed to determine the concentration at which the drug precipitates.

Nephelometry is commonly used to determine the kinetic solubility of a drug by measuring the turbidity of each well on the micro-well plate. A nephelometer passes light through each well on the plate and measures light scattering as a function of turbidity. If the drug is soluble at a given concentration, the light passes through the well without being scattered. Alternatively, if a drug is not soluble, it precipitates and the particles in solution cause the light to be scattered. Therefore, the concentration at which the nephelometer detects a significant amount of light scattering is the kinetic solubility of the drug. This process is summarized in Figure 63.
The kinetic solubility of various CADA analogs was analyzed using 20mM stock solutions in DMSO, which were diluted with PBS in a number of serial dilutions on a 96-well plate to produce wells with concentrations from 500-0.78 µM and 400-0.38 µM. The plate was shaken on an orbital shaker for 5 min and incubated at 37°C for one hour before being analyzed using a Nepheloskan Ascent nephelometer (ThermoScientific). An overview of the kinetic solubilities of various CADA analogs are shown in Table 9.

Alternatively, thermodynamic solubility is typically determined using the shake-flask method, in which an over-saturated solution of the drug in an aqueous buffer is agitated for 24-72 h. The solid particles are subsequently filtered off and the filtrate is analyzed to determine the concentration of the dissolved drug. In contrast with kinetic solubility, the over-saturated solutions are agitated at room temperature for a length of time sufficient to achieve equilibrium between the crystalline and dissolved state.

**Figure 63.** An overview of determining kinetic solubility using nephelometry. Light is passed through wells containing the drug at various concentration. The kinetic solubility is reached when the drug precipitates from solution, resulting in light scattering which is detected by the nephelometer.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Kinetic Solubility (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA HCl</td>
<td>27</td>
</tr>
<tr>
<td>DJ010 HCl</td>
<td>194</td>
</tr>
<tr>
<td>DJ011 HCl</td>
<td>34</td>
</tr>
<tr>
<td>DJ012 HCl</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>DJ013 HCl</td>
<td>183</td>
</tr>
<tr>
<td>DJ014 HCl</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>DJ015 HCl</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>DJ016 HCl</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>CK147 HCl</td>
<td>101</td>
</tr>
<tr>
<td>VGD020 HCl</td>
<td>-</td>
</tr>
<tr>
<td>TL020 HCl</td>
<td>-</td>
</tr>
<tr>
<td>LAL014 HCl</td>
<td>141</td>
</tr>
<tr>
<td>LAL016 HCl</td>
<td>-</td>
</tr>
<tr>
<td>LAL020 HCl</td>
<td>213</td>
</tr>
<tr>
<td>LAL024 HCl</td>
<td>255</td>
</tr>
<tr>
<td>CK075 2HCl</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>VGD040 2HCl</td>
<td>&gt; 500</td>
</tr>
</tbody>
</table>

Table 9. Kinetic solubility of various CADA analogs determined by nephelometry
Determination of the molar absorptivity of various CADA analogs

The concentration of various CADA analogs in thermodynamic solubility and partition coefficient assays was analyzed using UV-Vis spectroscopy in accordance to the Beer-Lambert law:\textsuperscript{66}

\begin{equation}
A = \varepsilon \cdot c \cdot l
\end{equation}

where \( A \) = absorbance, \( \varepsilon \) = the molar extinction coefficient, \( c \) = concentration (M), and \( l \) = pathlength (usually 1 cm) of the cuvette used in the experiment. The molar extinction coefficient (also known as the molar absorptivity or the molar attenuation coefficient) is a measure of how well a molecule attenuates light at a given wavelength. If the \( \varepsilon \) for a molecule is known, the concentration of a particular solution can be calculated by measuring the UV-vis spectrum and solving for \( c \). As such, UV-vis spectroscopy provides a useful method of analyzing unknown concentrations of various CADA analogs in aqueous solvents.

However, CADA compounds are novel and, therefore, the \( \varepsilon \) values in water were previously unknown. In order to determine \( \varepsilon \) for a given compound, one must know the remaining variables in the Beer-Lampert equation. Since the aqueous solubility of most CADA analogs was also unknown, the \( \varepsilon \) in an aqueous solution could not be directly calculated using the Beer-Lampert equation. Instead, solutions of known concentrations of CADA analogs in methanol were prepared and analyzed by UV-vis spectroscopy, as methanol is the most similar solvent to water in terms of polarity and solvating ability. Therefore, a major assumption in using the Beer-Lampert law to calculate aqueous
concentrations of CADA analogs in various assays is that the $\varepsilon$ value calculated in methanol will be similar to the unknown $\varepsilon$ value in water.

Solutions of CADA analogs in methanol at various concentrations (typically 10, 20, and 30 µM) were prepared and analyzed by UV-vis spectroscopy. The $\varepsilon$ values at various concentrations were determined and plotted to analyze the linearity of the results; the $\varepsilon$ should remain constant at all concentrations if the results were linear, where non-linear results would suggest otherwise. The molar absorptivity of various CADA analogs are shown in Table 10.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\varepsilon$ (MeOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA HCl</td>
<td>183164</td>
</tr>
<tr>
<td>VGD019 HCl</td>
<td>144622</td>
</tr>
<tr>
<td>VGD020 HCl</td>
<td>213600</td>
</tr>
<tr>
<td>VGD040 HCl</td>
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</tr>
<tr>
<td>CK207 2HCl</td>
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</tr>
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<td>DJ010 HCl</td>
<td>24468</td>
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<tr>
<td>DJ011 HCl</td>
<td>214622</td>
</tr>
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<td>DJ012 HCl</td>
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<td>DJ014 HCl</td>
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<td>DJ015 HCl</td>
<td>251267</td>
</tr>
<tr>
<td>DJ016 HCl</td>
<td>26170</td>
</tr>
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<td>LAL014 HCl</td>
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<td>LAL018 HCl</td>
<td>37317</td>
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<td>LAL020 HCl</td>
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<tr>
<td>LAL024 HCl</td>
<td>30417</td>
</tr>
<tr>
<td>LAL026 HCl</td>
<td>35550</td>
</tr>
<tr>
<td>Compound</td>
<td>Molar Absorptivity (ε)</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------</td>
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<tr>
<td>TL020 HCl</td>
<td>205933</td>
</tr>
</tbody>
</table>

Table 10. Molar absorptivity (ε) of various CADA analogs in methanol as determined by the Beer-Lampert equation.

Determination of thermodynamic solubility of biologically active CADA analogs

Thermodynamic solubility is typically determined using the shake-flask method (Figure 64), in which an over-saturated solution of the drug in an aqueous buffer is agitated for 24-72 h. The solid particles are filtered off and the filtrate is analyzed to determine the concentration of the dissolved drug. In contrast with kinetic solubility, the over-saturated solutions are agitated at room temperature for a length of time sufficient to

![Figure 64](image-url)  

Figure 64. An overview of the shake-flask method to determine thermodynamic solubility. Saturated solutions of a drug in an aqueous medium is agitated for a length of time suitable for reaching equilibrium between the crystalline state and the dissolved state. The solution is filtered and the concentration of the drug is analyzed using UV-Vis or HPLC to determine the concentration.
achieve equilibrium between the crystalline and dissolved state. The thermodynamic solubility of various CADA analogs are given in Table 11.

<table>
<thead>
<tr>
<th>ID</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>A</th>
<th>$\varepsilon$</th>
<th>Thermo. Sol. ($\mu$M)</th>
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<td>CADA HCl</td>
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<tr>
<td>DJ016 HCl</td>
<td>236.00</td>
<td>1.46014</td>
<td>26170</td>
<td>28000</td>
</tr>
<tr>
<td>LAL005 HCl</td>
<td>266.32</td>
<td>0.691</td>
<td>35533</td>
<td>2.48</td>
</tr>
<tr>
<td>LAL020 HCl</td>
<td>264.54</td>
<td>0.927</td>
<td>29078</td>
<td>4.07</td>
</tr>
<tr>
<td>LAL024 HCl</td>
<td>250.00</td>
<td>0.866</td>
<td>30417</td>
<td>35.4</td>
</tr>
<tr>
<td>LAL026 HCl</td>
<td>263.65</td>
<td>0.601</td>
<td>35550</td>
<td>2.16</td>
</tr>
<tr>
<td>LAL030 HCl</td>
<td>262.46</td>
<td>0.731</td>
<td>37617</td>
<td>389</td>
</tr>
<tr>
<td>LAL032 HCl</td>
<td>223.09</td>
<td>0.539</td>
<td>143917</td>
<td>3.75</td>
</tr>
<tr>
<td>LAL039 HCl</td>
<td>265.41</td>
<td>0.681</td>
<td>3628</td>
<td>7,500</td>
</tr>
</tbody>
</table>
Table 11. Thermodynamic solubility of various CADA analogs using UV-Vis spectroscopy and the Beer-Lampert law (A: absorbance (UV-Vis); ε: molar absorptivity in CH3OH)

<table>
<thead>
<tr>
<th></th>
<th>[A]</th>
<th>ε</th>
<th>[Drug]_octanol</th>
<th>[Drug]_water</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL020 HCl</td>
<td>243.36</td>
<td>0.881</td>
<td>205933</td>
<td>29.6</td>
</tr>
<tr>
<td>AR001 HCl</td>
<td>227.00</td>
<td>0.333</td>
<td>7074</td>
<td>47</td>
</tr>
</tbody>
</table>

The partition and distribution coefficients

One of the key parameters of the Lipinski rule of five, the partition coefficient (P) is a common assay to assess the lipophilicity of neutral drugs in the development stage. Lipophilicity is defined as tendency of a compound to partition into a non-polar solution rather than an aqueous solution. The partition coefficient (P) is defined as the equilibrium distribution of a neutral drug in a biphasic environment consisting of a hydrophobic and hydrophilic solvent. Typically, n-octanol and water are used as the hydrophobic and hydrophilic solvents, respectively (n-octanol structurally and chemically resembles the phospholipids present in lipid membranes). The partition coefficient is normally expressed in logarithmic terms, as shown below:

$$ \text{Log}(P) = \text{Log} \left( \frac{\text{[Drug]}_{\text{octanol}}}{\text{[Drug]}_{\text{water}}} \right) $$

Therefore, a partition coefficient of 0 would indicate that the compound is present in equal concentrations in both octanol and water, and a partition coefficient of 3 would indicate that the concentration of the compound in the octanol phase is 1,000 times great than in the aqueous phase.
The determination of the partition coefficient in the drug development process is crucial, as lipophilicity affects other ADME properties. Fortunately, a significant amount of effort has been put forth in the development of accurate in silico methods of calculating the partition coefficient, enabling medicinal chemists to rapidly assess the lipophilicity of drugs in early developmental stages. The calculated partition coefficient (cLog(P)) can be reliably determined thanks to the vast amount of experimentally determined P values. Thus, the cLog(P) for is typically predicted using a fragment-based approach, in which individual fragments of the compound are compared to experimentally determined P values to generate an final value for the overall structure.

The partition coefficient can also be determined experimentally, which is most commonly performed using the shake-flask method. A known amount of compound is added to a flask containing equal volumes of 1-octanol and water, which is agitated on a mechanical shaker for 24-72 h. The concentrations of the drug in each layer are determined using the Beer-Lampert equation or HPLC to determine the Log(P).

However, many drug candidates contain ionizable groups. Therefore, which themselves are in equilibrium between their ionized and neutral forms. Analogous to the partition coefficient (P), the distribution coefficient (D) is the tendency for an ionizable compound to exist in the hydrophilic or aqueous phase at a specified pH. This differs from P because a portion of the compound may be in the ionized form and a portion of the compound may be in the neutral form, and each will have different solubilizing tendencies. The distribution coefficient is expressed in logarithmic terms, as shown below:
Log(D)_{pH} = \log \left( \frac{[\text{Drug}]{_{1\text{-octanol}}}}{[\text{Drug}]_{\text{water}}} \right)

Unlike Log(P), Log(D) depends on the amount ionized compound as a result of the pK_a of the compound and the pH of the solution. Generally, the Log(D) of bases increases with increasing pH while the Log(D) of acids decreases with increasing pH. An overview of the Log(D) values of various FDA-approved drugs is given in Table 12.93

**Determination of cLogP of biologically active CADA analogs**

A host of programs for predicting cLogP of novel compounds are available, both commercially and for academic purposes. Calculating cLog(P) typically uses a fragment based approach, in which individual structural features of a novel structure are compared to known Log(P) values to generate a predicted Log(P) for the overall structure. The cLogP for a vast number of neutral CADA analogs were calculated and are shown in Tables 13-15. Notably, these values were calculated for the free base forms of CADA analogs. cLog(P) values were obtained using three commonly available software suites that were free of charge, which are shown below:

<table>
<thead>
<tr>
<th>Software Suite</th>
<th>Method</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(MI)</strong>: cLogP obtained using Molinspiration calculator</td>
<td>Available for free via: <a href="http://www.molinspiration.com/services/logp.html">http://www.molinspiration.com/services/logp.html</a></td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Trade Name</td>
<td>Use(s)</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>L-3,4-dihydroxy-phenylalanine</td>
<td>L-DOPA</td>
<td>Parkinson’s disease &amp; dopamine-responsive dystonia</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Aleve</td>
<td>Anti-inflammatory agent</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Nexterone</td>
<td>Antiarrhythmic agent</td>
</tr>
<tr>
<td>Desipramine</td>
<td>Norpramin</td>
<td>Antidepressant</td>
</tr>
<tr>
<td>Sulfamerazine</td>
<td>Sulfamerazine</td>
<td>Antibacterial agent</td>
</tr>
</tbody>
</table>

Table 12. Calculated Log(D) values for various FDA-approved drugs.\(^9^3\)
<table>
<thead>
<tr>
<th>ID</th>
<th>cLogP (CD)</th>
<th>cLogP (MI)</th>
<th>cLogP (ALOGPS)</th>
<th>Average cLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>5.38</td>
<td>5.50</td>
<td>3.57</td>
<td>4.82</td>
</tr>
<tr>
<td>VGD019</td>
<td>4.80</td>
<td>6.00</td>
<td>3.96</td>
<td>4.92</td>
</tr>
<tr>
<td>VGD020</td>
<td>5.01</td>
<td>6.00</td>
<td>4.03</td>
<td>5.01</td>
</tr>
<tr>
<td>VGD039</td>
<td>5.93</td>
<td>5.85</td>
<td>4.04</td>
<td>5.27</td>
</tr>
<tr>
<td>VGD040</td>
<td>3.69</td>
<td>4.44</td>
<td>3.05</td>
<td>3.73</td>
</tr>
<tr>
<td>CK147</td>
<td>5.42</td>
<td>6.04</td>
<td>4.29</td>
<td>5.25</td>
</tr>
<tr>
<td>CK207</td>
<td>5.42</td>
<td>6.02</td>
<td>4.26</td>
<td>5.23</td>
</tr>
<tr>
<td>TL020</td>
<td>4.81</td>
<td>5.65</td>
<td>4.06</td>
<td>4.84</td>
</tr>
<tr>
<td>AR001</td>
<td>5.58</td>
<td>6.38</td>
<td>4.53</td>
<td>5.50</td>
</tr>
</tbody>
</table>

Table 13. Summary of cLogP for various biologically active CADA analogs
<table>
<thead>
<tr>
<th>ID</th>
<th>cLogP (CD)</th>
<th>cLogP (MI)</th>
<th>cLogP (ALOGPS)</th>
<th>Average cLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ010</td>
<td>4.49</td>
<td>5.37</td>
<td>3.76</td>
<td>4.54</td>
</tr>
<tr>
<td>DJ011</td>
<td>4.86</td>
<td>5.89</td>
<td>3.89</td>
<td>4.88</td>
</tr>
<tr>
<td>DJ012</td>
<td>4.34</td>
<td>5.16</td>
<td>3.46</td>
<td>4.32</td>
</tr>
<tr>
<td>DJ013</td>
<td>N/A</td>
<td>5.68</td>
<td>4.01</td>
<td>4.85</td>
</tr>
<tr>
<td>DJ014</td>
<td>N/A</td>
<td>4.96</td>
<td>3.49</td>
<td>4.23</td>
</tr>
<tr>
<td>DJ015</td>
<td>N/A</td>
<td>5.48</td>
<td>4.21</td>
<td>4.85</td>
</tr>
<tr>
<td>DJ016</td>
<td>N/A</td>
<td>4.75</td>
<td>3.63</td>
<td>4.19</td>
</tr>
<tr>
<td>DJ017</td>
<td>N/A</td>
<td>5.28</td>
<td>4.4</td>
<td>4.84</td>
</tr>
<tr>
<td>DJ018</td>
<td>N/A</td>
<td>4.55</td>
<td>3.76</td>
<td>4.16</td>
</tr>
<tr>
<td>DJ019</td>
<td>N/A</td>
<td>5.07</td>
<td>4.37</td>
<td>4.72</td>
</tr>
<tr>
<td>DJ020</td>
<td>N/A</td>
<td>4.35</td>
<td>3.83</td>
<td>4.09</td>
</tr>
<tr>
<td>DJ021</td>
<td>N/A</td>
<td>4.87</td>
<td>4.33</td>
<td>4.60</td>
</tr>
</tbody>
</table>

**Table 14.** Summary of cLogP for CADA and various PEGylated CADA analogs.
<table>
<thead>
<tr>
<th>ID</th>
<th>cLogP (CD)</th>
<th>cLogP (MI)</th>
<th>cLogP ( ALOGPS)</th>
<th>Average cLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL001</td>
<td>5.32</td>
<td>5.59</td>
<td>3.18</td>
<td>4.70</td>
</tr>
<tr>
<td>LAL005</td>
<td>6.54</td>
<td>6.84</td>
<td>4.06</td>
<td>5.81</td>
</tr>
<tr>
<td>LAL014</td>
<td>6.51</td>
<td>6.93</td>
<td>4.37</td>
<td>5.94</td>
</tr>
<tr>
<td>LAL016</td>
<td>5.05</td>
<td>5.57</td>
<td>3.43</td>
<td>4.68</td>
</tr>
<tr>
<td>LAL018</td>
<td>6.44</td>
<td>6.79</td>
<td>4.31</td>
<td>5.85</td>
</tr>
<tr>
<td>LAL020</td>
<td>4.71</td>
<td>5.20</td>
<td>3.19</td>
<td>4.37</td>
</tr>
<tr>
<td>LAL022</td>
<td>6.57</td>
<td>7.00</td>
<td>4.08</td>
<td>5.88</td>
</tr>
<tr>
<td>LAL024</td>
<td>6.49</td>
<td>6.44</td>
<td>3.78</td>
<td>5.57</td>
</tr>
<tr>
<td>LAL026</td>
<td>6.16</td>
<td>6.44</td>
<td>3.71</td>
<td>5.44</td>
</tr>
<tr>
<td>LAL028</td>
<td>5.74</td>
<td>6.05</td>
<td>3.45</td>
<td>5.08</td>
</tr>
<tr>
<td>LAL030</td>
<td>4.44</td>
<td>4.66</td>
<td>2.84</td>
<td>3.98</td>
</tr>
<tr>
<td>LAL032</td>
<td>6.62</td>
<td>6.30</td>
<td>3.93</td>
<td>5.62</td>
</tr>
<tr>
<td>LAL036</td>
<td>6.68</td>
<td>7.48</td>
<td>4.77</td>
<td>6.31</td>
</tr>
<tr>
<td>LAL039</td>
<td>6.21</td>
<td>6.26</td>
<td>3.84</td>
<td>5.44</td>
</tr>
<tr>
<td>LAL042</td>
<td>5.33</td>
<td>5.36</td>
<td>3.77</td>
<td>4.82</td>
</tr>
</tbody>
</table>

Table 15. Summary of cLogP for various LAL compounds containing pyridine moieties
Determination of Log(D)$_{7.4}$ of biologically active CADA analogs using the shake-flask method

Since CADA and CADA analogs are almost exclusively tested in biological assays as the corresponding HCl salt, the experimental determination of the distribution coefficient (D) is a more accurate analysis of lipophilicity than the partition coefficient (P). Each analog was assayed as the HCl salt by dissolving the compound in equal parts 1-octanol and PBS buffer ($pH = 7.4$) (Figure 65). The concentration of the drug in the aqueous phase after 72 hours of agitation on a mechanical shaker was analyzed using UV-Vis spectroscopy and the Beer-Lampert equation using the molar absorptivities listed in Table 16.
<table>
<thead>
<tr>
<th>Compound</th>
<th>cLog(P)</th>
<th>Log(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA HCl</td>
<td>4.82</td>
<td>2.1</td>
</tr>
<tr>
<td>VGD019 HCl</td>
<td>4.92</td>
<td>1.3</td>
</tr>
<tr>
<td>VGD020 HCl</td>
<td>5.01</td>
<td>2.4</td>
</tr>
<tr>
<td>VGD040 HCl</td>
<td>3.73</td>
<td>1.8</td>
</tr>
<tr>
<td>CK147 HCl</td>
<td>5.25</td>
<td>0.28</td>
</tr>
<tr>
<td>CK207 2HCl</td>
<td>5.23</td>
<td>-0.36</td>
</tr>
<tr>
<td>DJ010 HCl</td>
<td>4.54</td>
<td>1.03</td>
</tr>
<tr>
<td>DJ011 HCl</td>
<td>4.88</td>
<td>2.56</td>
</tr>
<tr>
<td>DJ012 HCl</td>
<td>4.32</td>
<td>0.39</td>
</tr>
<tr>
<td>DJ013 HCl</td>
<td>4.85</td>
<td>1.48</td>
</tr>
<tr>
<td>DJ014 HCl</td>
<td>4.23</td>
<td>1.40</td>
</tr>
<tr>
<td>DJ015 HCl</td>
<td>4.85</td>
<td>1.50</td>
</tr>
<tr>
<td>DJ016 HCl</td>
<td>4.19</td>
<td>0.42</td>
</tr>
<tr>
<td>DJ017 HCl</td>
<td>4.84</td>
<td>n/d</td>
</tr>
<tr>
<td>LAL014 HCl</td>
<td>5.94</td>
<td>0.48</td>
</tr>
<tr>
<td>TL020 HCl</td>
<td>4.84</td>
<td>2.37</td>
</tr>
<tr>
<td>AR001 HCl</td>
<td>5.50</td>
<td>0.84</td>
</tr>
</tbody>
</table>

**Table 16.** A comparison of cLog(P) and Log(D) values for various biologically active CADA analogs.
Analyzing cell permeability using the Caco-2 cell permeability assay

A majority of drug absorption of an orally administered drug takes place in the small intestine, where a single layer of simple columnar epithelial cells located on the luminal surface function to absorb nutrients into the bloodstream for distribution throughout the body. For an orally administrated drug, this cell layer is the final barrier that a drug encounters in the GI tract. The general structure of the intestinal epithelium is shown in Figure 66.100,101

![Diagram of Columnar Intestinal Epithelium Cells and Drug Transport Modes](image)

**Figure 66.** The general structural features of columnar intestinal epithelium cells and the modes of drug transport across the monolayer.100,101

The apical membrane resides on the lumen of the small intestine and is lined with microvilli, which further increase the surface area of the small intestine surface to enhance the absorption of nutrients. Alternatively, the basolateral membrane is attached to the basement membrane, which is rich in veins and arteries that absorb molecules that effectively traverse the monolayer (transcellular transport). The epithelial cells are columnar in shape with tight junctions that bring adjacent epithelial cells close together to
prevent the passage of molecules or ions across the monolayer by passing between individual cells (paracellular transport).

Cell permeability is an important pharmacokinetic property to consider in the drug development process. Fortunately, Caco-2 cells have emerged as a convenient *in vitro* method of predicting cell permeability of orally administered drugs. Originally derived from human epithelial colorectal adenocarcinoma cells, Caco-2 cells can be cultured to morphologically and physiologically mimic the epithelial cells of the small intestine, displaying microvilli and various transport proteins on the apical membrane.\(^9_3,10_0,10_1^\)

The general experimental set-up of a Caco-2 cell permeability assay is shown in Figure 67. Caco-2 cells are cultured on a plastic insert containing a semi-permeable membrane, which is placed in a well containing cell culture medium to create two compartments that are separated by a monolayer of Caco-2 cells. The apical compartment mimics the small intestine lumen while the basolateral compartment represents the

![Figure 67](image-url)
basement membrane (and presumably access to blood vessels), thereby creating an in vitro environment that accurately mimics the molecular structures of intestinal epithelium cells in vivo.\textsuperscript{100,101}

Cell permeability is calculated by administering the compound to one of the compartments and analyzing the concentration of the compound in aliquots taken from the opposite compartment at incremental time points. Apparent permeability ($P_{\text{app}}$) is calculated according to the following equation:

$$P_{\text{app}} = \frac{(dQ/dt)}{(C_0 \times A)}$$

where $dQ/dt$ is equal to the concentration of the compound in one compartment as a function of time, $C_0$ is the initial concentration of the compound in the donor compartment, and $A$ is the area of the filter on which the monolayer is cultured. General permeability ranges and their significance is shown in Figure 68.\textsuperscript{93,101}

- **Low permeability**: $P_{\text{app}} < 2 \times 10^6$ cm/s
- **Moderate permeability**: $2 \times 10^6$ cm/s < $P_{\text{app}} < 20 \times 10^6$ cm/s
- **High permeability**: $P_{\text{app}} > 20 \times 10^6$ cm/s

**Figure 68.** Apparent permeability ranges determined by the Caco-2.\textsuperscript{93,101}
The apparent permeability is studied in both directions; that is, the movement of the compound from the apical to basolateral compartment and vice versa. If the $P_{\text{app}}$ from the apical to basolateral compartment is the same as it is from the basolateral to apical compartment, the drug is likely crossing the membrane via passive transcellular transport. However, if the $P_{\text{app}}$ differs, the drug is likely being transported across the membrane via protein channels and/or transport (facilitated transport). Therefore, Caco-2 cell permeability assays also gain insight on the mode of transport across the epithelial membrane.

**Metabolism & in vitro microsomal assay for metabolic stability**

Metabolism collectively entails a set of chemical reactions that take place within the body in order to convert food into usable energy that fuels cellular/biological processes. These chemical reactions are split into two main categories: catabolic and anabolic, where the former consists of chemical degradation of molecules and the latter consists of chemical reactions that produce new biomolecules.\(^\text{93,102}\)

An orally administered drug will almost immediately face a series of metabolic reactions which challenges its chemical stability. The pancreas secretes a variety of enzymes into the stomach and small intestine that aid in the chemical degradation of proteins into amino acids and a number of hydrolysis reactions. Furthermore, a drug that survives the chemical environment of the GI tract is susceptible to further metabolic reactions in the blood stream, such as hydrolysis, and to a host of metabolic reactions in the liver and kidney; the primary organs where metabolism takes place.
Blood flows into the liver through the portal vein, which branches into consecutively smaller veins that eventually come into contact with hepatocytes, which are specialized cells that permeate throughout the liver and contain a host of metabolic enzymes. There are two major modes of metabolism that occur in the liver: phase I metabolism consists of enzymatic chemical modification of the drug (such as a variety of oxidative modifications by cytochrome P450 enzymes), and phase II metabolism involves the addition of polar chemical functionalities to the drug, such as phosphorylation.

Thus, a successful drug must have a chemical structure that results in a specific biological response while being chemically stable to a variety of chemical degradation reactions. An in vitro method of analyzing metabolic stability utilizing liver microsomes has found appreciable use in the drug development process. A solution of the drug in dimethylsulfoxide (DMSO) and liver microsomes is incubated at 37 °C with a buffered NADPH regenerating system. Liver microsomes contain the basic metabolic enzymes found in the liver, while the NADPH system provides the electrons needed for the oxidative processes of various enzymatic reactions. Aliquots are taken at incremental time points and quenched using cold acetonitrile, which inactivates the enzymes. The concentration of the drug is analyzed by HPLC or LC/MS/MS to calculate a half-life for the drug.

The metabolic stability of CADA and CADA analogs DJ010 and CK147 were studied using the previously described method and are currently awaiting analysis by HPLC to determine the metabolic half-life for these substrates.
Establishing pharmacokinetic profiles of various biologically active CADA analogs

Until recently, research focused on analyzing the pharmacokinetics of various CADA analogs has been limited; likely a result of a more pressing focus on SAR experiments and understanding CADA’s unique mechanism of action. As the lead compound in this research, the pharmacokinetics of CADA were of interest for comparison to CADA analogs synthesized with hypothesized enhanced drug-like properties. Several pharmacokinetic parameters of CADA are given in Figure 69.

![Chemical structure of CADA](image)

<table>
<thead>
<tr>
<th>ID</th>
<th>Kinetic Solubility (µM)</th>
<th>Thermodynamic Solubility (µM)</th>
<th>Log(D)₇.₄</th>
<th>cLog(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>27</td>
<td>8.3</td>
<td>2.1</td>
<td>4.82</td>
</tr>
</tbody>
</table>

**Figure 69.** A summary of the pharmacokinetic properties of CADA.
Pharmacokinetic profile of DJ CADA analogs

The covalent incorporation of polyethylene- and methoxypolyethylene-glycol (PEGylation and mPEGylation, respectively) has been shown to increase the metabolic stability and solubility of small molecules and biological macromolecules. A series of mPEGylated and PEGylated CADA analogs were synthesized with the intention of producing potent CADA analogs with enhanced biological activity. mPEGylated and PEGylated CADA analogs were analyzed utilizing a variety of previously described pharmacokinetic assays, a summarized in Table 17.

mPEGylated and PEGylated CADA analogs (Figure 70) exhibit exceptionally better kinetic and thermodynamic solubility compared to CADA. In general, analogs with a terminal hydroxyl group are more soluble than the corresponding analog with a terminal methoxy group. Additionally, mPEGylated analogs exhibit higher Log(D) values than PEGylated CADA analogs. Taken together with the observed hCD4 and sortilin down-modulation potencies of mPEGylated and PEGylated CADA analogs, the results shown in Table 18 suggest that these analogs may prove useful for in vivo studies in the future.
Figure 70. Structures of various PEGylated and mPEGylated CADA analogs.
<table>
<thead>
<tr>
<th>ID*</th>
<th>MW*</th>
<th>Kinetic Solubility (µM)</th>
<th>Thermodynamic Solubility (µM)</th>
<th>cLog(P)</th>
<th>Log(D) 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>618.25</td>
<td>27</td>
<td>8.3</td>
<td>4.82</td>
<td>2.1</td>
</tr>
<tr>
<td>DJ010</td>
<td>670.32</td>
<td>194</td>
<td>120</td>
<td>4.33</td>
<td>1.03</td>
</tr>
<tr>
<td>DJ011</td>
<td>684.35</td>
<td>34</td>
<td>20</td>
<td>4.56</td>
<td>2.56</td>
</tr>
<tr>
<td>DJ012</td>
<td>714.37</td>
<td>&gt; 500</td>
<td>340</td>
<td>4.21</td>
<td>0.39</td>
</tr>
<tr>
<td>DJ013</td>
<td>728.40</td>
<td>183</td>
<td>26.4</td>
<td>4.68</td>
<td>1.48</td>
</tr>
<tr>
<td>DJ014</td>
<td>758.43</td>
<td>&gt; 500</td>
<td>407</td>
<td>4.24</td>
<td>1.40</td>
</tr>
<tr>
<td>DJ015</td>
<td>772.45</td>
<td>&gt; 500</td>
<td>4,710</td>
<td>4.83</td>
<td>n/d</td>
</tr>
<tr>
<td>DJ016</td>
<td>802.48</td>
<td>&gt; 500</td>
<td>28,000</td>
<td>4.34</td>
<td>0.42</td>
</tr>
<tr>
<td>DJ017</td>
<td>816.51</td>
<td>-</td>
<td>-</td>
<td>4.95</td>
<td>-</td>
</tr>
</tbody>
</table>

* MW are given as HCl salts

Table 17. An overview of several pharmacokinetic parameters of various PEGylated and mPEGylated CADA analogs
A pharmacokinetic profile of LAL CADA analogs

A series of CADA analogs containing a fused pyridine ring were recently synthesized by Dr. Lumangtad and found to exhibit comparable biological activity to CADA. Since pyridine is miscible with water, these analogs were reasoned to exhibit more favorable drug-like properties compared to CADA. Although Dr. Lumangtad was able to develop feasible synthetic routes to access the analogs shown in Figure 71, their pharmacokinetic properties were never investigated.

Dr. Lumangtad had synthesized an appreciable amount of serveral LAL compounds, which were analyzed using kinetic solubility, thermodynamic solubility, and lipophilicity assays. The results of these experiments are shown in Table 18. In general, CADA analogs containing a fused pyridine exhibit more favorable drug-like properties compared to CADA, as had been previously hypothesized. In particular, LAL020 and LAL024 exhibit the highest kinetic solubility. Although the establishment of a comprehensive pharmacokinetic profile was ultimately hindered due to limited access to synthetic samples, LAL compounds appear to exhibit more favorable drug-like properties compared to CADA and their utility for studying the down-modulation of hCD4 and sortilin in vivo may eventually prove useful.
Figure 71. Structures of various LAL CADA analogs which were analyzed using various pharmacokinetic assays.
<table>
<thead>
<tr>
<th>ID</th>
<th>MW</th>
<th>Kinetic Solubility (µM)</th>
<th>Thermodynamic Solubility (µM)</th>
<th>cLog(P)</th>
<th>Log(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL001</td>
<td>576.17</td>
<td>n/d</td>
<td>n/d</td>
<td>4.18</td>
<td>-</td>
</tr>
<tr>
<td>LAL005</td>
<td>618.25</td>
<td>n/d</td>
<td>n/d</td>
<td>4.81</td>
<td>-</td>
</tr>
<tr>
<td>LAL014</td>
<td>638.24</td>
<td>141</td>
<td>-</td>
<td>5.13</td>
<td>0.48</td>
</tr>
<tr>
<td>LAL016</td>
<td>606.19</td>
<td>-</td>
<td>-</td>
<td>4.30</td>
<td>-</td>
</tr>
<tr>
<td>LAL018</td>
<td>668.26</td>
<td>100</td>
<td>-</td>
<td>5.07</td>
<td>-</td>
</tr>
<tr>
<td>LAL020</td>
<td>578.14</td>
<td>213</td>
<td>4.07</td>
<td>4.12</td>
<td>-</td>
</tr>
<tr>
<td>LAL022</td>
<td>618.25</td>
<td>n/d</td>
<td>n/d</td>
<td>4.85</td>
<td>-</td>
</tr>
<tr>
<td>LAL024</td>
<td>618.25</td>
<td>255</td>
<td>35.4</td>
<td>4.66</td>
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</tr>
<tr>
<td>LAL026</td>
<td>604.22</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>LAL028</td>
<td>590.19</td>
<td>-</td>
<td>-</td>
<td>4.39</td>
<td>-</td>
</tr>
<tr>
<td>ID</td>
<td>MW</td>
<td>Kinetic Solubility (µM)</td>
<td>Thermodynamic Solubility (µM)</td>
<td>cLog(P)</td>
<td>Log(D)</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------------------------</td>
<td>-------------------------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>LAL030</td>
<td>592.17</td>
<td>-</td>
<td>389</td>
<td>4.21</td>
<td>-</td>
</tr>
<tr>
<td>LAL032</td>
<td>642.27</td>
<td>-</td>
<td>3.75</td>
<td>4.71</td>
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</tr>
<tr>
<td>LAL036</td>
<td>674.31</td>
<td>-</td>
<td>-</td>
<td>5.37</td>
<td>-</td>
</tr>
<tr>
<td>LAL039</td>
<td>592.21</td>
<td>n/d</td>
<td>7,508</td>
<td>4.78</td>
<td>-</td>
</tr>
<tr>
<td>LAL042</td>
<td>648.28</td>
<td>n/d</td>
<td>n/d</td>
<td>4.22</td>
<td>n/d</td>
</tr>
</tbody>
</table>

*MW are given as HCl salts

**Table 18.** An overview of several pharmacokinetic parameters of various pyridine fused CADA analogs
A pharmacokinetic profile of VGD CADA analogs

The pharmacokinetics of several unsymmetrical CADA analogs previously synthesized by Dr. Demillo were analyzed using thermodynamic solubility, kinetic solubility, and lipophilicity assays. These analogs were not designed with the intent of obtaining CADA analogs with enhanced drug-like properties; rather, VGD analogs were synthesized in order to identify chemical and physical properties associated with potent hCD4 down-modulation. The structures of these analogs are shown in Figure 72, which includes the highly-potent CADA analog VGD020.

As shown in Table 19, the VGD analogs analyzed did not contain structural features associated with favorable drug solubility. Accordingly, the pharmacokinetic properties of these analogs are not particularly impressive with the exception of VGD040, which likely exists as the diprotic salt and exhibits high kinetic and thermodynamic solubility.
Figure 72. Structures of various VGD CADA analogs tested for their pharmacokinetic parameters.

<table>
<thead>
<tr>
<th>ID</th>
<th>MW</th>
<th>Kinetic Solubility (µM)</th>
<th>Thermodynamic Solubility (µM)</th>
<th>cLog(P)</th>
<th>Log(D)7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>618.25</td>
<td>27</td>
<td>8.3</td>
<td>4.82</td>
<td>2.1</td>
</tr>
<tr>
<td>VGD019</td>
<td>624.86</td>
<td>-</td>
<td>1.8</td>
<td>4.92</td>
<td>1.3</td>
</tr>
<tr>
<td>VGD020</td>
<td>640.30</td>
<td>-</td>
<td>1.142</td>
<td>5.01</td>
<td>2.4</td>
</tr>
<tr>
<td>VGD039</td>
<td>655.27</td>
<td>-</td>
<td>-</td>
<td>5.85</td>
<td>1.7</td>
</tr>
<tr>
<td>VGD040</td>
<td>506.57</td>
<td>&gt; 500</td>
<td>310</td>
<td>3.73</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* MW are given as HCl salts, except for VGD040, which is given as the diprotic HCl salt.

Table 19. An overview of several pharmacokinetic parameters of various unsymmetrical VGD CADA analogs
A pharmacokinetic profile of miscellaneous biologically active CADA analogs

Analogous to the VGD series of CADA analogs, several CADA analogs shown in Figure 73 have recently been synthesized in efforts geared towards obtaining more potent CADA analogs, including the highly potent analogs CK147 and TL020. The pharmacokinetic properties of these analogs are shown in Table 20.

In general, these CADA analogs exhibit enhanced kinetic and thermodynamic solubilities compared to CADA. In particular, CK075 exhibits a kinetic solubility of > 500 µM, which is thought to be the result of its existence as a diprotic HCl salt and is consistent with the observed pharmacokinetic properties of the structurally similar VGD040. Interestingly, the thermodynamic solubility of CK207 is much higher than that of CK147. This observation may be a result of the dimethylamino moiety of CK207 being protonated, whereas the dimethylamino moiety of CK147 is not protonated. This difference in basicity can be rationalized by conjugation with the electron-withdrawing effects of the sulfonamide group experienced to a greater degree by CK147 compared to CK207. Furthermore, CK207 is the only tested CADA analog that was found to have a negative Log(D), which indicates that it is slightly more soluble in an aqueous medium and further suggests that it exists as a deprotonated salt.
Figure 73. Structures of various biologically active CADA analogs analyzed for their pharmacokinetic properties

<table>
<thead>
<tr>
<th>ID</th>
<th>MW*</th>
<th>Kinetic Solubility (µM)</th>
<th>Thermodynamic Solubility (µM)</th>
<th>cLog(P)</th>
<th>Log(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CADA</td>
<td>618.25</td>
<td>27</td>
<td>8.3</td>
<td>4.82</td>
<td>2.1</td>
</tr>
<tr>
<td>CK147</td>
<td>653.34</td>
<td>101</td>
<td>1.7</td>
<td>5.25</td>
<td>0.28</td>
</tr>
<tr>
<td>CK075</td>
<td>570.63</td>
<td>&gt; 500</td>
<td>n/d</td>
<td>4.20</td>
<td>n/d</td>
</tr>
<tr>
<td>CK207</td>
<td>689.80</td>
<td>-</td>
<td>860</td>
<td>5.23</td>
<td>-0.36</td>
</tr>
<tr>
<td>AR001</td>
<td>656.36</td>
<td>-</td>
<td>47</td>
<td>5.50</td>
<td>0.84</td>
</tr>
<tr>
<td>TL020</td>
<td>669.34</td>
<td>-</td>
<td>-</td>
<td>4.84</td>
<td>2.37</td>
</tr>
</tbody>
</table>

*MW given as HCl salts, except for CK075 & CK207, which are given as diprotic HCl salts

Table 20. An overview of several pharmacokinetic parameters of various unsymmetrical CADA analogs
Summary

The pharmacokinetics of approximately 30 CADA analogs were analyzed utilizing a variety of common pharmacokinetic assays, including kinetic solubility, thermodynamic solubility, and lipophilicity assays. Many CADA analogs were found to exhibit significantly enhanced solubility compared to CADA, including many mPEGylated, PEGylated, and pyridine-fused CADA analogs that were synthesized with that very purpose.

CADA has traditionally suffered from poor pharmacokinetic properties, which has hindered the ability to study its unique mechanism of action and therapeutic potential in \textit{in vivo} models. This research aimed to establish a pharmacokinetic lab that could routinely assess the drug-like properties of potent CADA analogs. A new lab capable of analyzing drug solubility, cell permeability, and metabolic stability has been constructed and will certainly find extensive use in future research endeavors.
Experimental

General experimental procedure for kinetic solubility experiments

The following procedure\textsuperscript{93,97} was used to analyze the kinetic solubility of each CADA analog tested in this research. Serial dilutions of CADA analogs with starting concentrations of 500 µM and 400 µM in 2.5% DMSO with a final volume of 100 µL were prepared in duplicate on sterile, 96-well microtiter plates, as shown below. Plates were shaken for 5 minutes and incubated for 1 h at 37°C before being analyzed using a nephelometer.

![Diagram of 96-well microtiter plate]

Preparing 500 µM solutions for serial dilution

Using a micropipette, 100 µL of a 2.5% DMSO in PBS buffer (pH = 7.5) solution was added into wells (A-B)1-11. Into wells (A-B)12 was added 195 µL of PBS buffer (pH = 7.5). 5 µL of a 20 mM stock solution (in DMSO) for each CADA analog tested was added to wells (A-B)12 and the solution was mixed by pipetting 100 µL up and down five times before removing 100 µL and transferring it to the adjacent well in column (A-B)11, which
was mixed by pipetting 100 µL up and down three times before removing 100 µL and again transferring it to well (A-B)10, and so on and so forth until (A-B)3, after which 100 µL was removed and placed in the appropriate waste container. (A-B)1-2 served as blanks, containing only 100 µL of a 2.5% DMSO in PBS buffer (pH = 7.5) solution. Therefore, serial dilutions were performed from 500 to 0.98 µM, with each well containing a final volume of 100 µL.

**Preparing 400 µM solutions for serial dilution**

Using a micropipette, 100 µL of a 2.5% DMSO in PBS buffer (pH = 7.5) solution was added into wells (C-D)1-11. Into wells (C-D)12 was added 40 µL of PBS buffer (pH = 7.5). In order to keep the DMSO concentration constant, 0.4 mL of a 500 µM solution of each CADA analog was prepared by adding 290 µL of PBS buffer (pH = 7.5) and 10 µL of a 20 mM stock solution (in DMSO). The 500 µM solution was vortexed and 160 µL was immediately added to wells (C-D)12 and mixed by pipetting 100 µL up and down five times before removing 100 µL and transferring it to the adjacent well in column (C-D)11, which was mixed by pipetting 100 µL up and down three times before removing 100 µL and again transferring it to well (C-D)10, and so on and so forth until (C-D)3, after which 100 µL was removed and placed in the appropriate waste container. (C-D)1-2 served as blanks, containing only 100 µL of a 2.5% DMSO in PBS buffer (pH = 7.5) solution. Therefore, serial dilutions were performed from 0.39 to 0.98 µM, with each well containing a final volume of 100 µL.
Analyzing microtiter plates using nephelometry

Prior to use, the nephelometer was allowed to warm up for at least 0.5 h. Each plate was placed in the Nepheloskan Ascent nephelometer with the lid off, shaken for 5 min, and incubated for 1 h at 37°C before measuring each well. The raw data was analyzed using Microsoft Excel. The raw data was adjusted by subtracting the blank value in each row from the measurements of each well in the same row.

General experimental procedure for thermodynamic solubility experiments

Thermodynamic solubility was analyzed using the traditional shake-flask method. For each CADA analog tested in this assay, a small amount (3-5 mg) of the analog was added to a glass vial containing 5 mL of PBS buffer (pH = 7.5) and agitated on a mechanical shaker for 72 h. In order to insure the solutions remained saturated, the solutions were visually inspected at 24 and 48 h to ensure some amount of the CADA analog remained insoluble. After 72 h, the solutions were filtered using a PTFE syringe filter (0.45 μL) and the filtrate was analyzed using UV-Vis spectroscopy in conjunction with the Beer-Lambert law to calculate the concentration of each CADA analog.

General experimental procedure for Log(D)7.4 experiments

The distribution coefficient (D) was determined in the following manner: first, a known concentration of each CADA analog in 1-octanol was prepared (typically 0.1 mM). An aliquot of the 1-octanol solution was placed in a vial containing an equal amount of phosphate buffered saline (PBS, pH = 7.4) and the vial was agitated with a mechanical shaker for 48 h.
After 48 h on the shaker, the biphasic solution was allowed to settle for an hour before being transferred to a separatory funnel. The aqueous and organic layers were separated; the former was analyzed using UV-Vis spectroscopy to determine the unknown concentration of the CADA analog using the Beer-Lampert law, analogous to the previously described thermodynamic solubility experiments. Assuming the difference between the starting concentration and the concentration found in the aqueous layer was equal to the concentration in the 1-octanol layer, the partition coefficient was determined taking the log of the ratio of the concentration in each layer, as shown below:

$$\text{Log}(D)_{7.4} = \frac{[\text{CADA analog}]_{1\text{-octanol}}}{[\text{CADA analog}]_{\text{PBS}}}$$

**General experimental procedure for determining standard curves for various CADA analogs by HPLC**

Each curve was generated by analyzing the area of various CADA analogs at five different known concentrations. Each analog required its own unique HPLC method and each concentration was tested in triplicate. The average area of each concentration was used to generate the standard curve. The data was fit with a linear regression line with the stipulation of an $R^2$ value of less than 0.95 was to be considered erroneous data.

In general, it seems that solutions in the 0.5-2.0 mM range gave consistent results; concentrations of less than 0.5 mM have been somewhat inconsistent. These standard curves will be used to analyze the unknown concentrations of various CADA analogs in future pharmacokinetic assays, such as thermodynamic solubility and metabolic stability.
General experimental procedure for in vitro assays for analyzing metabolic stability

The metabolic stability of CADA HCl, **DJ010 HCl**, and **CK147 HCl** was analyzed according to the procedure reported by Pogorzelska, *et al.* 0.5 mL of 100 µM solutions in PBS buffer (pH = 7.4) were prepared for each compound from 20 mM stock solutions (DMSO). Each compound was tested in triplicate at 6 time points (5, 10, 15, 30, 45, and 60 minutes) on a microtiter plate; 18 wells were prepared for each compound. Into each well was added 180 µL PBS (pH = 7.4) buffer, 5 µL of microsomes (human microsomes pooled from 50 donors; ThermoFisher Scientific catalog #: HMMCPL), and 10 µL NADPH regenerating system (Promega catalog #: V9510). The microtiter plate was incubated at 37 °C for 10 minutes before 2 µL of test compound was added. 200 µL of ice-cold acetonitrile was added to the wells at the previously mentioned time points to quench metabolic activity. The contents of the well were centrifuged at 7500 g for 10 minutes and the supernatant was removed for analysis by HPLC.
Conclusions

The primary goal of this research has been to advance the understanding of the feasibility of targeting signal peptides as a means of modulating protein expression. Research efforts towards understanding CADA’s mechanism of action may allow scientists to design small molecules to target signal peptides, thereby modulating protein expression with extremely high specificity.

Photoaffinity labeling (PAL) has been shown to be useful in identifying a number of ligand-binding site interactions (specific and otherwise) and, despite the results of this research, may still be a promising avenue towards understanding interactions between CADA and the hCD4 or sortilin signal peptides. The immediate problem that needs to be addressed is designing a biological assay to perform PAL experiments using the aryl azide RA018, which has been demonstrated to photolyze and down-modulate hCD4 expression with a similar potency to CADA. Cotranslational translocation is often difficult to study because it is a transient process, which makes designing a PAL assay particularly difficult in this research. It may be valuable to pursue an aryl azide analog with a fluorophore or the ability to bind to a fluorophore post-photolysis.

As was addressed in this research, the design of CADA analogs with a diazirines functionality will likely be the ideal candidate for PAL experiments, especially given the tendency for aryl nitrenes to undergo rearrangements. The synthesis of the diazirine CADA analog DJ009 using traditional methods of synthesizing diazirines from ketones ultimately proved unsuccessful for several reasons. First, the 9-step synthesis was entirely linear with the bottle-neck occurring approximately half-way through the synthetic pathway, making
obtaining appreciable amounts of late-stage intermediates particularly difficult. Indeed, the oxidation of the diaziridine DJ008 to DJ009 may have occurred and produced a small amount of diazirines, but not an appreciable amount that could characterized and analyzed for biological activity. However, a number of other methods for synthesizing diazirines (Scheme 11) may be useful if they could avoid these shortcomings, and commercial diazirines are becoming increasingly available. It seems the latter would be the ideal approach to incorporating a diazirines functionality onto a CADA analog.

The design of CADA analogs with favorable drug-like properties and potency has become increasingly more feasible in recent years; PEGylated and mPEGylated CADA analogs were hypothesized to be favorable candidates for these qualities. Indeed, they exhibited comparable (or more potent) biological activity than CADA. Furthermore, PEGylated and mPEGylated CADA analogs displayed higher aqueous solubility than CADA and may be useful for in vivo experiments. Furthermore, this research suggests that a PEG or mPEG group may be incorporated on to potent analogs such as CK147, as shown in Figure S1.

Studying the pharmacokinetics of various CADA analogs was a frustratingly awarding experience, requiring the construction of a new laboratory, collaborating with other scientists, and continuously learning from mistakes. However, the long-term value of setting up a pharmacokinetic lab will only grow, enabling the rapid in-house identification of CADA analogs with favorable drug-like properties and allowing collaborators working

![Figure 74. A PEGylated or mPEGylated CADA may exhibit potent biological activity and enhanced drug-like properties.](image)
with CADA analogs *in vivo* to have a better understanding of how the analogs behave in a biological system.
References


Current Drug Metabolism 2008, 9, 879-885.


(100) Van Breemen, R. B.; Li, Y. Caco-2 cell permeability assays to measure drug absorption. Expert Opin Drug Metab Toxicol. 2005, 2, 175-185.


Appendix A: $^1$H NMR & $^{13}$C NMR spectra of DJ CADA analogs

Figure S1. $^1$H- and $^{13}$C-NMR spectra of DJ001
Figure S2. $^1$H- and $^{13}$C-NMR spectra of DJ002
Figure S3. $^1$H- and $^{13}$C-NMR spectra of DJ003
Figure S4. $^1$H- and $^{13}$C-NMR spectra of DJ004
Figure S5. $^1$H- and $^{13}$C-NMR spectra of DJ005
Figure S6. $^1$H- and $^{13}$C-NMR spectra of DJ006
Figure S7. $^1$H- and $^{13}$C-NMR spectra of DJ007
Figure S8. $^1$H- spectrum of DJ008
Figure S9. $^1$H- and $^{13}$C-NMR spectra of DJ010
Figure S10. $^1$H- and $^{13}$C-NMR spectra of DJ011
Figure S11. $^1$H- and $^{13}$C-NMR spectra of DJ012
Figure S12. $^1$H- and $^{13}$C-NMR spectra of DJ013
Figure S13. $^1$H- and $^{13}$C-NMR spectra of DJ014
Figure S14. $^1$H- and $^{13}$C-NMR spectra of DJ015
Figure S15. $^1$H- and $^{13}$C-NMR spectra of DJ016
Appendix B: Molar absorptivities of various CADA analogs

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>A</th>
<th>$\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
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<td>0.24608</td>
<td>24608</td>
</tr>
<tr>
<td>20</td>
<td>234.45</td>
<td>0.49522</td>
<td>24761</td>
</tr>
<tr>
<td>30</td>
<td>233.9</td>
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</table>

Average $\varepsilon$  
37317

Figure S16. Molar absorptivity of DJ010

<table>
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<th>Concentration (µM)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>A</th>
<th>$\varepsilon$</th>
</tr>
</thead>
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<tr>
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<td>238.12</td>
<td>1.014</td>
<td>202800</td>
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<td>7.5</td>
<td>238.12</td>
<td>1.55</td>
<td>206667</td>
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</table>

Average $\varepsilon$  
214622

Figure S17. Molar absorptivity of DJ011
<table>
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<th>Concentration (µM)</th>
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<th>A</th>
<th>$\varepsilon$</th>
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<tbody>
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<td>10</td>
<td>236.9</td>
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<tr>
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**Figure S18.** Molar absorptivity of DJ012

<table>
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<th>$\lambda_{\text{max}}$ (nm)</th>
<th>A</th>
<th>$\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
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<td>111600</td>
</tr>
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<td>236.2</td>
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<tr>
<td>7.5</td>
<td>235.64</td>
<td>1</td>
<td>133333</td>
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</table>

**Average $\varepsilon$**

25253

**Figure S19.** Molar absorptivity of DJ013
<table>
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<th>Concentration (µM)</th>
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<th>ε</th>
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</thead>
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<td>235.64</td>
<td>0.693</td>
<td>277200</td>
</tr>
<tr>
<td>5</td>
<td>236.57</td>
<td>1.321</td>
<td>264200</td>
</tr>
<tr>
<td>7.5</td>
<td>237.19</td>
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<td>212400</td>
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</table>

Average ε

251267

**Figure S20.** Molar absorptivity of DJ015

<table>
<thead>
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<th>ε</th>
</tr>
</thead>
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<td>0.25255</td>
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</tr>
<tr>
<td>20</td>
<td>236.85</td>
<td>0.54156</td>
<td>27078</td>
</tr>
<tr>
<td>30</td>
<td>240.2</td>
<td>0.78532</td>
<td>26177</td>
</tr>
</tbody>
</table>

Average ε

26170

**Figure S21.** Molar absorptivity of DJ016
Concentration (µM) | \( \lambda_{\text{max}} \) (nm) | \( A \) | \( \varepsilon \)  
--- | --- | --- | ---  
10  | 229.811 | 0.346 | 34600  
20  | 230.284 | 0.740 | 37000  
30  | 229.811 | 1.050 | 35000  

**Average \( \varepsilon \)**  
35533  

**Figure S22.** Molar absorptivity of LAL005

\[ y = 0.0352x + 0.008 \]  
\[ R^2 = 0.9953 \]  

\[
\text{LAL05 HCl in MeOH}
\]

Concentration (µM) | \( \lambda_{\text{max}} \) (nm) | \( A \) | \( \varepsilon \)  
--- | --- | --- | ---  
10  | 227.4 | 0.35175 | 35175  
20  | 231.45 | 0.74083 | 37041  
30  | 234.75 | 1.09372 | 36457  

**Average \( \varepsilon \)**  
36224  

**Figure S23.** Molar absorptivity of LAL014

\[ y = 0.0371x - 0.0132 \]  
\[ R^2 = 0.9992 \]  

\[
\text{LAL014 HCl in MeOH}
\]
Concentration (µM) | λ_{max} (nm) | A | ε
---|---|---|---
10 | 228.864 | 0.403 | 40300
20 | 229.338 | 0.733 | 36650
30 | 229.811 | 1.05 | 35000

**Average ε**

37317

**Figure S24.** Molar absorptivity of LAL018

Concentration (µM) | λ_{max} (nm) | A | ε
---|---|---|---
10 | 228.932 | 0.275 | 27500
20 | 228.487 | 0.572 | 28600
30 | 230.712 | 0.934 | 31133

**Average ε**

29078

**Figure S25.** Molar absorptivity of LAL020
**Figure S26.** Molar absorptivity of LAL024

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>A</th>
<th>(\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>229.646</td>
<td>0.307</td>
<td>30700</td>
</tr>
<tr>
<td>20</td>
<td>227.434</td>
<td>0.611</td>
<td>30550</td>
</tr>
<tr>
<td>30</td>
<td>229.204</td>
<td>0.9</td>
<td>30000</td>
</tr>
</tbody>
</table>

Average \(\varepsilon\)  

**Figure S27.** Molar absorptivity of LAL026

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>A</th>
<th>(\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>229.338</td>
<td>0.373</td>
<td>37300</td>
</tr>
<tr>
<td>20</td>
<td>229.338</td>
<td>0.685</td>
<td>34250</td>
</tr>
<tr>
<td>30</td>
<td>230.712</td>
<td>1.053</td>
<td>35100</td>
</tr>
</tbody>
</table>

Average \(\varepsilon\)
<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ_{max} (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>228.042</td>
<td>0.438</td>
<td>43800</td>
</tr>
<tr>
<td>20</td>
<td>229.882</td>
<td>0.727</td>
<td>36350</td>
</tr>
<tr>
<td>30</td>
<td>228.042</td>
<td>0.981</td>
<td>32700</td>
</tr>
</tbody>
</table>

**Average ε**

37617

**Figure S28.** Molar absorptivity of LAL030

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ_{max} (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>254.425</td>
<td>1.666</td>
<td>166600</td>
</tr>
<tr>
<td>20</td>
<td>255.31</td>
<td>2.669</td>
<td>133450</td>
</tr>
<tr>
<td>30</td>
<td>256.195</td>
<td>3.951</td>
<td>131700</td>
</tr>
</tbody>
</table>

**Average ε**

143917

**Figure S29.** Molar absorptivity of LAL032
Figure S30. Molar absorptivity of LAL038

Figure S31. Molar absorptivity of LAL039
### LAL044 HCl in MeOH

![Graph showing the relationship between concentration (µM) and absorbance (A).](image)

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>229.338</td>
<td>0.373</td>
<td>37300</td>
</tr>
<tr>
<td>20</td>
<td>229.338</td>
<td>0.685</td>
<td>34250</td>
</tr>
<tr>
<td>30</td>
<td>230.712</td>
<td>1.053</td>
<td>35100</td>
</tr>
</tbody>
</table>

Average ε

40624

**Figure S32.** Molar absorptivity of LAL044

### VGD019 HCl in MeOH

![Graph showing the relationship between concentration (µM) and absorbance (A).](image)

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>217</td>
<td>0.70305</td>
<td>140610</td>
</tr>
<tr>
<td>15</td>
<td>217</td>
<td>2.12315</td>
<td>141543</td>
</tr>
<tr>
<td>25</td>
<td>220</td>
<td>3.79285</td>
<td>151714</td>
</tr>
</tbody>
</table>

Average ε

144622

**Figure S33.** Molar absorptivity of VGD019
<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ_{max} (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>235.021</td>
<td>0.486</td>
<td>194400</td>
</tr>
<tr>
<td>5</td>
<td>237.5</td>
<td>1.118</td>
<td>223600</td>
</tr>
<tr>
<td>7.5</td>
<td>237.81</td>
<td>1.671</td>
<td>222800</td>
</tr>
</tbody>
</table>

Average ε
213600

**Figure S34.** Molar absorptivity of **VDG020**

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ_{max} (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>225.723</td>
<td>0.579</td>
<td>231600</td>
</tr>
<tr>
<td>5</td>
<td>225.413</td>
<td>1.279</td>
<td>255800</td>
</tr>
<tr>
<td>7.5</td>
<td>225.413</td>
<td>1.729</td>
<td>230533.3</td>
</tr>
</tbody>
</table>

Average ε
239311

**Figure S35.** Molar absorptivity of **VDG039**
### Figure S36. Molar absorptivity of VGD040

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>240</td>
<td>1.10504</td>
<td>110504</td>
</tr>
<tr>
<td>20</td>
<td>242</td>
<td>2.28914</td>
<td>114457</td>
</tr>
<tr>
<td>30</td>
<td>237</td>
<td>3.45226</td>
<td>115075</td>
</tr>
</tbody>
</table>

**Average ε**

### Figure S37. Molar absorptivity of VGD072

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>280</td>
<td>0.14</td>
<td>28000</td>
</tr>
<tr>
<td>15</td>
<td>277</td>
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<td>27855</td>
</tr>
<tr>
<td>25</td>
<td>278</td>
<td>0.72673</td>
<td>29069</td>
</tr>
</tbody>
</table>

**Average ε**

28308
Concentration (µM) | $\lambda_{max}$ (nm) | $A$ | $\varepsilon$  
--- | --- | --- | ---  
10 | 282.35 | 0.235 | 23500  
20 | 282 | 0.47758 | 23879  
30 | 283.85 | 0.72438 | 24146

**Average $\varepsilon$**

**26930**

**Figure S38.** Molar absorptivity of CK147

Concentration (µM) | $\lambda_{max}$ (nm) | $A$ | $\varepsilon$  
--- | --- | --- | ---  
10 | 260 | 0.076 | 7600  
20 | 262 | 0.14186 | 7093  
30 | 259 | 0.21283 | 7094

**Average $\varepsilon$**

**7262**

**Figure S39.** Molar absorptivity of CK207
Figure S40. Molar absorptivity of AR001

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>260</td>
<td>0.076</td>
<td>7600</td>
</tr>
<tr>
<td>20</td>
<td>262</td>
<td>0.14186</td>
<td>7093</td>
</tr>
<tr>
<td>30</td>
<td>259</td>
<td>0.21283</td>
<td>7094</td>
</tr>
</tbody>
</table>

Average ε

7262

Figure S41. Molar absorptivity of TL020

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>A</th>
<th>ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>260</td>
<td>0.076</td>
<td>7600</td>
</tr>
<tr>
<td>20</td>
<td>262</td>
<td>0.14186</td>
<td>7093</td>
</tr>
<tr>
<td>30</td>
<td>259</td>
<td>0.21283</td>
<td>7094</td>
</tr>
</tbody>
</table>

Average ε

205933
Appendix C: Standard curves for CADA, CK147, & DJ010 by high performance liquid chromatography

Although determining unknown concentrations of CADA analogs in solubility and lipophilicity assays using the Beer-Lampert equation and UV-Vis spectroscopy have proved useful in our research, pharmacokinetic assays that contain molecular relics of biological systems (ex. Caco-2 cells or liver microsomes in cell permeability and metabolic stability assays, respectively) render UV-Vis an insufficient method for analyzing unknown concentrations of CADA analogs. Presumably, biological macromolecules would likely absorb in the same region of CADA analogs, which would not allow for an accurate assessment of the spectroscopic values needed to calculate concentration from the Beer-Lampert equation.

Alternatively, high-performance liquid chromatography (HPLC) allows the separation and individual spectroscopic analysis of various components of a mixture. Similar to our approach in the determination of the molar absorptivities of various CADA analogs, known concentrations of CADA, DJ010, and CK147 in acetonitrile were prepared and analyzed as a function of peak area using HPLC. Each concentration tested was run in triplicate and the standard curves generated for each compound are shown in Figures 73-75. These standard curves will prove useful in future experiments to analyze the metabolic stability and cell permeability of CADA, DJ010, and CK147.
Figure S42. HPLC standard curve data generated for CADA.
**Figure S43.** HPLC standard curve data generated for the highly potent CADA analog CK147.
**Figure S44.** Standard curve data generated for the PEGylated CADA analog **DJ010.**