Chemical inhibitors of protein-protein interactions involved in G protein-mediated signaling events as potential therapeutics

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CHEMICAL INHIBITORS OF PROTEIN-PROTEIN INTERACTIONS INVOLVED IN G PROTEIN-MEDIATED SIGNALING EVENTS AS POTENTIAL THERAPEUTICS

by

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacy (Medicinal & Natural Products Chemistry) in the Graduate College of The University of Iowa

December 2017

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This is to certify that the Ph.D. thesis of

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Abstract

G protein-coupled receptors (GPCRs) play a central role in numerous biological processes, from olfaction to vision to neurotransmission and more, leading to their classification as important drug targets. Signaling through GPCRs is carried out by a number of intracellular effector proteins, such as the $\alpha$ subunits of heterotrimeric G proteins and adenylyl cyclases (AC). In this work, inhibition of protein-protein interactions as a mechanism to reduce GPCR-mediated cAMP formation is explored to identify compounds with potential therapeutic value.

Regulators of G Protein Signaling (RGS) inhibit $G_{\alpha i/o}$ signaling, resulting in persistent AC-mediated cAMP formation. Fragment-based screening against RGS17, which has been implicated in a number of cancers, identified seven hits that bind RGS17, though only one leads to inhibition of RGS17 function in vitro. Importantly, these compounds represent a starting point for future structure-based drug discovery targeting RGS17 for the development of new chemotherapeutics.

As different RGS proteins serve different functions throughout the body, achieving isoform selectivity is important for reducing potentially deleterious side effects. Analysis of the selectivity of previously described RGS4 inhibitors revealed that several are more potent inhibitors of RGS14. This work also identified the RGS proteins for which inhibitors are likely to be found (RGS14, RGS4, RGS1) and those which are likely to be more difficult drug targets (RGS6 and RGS7).

Finally, inhibition of AC8 as a means to decrease intracellular levels of cAMP is explored. AC1 and AC8 are robustly stimulated by calmodulin (CaM), so inhibition of the AC/CaM interaction was explored as a novel mechanism of AC inhibition.
Biochemical and cell-based assays were developed, and a pilot screen of 1,000 FDA-approved compounds identified six capable of inhibiting the AC8/CaM interaction. This ultimately resulted in reduced AC8 activity and cAMP accumulation, validating this interaction as a druggable target. The compounds identified were not selective for AC8 over AC1, but they have pre-clinical utility in evaluating the biology of CaM-stimulated AC activity.
Public Abstract

Progression of a drug candidate from the laboratory to the clinic and, ultimately, to the patient is a long, and often arduous process. Recent advances in chemistry, biology, and medicine have provided more insight into the causes and effects of diseases than ever before. Much of this work involves the identification of specific proteins that have altered activity between healthy and affected individuals. Chemicals that can restore activity in afflicted individuals to that observed in healthy patients could be used as drugs. The identification and further testing of chemicals capable of achieving these desired outcomes is often the largest obstacle to surmount in the development of new medicines.

In my doctoral studies, I have been working to identify chemicals that can change the activity of two different classes of proteins. The first class is Regulator of G Protein Signaling proteins, which regulate a number of processes throughout the body. One of these proteins, RGS17, is overactive in prostate, lung, and breast cancers, among others. Finding molecules that can reduce its activity in cancer has been difficult but my work begins to explore an alternative drug discovery method based on the structure of the RGS17 protein. The second class of proteins is adenylyl cyclases, which are important neurological signaling molecules. These proteins have been identified as overactive in pain, stress, and anxiety, so I have attempted to find chemicals that reduce adenylyl cyclase activity to alleviate the effects of these conditions.
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Chapter 1. Introduction

Portions of the following chapter are adapted from Hayes, M.P. and Roman, D.L. *Regulator of G Protein Signaling 17 as a Negative Modulator of GPCR Signaling in Multiple Human Cancers.* AAPS J, 2016, 18(3): p. 550-9.

Approximately 30% of FDA approved drugs exert their effects by modulating guanine nucleotide-binding protein (G protein) signaling cascades. Most of these compounds act by either activating or inhibiting G protein-coupled receptors (GPCRs). GPCRs facilitate the exchange of GDP for GTP on Ga subunits, allowing Ga to dissociate from the Gβγ heterodimer. Both Ga and Gβγ are then free to interact with downstream effectors resulting in altered intracellular responses to extracellular stimuli. Signaling is terminated by hydrolysis of GTP to GDP by the intrinsic GTPase activity of Ga, resulting in the reformation of the inactive Gaβγ heterotrimer. This cycle is outlined in Figure 1. Ga subunits are largely divided based on effect on downstream effectors. For example, Ga₁s and Ga₁olf stimulate adenylyl cyclase (AC)-mediated cAMP formation, whereas Ga₁o, Ga₁i, and Ga₂ inhibit AC activity.

1.1 Regulator of G Protein Signaling Proteins

Regulator of G Protein Signaling proteins (RGS) act as GTPase activating proteins (GAP) for activated Ga₀, Ga₁, Ga₂, and Ga₁q/11 subunits. RGS proteins act to stabilize transition states of Ga that result in increased GTP hydrolysis to GDP, leading to association of Ga with Gβγ and signal termination [1]. The hallmark of RGS family members is the approximately 120 residue RH domain that is responsible for the direct interaction of RGS proteins with GTP-bound Ga. This domain of nine α-helices is
arranged into a structure that contains two basic regions, termed the terminal and bundle subdomains. The terminal subdomain contains \( \alpha_1, \alpha_2, \alpha_3, \alpha_8, \) and \( \alpha_9 \), which includes both the N- and C-termini. The bundle domain contains \( \alpha_4-\alpha_7 \) arranged in an anti-parallel bundle (Figure 2a) [1].

**Figure 1. G Protein Signaling Mechanism** Ligand binding results in GPCR-mediated activation of G\( \alpha \) and G\( \beta \gamma \) signaling cascades. Signaling is terminated by G\( \alpha \) hydrolysis of GTP, which is accelerated by RGS proteins. Figure previously published in [2].

The RGS family of proteins is composed of approximately 20 members that are divided by sequence similarity into four sub-families (Figure 2b). RGS proteins can be relatively simple, containing very short regions outside the RH domain (RZ, R4). Alternatively, RGS proteins can be very complex, capable of binding multiple other protein partners through the inclusion of accessory domains (R7, R12). The domain composition of different RGS protein sub-families is shown in Figure 2b. Additionally,
RGS homology (RH) domains are found in a number of proteins that are not considered members of the RGS family due to other functional roles or lack of detectable RH domain Ga binding. These proteins include G protein-coupled receptor kinases, axins, sorting nexins, and RhoGEFs.

RGS proteins are able to regulate signaling that occurs due to activation of a number of receptors, including µ opioid, 5-HT1A serotonin, D2 dopamine, and AT1 angiotensin receptors [3-13]. Due to this ability to negatively regulate intracellular signaling of GPCRs coupled to Gaαs, Gaαi, Gaαz, and Gaαq/11 subunits, RGS proteins have been identified as attractive targets for drug development. Genetic deletion and knockout models of RGS proteins have been particularly useful in identifying disease states in which altering RGS
activity could be therapeutically beneficial. *RGS4*\(^{+/-}\) mice have been useful in elucidating RGS4’s function in Parkinson’s disease, as these mice are less sensitive to 6-OHDA impairment models and exhibit reduced L-DOPA-induced involuntary movement [14, 15], but recent findings challenge these results [16]. *RGS2*\(^{+/-}\) and *RGS5*\(^{+/-}\) mice identified the opposing roles of these isoforms in vascular function, as deletion of *RGS2* results in hypertension, whereas lack of *RGS5* results in hypotension [17-19]. *RGS6*\(^{+/-}\) mice have identified its role in anxiety, depression, alcoholism, and Parkinson’s disease [20-22].

1.2 Regulator of G Protein Signaling 17

One member of the RGS family that has recently gained attention as an attractive drug target is RGS17, a member of the RZ sub-family, so named because each member of this family exhibits GAP activity towards G\(\alpha_z\) [10, 23, 24]. The other RZ family members, RGS19 (GAIP), RGS20 (RGSZ1), and Ret-RGS, share 50%, 53%, and 33% sequence identity with RGS17, respectively.

The endogenous tissue distribution of *Rgs17* under non-pathological conditions varies between the animal species and methodologies commonly used, but the consensus finds that it is primarily located within the central nervous system. In humans, *Rgs17* mRNA is found in the nucleus accumbens (NAc), parahippocampal gyrus, putamen, and the highest levels of expression are observed in the cerebellum. Low levels of *Rgs17* are also detectable in human testis [10, 25]. Previous work has identified that *Rgs17* expression levels are dynamic and can change in response to treatment with various stimuli. Treatment with the DD isoform of platelet-derived growth factor is sufficient to produce increased levels of *Rgs17* in cultured rat smooth muscle cells [26]. In rabbits, prenatal
exposure to cocaine results in statistically significant increased \textit{Rgs17} gene expression by approximately 33\%, whereas genetic deletion of D\textsubscript{1} dopamine receptors results in statistically significant decreased \textit{Rgs17} expression in the medical frontal cortex, striatum, and nucleus accumbens \[27\]. Increased \textit{Rgs17} expression can be induced in rats by treatment with D\textsubscript{2} dopamine receptor agonist quinpirriole \[28\]. Importantly, appreciable \textit{Rgs17} expression is not observed outside the CNS (aside from trace levels within testis) under normal conditions, but an increasing body of evidence has identified increased RGS17 protein activity in a number of cancer types.

\textit{Rgs17} was identified as a potential marker for familial lung cancer. A susceptibility locus was tracked to chromosome 6q23-25, the location of the \textit{Rgs17}. This was corroborated by the fact that RGS17 is often overexpressed in lung and prostate tumors by over 7.5-fold \[29, 30\]. More importantly, RNA interference (RNAi)-induced reduction of RGS17 protein levels reduced tumor volume by 59–75\% in a mouse xenograft cancer model. Moreover, transfection with cDNA to cause RGS17 overexpression increases levels of proteins with cAMP response elements (CRE)-dependent promoter regions. CRE binding protein (CREB) acts downstream of protein kinase A (PKA), which is activated by cAMP binding. These findings demonstrate that the anti-proliferative effect observed following RGS17 knockdown is likely due to RGS17’s GAP activity toward inhibitory $G\alpha$ subunits \[29\]. Increased RGS17 leads to decreased $G\alpha_{i/o/z}$ activity, increased AC activity, and, ultimately, increased AC-PKA-CREB pathway activation, altering the transcription of CRE-regulated genes (Figure 4) \[29\].

The exact mechanism by which RGS17 activity and expression are increased remains unknown. Evidence suggests that in lung cancer cell lines, RGS17 protein levels are
regulated by changes in microRNA (miRNA, miR) levels. These are short, non-coding RNA sequences capable of regulating translation of their target mRNA sequences by RNA silencing. One miRNA that can regulate RGS17 is mir-182, whose overexpression dramatically reduced available RGS17. Overexpression mir-182 recapitulated what was observed when RGS17 was specifically knocked down using RNAi, and increased mir-182 was sufficient to the proliferation and anchorage-independent growth of lung cancer cells in vitro [31]. Thus, one could hypothesize that increased RGS17 observed in lung and prostate cancers may be caused by loss of mir-182 expression.

Increased RGS17 results in excessive inhibition of $G_{ai/o}$, resulting in increased cAMP formation by AC, PKA activation, and transcription of CREB-regulated genes, causing increased growth and migration.

Increased RGS17 has been implicated in other types of cancer as well. In hepatocellular carcinoma (HCC), a statistically significant increase of $Rgs17$ was observed in five of seven patient-derived samples. Additionally, in a cancer cell line
model, knockdown of Rgs17 resulted in decreased intracellular cAMP and proliferation [32]. Unlike the miRNA regulation of RGS17 reported in some lung cancer cell lines, in HCC, RGS17 may be subject to epigenetic regulation, as it has been reported that there are changes in CpG site methylation in and around the Rgs17 gene [33]. In breast cancer, increased RGS17 is observed in patient samples, and knockdown results in decreased migration and invasion in breast cancer-derived cultured cells [34]. In breast cancer Rgs17 has reported to be regulated by miR-32, which is specifically down regulated in cancerous tissue as compared to surrounding normal tissue [34].

Due to RGS17’s relatively narrow pattern of expression in normal human tissue and its specific up-regulation in several cancers, the identification of RGS17 inhibitors deserves further exploration. A compound capable of recapitulating the reduction in invasion, migration, and tumor size that is observed during RGS17 RNAi could serve as a tool to further validate RGS17 as a drug target relevant in multiple types of cancer. As RGS17 is generally relegated to CNS tissues in healthy individuals, the predicted on-target side effects of an inhibitor could be mitigated by engineering compounds to have a poor blood-brain barrier penetration. Selection of inhibitors with large (> 400 Da) and hydrophilic structures could prevent blood-brain barrier permeability, preventing negative side effects associated with RGS17 inhibition in the CNS.

1.3 Inhibition of RGS Proteins with Small Molecules

As RGS proteins produce their effects by binding to accelerating the GTPase activity of activated Ga subunits, small molecule RGS inhibitors will necessarily need to be protein-protein interaction (PPI) inhibitors. One can envision achieving inhibition of
RGS/Gα interactions through two mechanisms: 1) small molecule binding directly to the RGS/Gα interface that prevents PPI 2) allosteric inhibition, where small molecule binding to a site distinct from the Gα interface causes a conformational change that reduces RGS affinity for Gα. Efforts to pursue the first strategy, as a proof of principle, used the crystal structure of RGS4-bound Gαi1 and structure-based drug design. This led to the generation of YJ34, a cyclic peptide that acts as a decoy and competitively inhibits RGS4 by mimicking regions of Gαi1 critical for RGS4 binding [35]. Following this discovery, high throughput screening (HTS) was employed to find more potent and specific RGS4 inhibitors, which ultimately yielded another peptide hit, 5nd, that was found to form a covalent complex with RGS4 through the formation of disulfide bonds [36]. As many of the pathologies in which modulation of RGS function could be therapeutically advantageous are disorders of the central nervous system (CNS) and development of CNS-penetrant peptides remains challenging, subsequent efforts were undertaken to find small molecules capable of inhibiting RGS activity.

![Figure 4 Cysteine-Dependent RGS Inhibitors](image)

Chemical structures of previously identified RGS inhibitors. For peptides, ‘Ac’ refers to N-terminal acetylation, and brackets indicate cyclical (disulfide bond) regions.
Screening of small molecule chemical libraries led to the identification of allosteric RGS inhibitors. CCG-4986, the first disclosed RGS4 inhibitor, was quickly found to interact with RGS4 through an allosteric, covalent mechanism, involving the modification of cysteine residues distal to the Ga interface [37-39]. Through a number of subsequent HTS efforts, several more compounds were identified that inhibit RGS4 (and RGS17) activity through covalent modification of cysteine residues or in a cysteine-dependent manner. These compounds include CCG-50014, CCG-59919, CCG-63802, CCG-63808, UI-5, and UI-1590 [40-44]. Additionally, RGS proteins can be inhibited by endogenous cysteine modifiers, such as 4-hydroxynonenal [45]. RGS inhibitor compound structures are presented in Figure 4.

1.4 Adenylyl Cyclase Proteins

ACs are downstream effectors of various GPCRs. The mammalian AC family of proteins is composed of ten family members (AC1-10), which catalyze formation of the signaling molecule cAMP from ATP. ACs 1-9 are membranous ACs (mAC) and confined to cellular membranes by two transmembrane regions (TM1 and TM2), each of which contains six transmembrane passes. In addition to these regions, mACs also contain five intracellular regions. A short, intracellular N-terminus is present, and following each TM region is a cytosolic, catalytic domain (C1a and C2a) and a cytosolic, regulatory domain (C1b and C2b). The composition of mACs is presented in Figure 5. The C1a and C2a regions associate to form a catalytic center that contains binding sites for ATP in all ACs and forskolin (FSK) in ACs 1-8. In fact, purified, recombinant C1 and C2 regions dimerize, and cAMP formation can be stimulated by either Ga, or FSK,
recapitulating results observed using overexpressed full length ACs in membrane- and cell-based assays [46]. Multiple crystal structures have been solved of the heterodimer formed between canine AC5 C1 and rat AC2 C2 regions, providing insight into the catalytic and regulatory functions of the cytosolic AC regions. The structures of these regions have been solved in the presence and absence of GTP-bound Gαs, various divalent metals (Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ca$^{2+}$), various nucleotides and analogs [ATP, PPi, 3'-O-(N'-methylanthraninoyl) GTP, etc.], and FSK and FSK analogs [47-52]. To date, little is known regarding the structure and function of the transmembrane regions.

As ACs represent a critical step in transforming extracellular stimuli into intracellular changes in cAMP levels, their activity is dynamically regulated by a number of other signaling molecules. This regulation is AC isoform specific, and even further complicating matters, some signaling proteins, like Gβγ, are able to activate some AC isoforms and inhibit others. Regulation of AC activity is summarized in Table 1. Based on their interactions with specific regulators, the AC family is divided into three groups.

Figure 5. Structure of mAC Mammalian ACs 1-9 contains two transmembrane domains (blue), two regulatory domains (red), and two catalytic domains (green). ATP and FSK bind between the catalytic domains.
Group I ACs (AC1, AC3, and AC8) are stimulated by interaction with Ca\(^{2+}\)-bound calmodulin (CaM/Ca\(^{2+}\)). Group II contains AC2, AC4, and AC7. Group III contains AC5 and AC6, which are robustly inhibited by G\(\alpha_{i/o}\) and Ca\(^{2+}\). AC9, the only member of Group IV, is unique among the membranous cyclases in that it is not activated by FSK.

<table>
<thead>
<tr>
<th>Regulator</th>
<th>AC Activity</th>
<th>Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSK</td>
<td>+</td>
<td>1-8</td>
</tr>
<tr>
<td>G(\alpha_s)</td>
<td>+</td>
<td>1 - 9</td>
</tr>
<tr>
<td>G(\alpha_i)</td>
<td>-</td>
<td>1, 5, 6(^a)</td>
</tr>
<tr>
<td>G(\alpha_o)</td>
<td>-</td>
<td>1(^b)</td>
</tr>
<tr>
<td>G(\beta\gamma)</td>
<td>-</td>
<td>1, 3, 8</td>
</tr>
<tr>
<td>G(\beta\gamma)</td>
<td>+</td>
<td>2, 4, 5, 6</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>-</td>
<td>5, 6</td>
</tr>
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<td>CaM/Ca(^{2+})</td>
<td>+</td>
<td>1, 8, 3</td>
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<tr>
<td>CaM Kinase</td>
<td>-</td>
<td>1, 3</td>
</tr>
<tr>
<td>Protein Kinase C</td>
<td>+</td>
<td>1, 2, 3, 5, 7</td>
</tr>
<tr>
<td>Protein Kinase C</td>
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<td>5, 6, 8</td>
</tr>
<tr>
<td>NO</td>
<td>-</td>
<td>5, 6</td>
</tr>
<tr>
<td>Raf kinase</td>
<td>+</td>
<td>2, 5, 6</td>
</tr>
<tr>
<td>RGS2</td>
<td>-</td>
<td>3, 5</td>
</tr>
</tbody>
</table>

Table 1. Regulators of AC Activity Modulators of AC activity. ‘+’ and ‘-’ indicate whether given effector stimulates or inhibits AC. Adopted from [53, 54]. \(^a\) Reported in [55]. \(^b\) Reported in [56].

1.5 Adenylly Cyclases 1 and 8

AC1 and AC8 have largely overlapping expression patterns. AC1 and AC8 are both expressed in rodent cerebellum, cortex, thalamus, and hippocampus, and AC8 is also found in the hypothalamus [57]. In humans, aside from the CNS, AC1 and AC8 are both found in peripheral blood leukocytes, and to a lesser degree in the heart, skeletal muscle, pancreas, kidney, ovary, and testis. AC1 is also found in the spleen, and AC8 is expressed in placental, lung, and liver tissues [58].
Due to the similar tissue distributions, knockout animal models have proven useful in elucidating the function of these individual AC isoforms in vivo. Mice lacking AC1, AC8, and both AC1 and AC8 (AC1/8\textsuperscript{-/-}) have been studied. AC1\textsuperscript{-/-} mice lack long-term synaptic changes following nerve injury that are indicative of neuropathic pain and show reduced acute and chronic muscle pain [59, 60]. Recent work has also identified a role for AC1 in Fragile X syndrome (FXS), which is caused by Fragile X mental retardation 1 (Fmr1). Fmr1\textsuperscript{-/-} mice exhibit higher levels of AC1 mRNA, but knockout of AC1 in these same animals reduced autism-like symptoms. These effects could also be recapitulated through pharmacological intervention, as Fmr1\textsuperscript{-/-} mice treated with AC1 inhibitor NB001 also exhibit reduced FXS symptoms.

AC8\textsuperscript{-/-} mice exhibit decreased ethanol intake at high concentrations compared to littermate controls. Though AC8\textsuperscript{-/-} mice are marginally more sensitive to the effects of alcohol, these effects are not as severe as those observed in AC1\textsuperscript{-/-} and AC1/8\textsuperscript{-/-} [61]. AC8\textsuperscript{-/-} mice also are more resistant to long-lasting anxiety and, though their anxiety levels in response to acute stress are similar to controls mice, their anxiety response following repeated stress is diminished [62, 63].

Similar to the AC1\textsuperscript{-/-} model, AC1/8\textsuperscript{-/-} mice are less responsive to inflammatory stimuli [64]. The double knockout mice also have diminished voluntary high concentration alcohol consumption, like mice lacking AC8 [61]. AC1/8\textsuperscript{-/-} mice are also much more sensitive to small doses of cocaine, while single knockout mice are not [65]. Mice lacking both isoforms have impaired long-term potentiation and have difficulty acquiring and retaining memory in water maze tests. This result is not unsurprising given the expression of both AC1 and AC8 isoforms in the hippocampus [57, 66]. Interestingly, treatment of
double knockout mice with FSK is able to rescue this loss in long-term memory, indicating that other AC isoforms may be able to compensate for loss of CaM-stimulated ACs [67]. Most importantly, memory and long-term potentiation deficits exhibited by AC1/8−/− mice are greatly reduced in animals lacking only a single AC isoform [67].

Taken together these results suggest that the development of specific AC1 inhibitors could be useful as non-opioid neuropathic pain treatments. Specific AC8 inhibitors could be therapeutically useful for reducing anxiety and stress and curbing excessive ethanol consumption.

1.6 Inhibition of ACs with Small Molecules

To date, efforts to identify inhibitors of ACs have identified molecules that fall into four classes: 1) ATP site competitive inhibitors 2) “P-site” inhibitors 3) allosteric inhibitors binding to FSK site 4) allosteric inhibitors binding sites distinct from FSK. Many ATP-site competitors and P-site inhibitors have adenine-like structures, giving rise to concerns about AC isoform specificity and potential off-target effects due to inhibition of other adenine nucleotide binding proteins. While P-site inhibitors are not competitive inhibitors, they suffer from lack of specificity between AC isoforms, poor cell permeability, and low potency [53, 68]. As the FSK binding site is highly conserved between AC isoforms, achieving selectivity with small molecule inhibitors has proved challenging. Little mechanistic information is known about the fourth class of AC inhibitors, other than they work through interactions at allosteric sites distinct from the FSK sites, but often with low potency [69, 70]. As all of these classes of compounds have
weaknesses as potential specific inhibitors of AC isoforms, the exploration of novel mechanisms of AC inhibition is needed.

Though CaM/Ca^{2+} can stimulate the activity of all Group I ACs, robust increases in cAMP generation is only observed for AC1 and AC8 (Table 1) [71, 72]. Increased AC3 activity in the presence of CaM/Ca^{2+} is only observed when AC3 is simultaneously stimulated using either FSK or G\alpha_s bound to GppNHp, a non-hydrolysable GTP analog [73]. Alternatively, CaM/Ca^{2+} alone is sufficient to increase cAMP formation by AC1 and AC8, even in the absence of FSK and/or G\alpha_s [71, 72]. Interestingly, the CaM interacting regions of AC1 and AC8 are not conserved. The C1b and C2a areas of AC1 bind CaM, whereas the N-terminus and C2b regions of AC8 bind CaM [74, 75]. Furthermore, the CaM-binding areas of AC8 interact with CaM through distinct mechanisms. The N-terminal region of AC8 contains a 1-5-8-14 CaM binding region (large, hydrophobic residues at indicated positions), whereas the AC8 C2b region contains an IQ-like motif. As such, it may be possible to achieve selectivity between AC1 and AC8 by identifying molecules that selectively bind the regions of the AC or CaM that are critical for association of either AC1 or AC8 without affecting association with CaM, and thus activity, of the other.

1.7 Small Molecule Inhibition of Protein-Protein Interactions

The context-dependent formation of PPIs is a critical step in many biological processes. Historically, the development of small molecule inhibitors of PPIs has proved difficult. Many proteins involved in PPIs are not enzymes but rather exert their effects solely through the formation of these context-dependent PPIs. The interfaces formed by
these complexes are often large, up to 2000 Å² surface area for a single interface, and “featureless”, lacking small molecule bind regions [76]. Recently it has been proposed that even on these “featureless” PPI surfaces, a disproportionately large amount of binding energy is due to small, discrete regions on the interface, termed “hot spots”, giving hope to the discovery of future PPI inhibitors [77]. These hopes are further bolstered by the entry of multiple PPI inhibitors into clinical use, such as tirofiban, which inhibits the extracellular fibrinogen/integrin IIbIIIa interaction [78], lifitegrast, which inhibits the extracellular LFA1/ICAM1 interaction [79], and venetoclax, which inhibits the Bcl-Bak interaction on mitochondrial membranes [80]. Many more PPI inhibitors are currently in clinical trials.

PPIs can be separated into distinct classes based on the nature of the protein regions that form inter-protein contacts. Two classes of protein-protein interactions relevant to the work described here are: 1) globular protein-peptide, like AC/CaM and 2) globular protein-globular protein, such as Gα/RGS [81]. Previous efforts have determined that the regions of AC8 and AC1 that mediate CaM binding are short, α helical sequences that can successfully be reduced to peptides, while retaining CaM binding [74, 82]. As PPIs formed by a globular protein (CaM) with peptides (derived from AC1 and AC8) are more easily targeted by small molecules, traditional HTS is a rational first step for the identification of novel AC/CaM interaction inhibitors. Alternatively, the RGS/Gα PPI occurs between two globular proteins, a PPI class that is more difficult to modulate with small molecules. The interactions between RGS proteins with Gα has been more thoroughly probed for inhibitors using HTS. These efforts have had little success in identifying potent, competitive RGS inhibitors, so alternative drug discovery methods
demand further attention. Additionally, the isoform selectivity of previously identified inhibitors across the RGS family has not been thoroughly examined.
Statement of Hypothesis

Selective disruption of protein-protein interactions downstream of GPCR signaling represents a putative drug mechanism of action that deserves further attention. These therapeutically relevant protein-protein interactions include inhibition of RGS17/Gα for the treatment of multiple types of cancer, AC8/CaM for relief from anxiety, stress, and excessive alcohol consumption, and AC1/CaM for use in chronic pain. Additionally, critical analysis of the selectivity and cysteine-dependence of current RGS family protein inhibitors will provide new insights into pathologies in which use of these inhibitors could be beneficial by identifying previously unknown inhibitor-RGS protein interactions.
Chapter 2. Fragment-based Lead Discovery Targeting RGS17

2.1 Introduction

RGS17 becomes inappropriately expressed in a number of human cancers, including lung, prostate, breast, and hepatocellular carcinoma [2]. Excessive RGS17 leads to inhibition of $G_{\alpha_i/o}$-mediated AC inhibition, causing increased levels of the intracellular second messenger cAMP (Figure 3) [29]. Increased cAMP causes increased release of the PKA catalytic subunit from the PKA regulatory subunit, allowing the catalytic subunit to phosphorylate and activate CREB, which leads to changes in transcription, such as increased Cyclin D1, overexpression of which is sufficient to cause some types of cancer [29, 83]. Furthermore, suppression of RGS17 using RNAi is sufficient to decrease tumorigenesis in a mouse xenograft model of cancer and prevent cancer cell migration [29, 34]. To date, information regarding the effect of global knockout of RGS17 in mice is lacking. Because of its relegation to the CNS under non-pathological conditions and its selective up-regulation in cancerous tissue as compared to patient-matched normal tissues, RGS17 is an attractive drug target.

Previous efforts by our lab using HTS have identified a limited number of RGS17 inhibitors. Initial efforts aimed at developing an HTS-compatible assay identified four compounds (RL1-4) capable of inhibiting the RGS17 interaction with $G_{\alpha_o}$ in vitro. These initial hit compounds were ultimately abandoned because they possessed reactive substructures (quinones, Michael acceptors) and were not potent, requiring 62 to 540-fold molar excess compound over RGS17 to achieve 50% inhibition [84]. Subsequent efforts identified four additional compounds capable of inhibiting RGS17/$G_{\alpha}$, UI-5, UI-1590, UI-1907, and UI-1907. Two of these compounds, UI-1590 and UI-1907, were found to
work through covalent mechanisms, as compound-adducted RGS17 species could be identified using mass spectrometry, while UI-5 and UI-1956 were found to be thiol reactive using other methods [43]. Additionally, each of these compounds lacked appreciable inhibition against a cysteine null RGS17 mutant (C117A), pointing to a cysteine-dependent mechanism, a commonality among RGS inhibitors that is undesirable and further explored in Chapter 3. Given the limited success of traditional HTS in identifying RGS17/G\(\alpha\) inhibitors, alternative screening methodologies deserve attention, such as fragment-based lead discovery (FBLD).

The goal of FBLD is to identify very small (usually <300 Da) compounds capable of eliciting a desired response. As these compound fragments are so small, they often have low affinity for their target of interest. Subsequent efforts then focus on growing or joining these fragments into larger, more complex compounds with increased affinity. FBLD differs from traditional HTS in a number of ways. HTS generally involves the screening of 100,000+ larger, more complex (~500 Da) compounds, whereas FBLD efforts usually focus on screening fewer (100-1000s), smaller (< 300 Da) compounds. This is possible because the size of “chemical space” (every possible chemical combination) increases with the number of non-hydrogen atoms. For example, it is estimated that by screening fragments with 11 non-hydrogen atoms or fewer chemical space is reduced from \(10^{60}\) to \(10^9\) compounds [85-87]. As fewer compounds need to be screened, the standard repertoire of HTS detection methodologies like fluorescence, absorption, and luminescence are exchanged for lower throughput, information rich methods, like nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), and even X-ray crystallography.
NMR has proven particularly useful for FBLD efforts, as it can provide unambiguous evidence of direct binding of a test compound to a protein of interest [88]. Additionally, it is capable of identifying the weak interactions typically observed in FBLD, while maintaining higher throughput than other biophysical methods. NMR allows for ligand-detected methods, in which changes in the NMR spectrum of a test compound in the presence and absence of the protein of interest is observed. Examples include WaterLOGSY and saturation transfer difference NMR [89, 90]. Alternatively, protein-detected methods record the spectrum of the protein of interest in the presence and absence of compound and changes in chemical shifts are monitored. If the chemical shifts of the protein have been previously assigned to individual residues, then detection of binding and binding site information can be derived from a single experiment. Protein-detected methods are generally limited by the fact that they require significant amounts of purified protein and that the protein of interest be smaller than 25-30 kD. Fortunately, the RH domain of RGS17, which is directly responsible for forming the complex with Gα, is approximately 17 kD in size, and 15N isotope-labeled protein can be purified in large quantities (> 100 mg/L culture) from E. coli culture.

Due to the shortcomings of traditional HTS in identifying RGS17 inhibitors, we embarked on an FBLD effort using protein-detected NMR screening to identify fragments capable of binding the RH domain of RGS17.
2.2 Results

Assignment of RGS17 15N-1H HSQC Chemical Shifts

In order to facilitate future structural efforts, we decided to employ a construct of RGS17 (Asn72 to Ser206) that had previously been optimized for increased recombinant expression in *E. coli* and crystallized by the Structural Genomics Consortium [91]. Purification of a $^1$H-$^{13}$C-$^{15}$N labeled sample of this construct allowed the unambiguous assignment of > 93% (120/129) of the RGS17 backbone amide resonances using a suite of multidimensional NMR experiments [92]. The assigned RGS17 $^1$H-$^{15}$N HSQC is shown in Figure 6.

![Figure 6. RGS17 Chemical Shift Assignments](image)

**Figure 6. RGS17 Chemical Shift Assignments** A) RGS17 RH domain $^1$H-$^{15}$N HSQC spectra with backbone residue assignments indicated with aliased peaks shown in red. Boxed region is expanded and peaks labeled in B.
DMSO Stability of RGS17 RH Domain

Fragments generally exhibit weak affinity, so high concentrations of fragments are required. As HTS and fragment libraries are often dissolved in the organic solvent dimethyl sulfoxide (DMSO), determination of RGS17 (residues 72-206, RGS17-RH) stability in the presence of various concentrations of DMSO was required to design a screening strategy. Examination of the RGS17-RH $^1$H-$^{15}$N HSQC in the presence of increasing concentrations of DMSO revealed that RGS17-RH is quite structurally stable in this solvent, as only small changes in chemical shifts were observed (Figure 7). Moreover, previous work by our lab had shown that no changes in RGS17-RH/Go PPI formation were observed at DMSO concentrations up to 3.3% (data not shown).

![Figure 7. DMSO Stability of RGS17 A) $^1$H-$^{15}$N HSQC in the presence of 0% (black), 5% (red), and 10% (green) DMSO. Boxed region is expanded and peaks labeled in B.]

Screening of Maybridge Ro3 Core Fragment Library

This stability, even at high concentrations of DMSO, allowed us to pursue a fragment pooling strategy to more efficiently screen the 1,000-fragment library. This was due to the fact that high concentrations of fragment were required, and the fragment library was provided as 100 mM stock solutions in DMSO. The combination of the fragment library into pools of fragments allowed the conservation of valuable time and resources, while increasing the throughput of the NMR screening method. Each pool
contained six fragments at a concentration of 1 mM (10-fold molar excess over RGS17-RH) with a final DMSO concentration of 6%. This reduced the number of samples from 1,000 to approximately 170. $^{1}\text{H} \text{-} ^{15}\text{N}$ HSQC spectra of 100 µM RGS17-RH in the presence of pooled fragments were then acquired. DMSO vehicle alone (6%) served as control. Spectra were then manually examined for fragment-induced chemical shift perturbations (CSP). Seventeen pooled fragment samples were identified as containing an RGS17-binding compound, meaning that 102 samples (6 fragments per pool x 17 pools) containing individual fragments were needed for further examination. $^{1}\text{H} \text{-} ^{15}\text{N}$ HSQC spectra were obtained for each individual fragment and manually inspected for CSPs. RGS17-RH binding was noted in 22 of these 102 samples, yielding an initial hit rate of 2.2%. Of these 22 hits, several only resulted in shifts of a single residue in the RGS17-RH $^{1}\text{H} \text{-} ^{15}\text{N}$ HSQC or binding was undetectable with aliquots of fragment freshly made from powder stocks upon reorder. These compounds were excluded from further work, reducing the hit rate from 2.2% to 0.7% (Figure 8).

**Figure 8. RGS17 Fragment Screening Results** A) Summary of fragment screen against RGS17 RH domain. B) Chemical structure of hit fragments identified in NMR screen.
Figure 9. Representative Fragment Titration  
a) Changes in chemical shifts of backbone amide peaks in the presence of 0 (red) up to 5 mM (blue) fragment.  
b) monitoring of fragment solubility at concentrations ranging from 0 (green) up to 5 mM (brown) fragment.

**Determination of Fragment Affinity for RGS17 RH Domain**

In order to prioritize hits from the NMR screen, the affinities of fragments for RGS17-RH were determined by obtaining $^1$H-$^{15}$N HSQC spectra in the presence of increasing concentrations of fragment. Increasing concentrations of fragment result in increased changes in chemical shifts. Throughout dissociation constant determination experiments, analysis was limited to fragment concentrations below their individual solubility limits. Fragment solubility limits were determined experimentally by monitoring height of fragment peaks in $^1$H NMR spectra. When peak height no longer increased with increasing fragment concentration, solubility limit was assumed to be
Concentrations up to 10 mM were attempted, but only 2 was found to be soluble at this concentration. An example fragment concentration-dependent increase in CSP with $^1$H NMR confirmation of fragment solubility is presented in Figure 9. To determine $K_D$ values, CSP was plotted as function of fragment concentration and fit to a rectangular hyperbolic, one-site binding model (Figure 10). This was performed for each residue that displayed concentration-dependent peak shifts. $K_D$ values were calculated for each residue, and these $K_D$ values were then averaged to determine the $K_{D,\text{Avg}}$ for each fragment (Figure 9, Table 2). $K_D$ values were found to range from $0.53 \pm 0.35$ mM for 4 to $5.5 \pm 4.7$ mM for 2, with the number of residues analyzed for each compound ranging from three to six. The identities of the analyzed residues are shown in Table 2.

*Figure 10. RGS17 Affinity for Hit Fragments* Determination of CSP at various increasing concentrations of fragment allow $K_D$ determination. Fragment being analyzed is indicated above plot, and residues being analyzed are labeled. Data was analyzed using one-site specific binding model using GraphPad Prism.

**Fragment 3 Inhibits RGS17 via Covalent Modification of C117**

During fragment titration experiments it was noted that 3 caused large changes in the $^1$H-$^{15}$N HSQC spectra independent of the concentration used (Figure 11a,b).
Table 2. Fragment Screening Summary

<table>
<thead>
<tr>
<th>Fragment</th>
<th>$K_{D,\text{avg}}$ (mM)</th>
<th>Residues Analyzed</th>
<th>L.E.</th>
<th>RGS4 Binding</th>
<th>RGS C117S Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.3 ± 2.0</td>
<td>K121, L163, N167, Y171</td>
<td>0.20</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>5.5 ± 4.7</td>
<td>L163, H169, Q175</td>
<td>0.18</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>0.5 ± 0.4</td>
<td>D139, N167, Q175</td>
<td>0.31</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>5.2 ± 8.5</td>
<td>E109, L163, N167, Q175</td>
<td>0.23</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>1.5 ± 0.3</td>
<td>E109, Y137, L163, N167</td>
<td>0.25</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>2.9 ± 2.4</td>
<td>E109, I159, L163, N167, A174, Q175</td>
<td>0.25</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

This is a phenomenon that is often observed due to irreversible binding. Many previous efforts have identified RGS inhibitors that act via covalent modification of cysteine residues. Covalent labeling of RGS17-RH by 3 was analyzed using N-(7-dimethylamino-4-methylcoumarin-3-yl) maleimide (DACM), a dye that exhibits an increase in fluorescence after covalently labeling cysteine residues in proteins. Decreased DACM labeling was observed by pre-incubating RGS17-RH with 10-fold molar excess 3 or N-ethyl maleimide, another cysteine reactive compound (Figure 11c,d). This lowered fluorescence is due to decreased availability of cysteine residues, as pre-incubation with either agent leads to irreversible modification of these residues prior to DACM addition. Furthermore, 3 binding was found to be cysteine-dependent, as it did not bind to a mutant.
in which C117 was mutated to serine (C117S) (Figure 12f). 3 was also found to bind RGS4-RH (Figure 12e).

Figure 11. Covalent Modification of RGS17 by Hit Fragment 3

a) ¹H-¹⁵N HSQC of RGS17 in the absence (yellow) and increasing concentration fragment 3 up to 5 mM (green). b) Expanded view of boxed region of a. c) Kinetic trace of DACM reaction with buffer alone or 2 µM RGS17 or 20 µM indicated compound d) Calculated slope from DACM kinetic traces in c. Data represents mean of n=3 ± S.D. ** p ≤ 0.01 one-way ANOVA with multiple comparisons.

Prioritization of RGS17-Binding Fragments

In order to prioritize the identified hit fragments, the ligand efficiency (LE) of each was calculated as described in Chapter 6.1. This is a simple metric used to relate the size of a given compound to the binding energy, allowing one to determine which fragments are making the most productive binding contacts per heavy (non-hydrogen) atom. This allows for the prioritization of fragments for future medicinal chemistry efforts. Generally, an acceptable starting point in FBLD is considered 0.3. The fragments
identified in this screen had LEs ranging from 0.18 for 2 to 0.31 for 4, which was the only compound that exhibited a LE value greater than the 0.3 threshold (Table 2).

Additionally, each fragment was tested for binding to RGS4-RH and RGS17-RH to evaluate the selectivity and cysteine dependence of each fragment. These are important metrics for RGS inhibitors, as will be explored further in Chapter 3. Each fragment except for 5 was found to bind RGS4-RH, and each compound except for 3 and 5 were found to bind RGS17-RH C117S (Table 2). Further work identified that at high fragment concentrations 4 resulted in a loss of observable spectra for RGS17-RH, RGS17-RH C117S, and RGS4-RH (Figure 12a-c), indicating that a loss in structural integrity of these proteins was occurring. This could be due to formation of large soluble aggregates and/or protein precipitation.
Fragment Effects on RGS17 GAP Activity and Thermal Stability

Efforts to this point focused on identifying compounds that were simply capable of binding to RGS17-RH. Next, it was assessed if this binding correlated to inhibition of RGS17-RH GAP activity, as measured using a previously developed phosphate detection assay [42]. Testing of each compound at 1 mM identified that only 3 inhibited RGS17-RH activity to a statistically significant degree (p \leq 0.0001, one way ANOVA with multiple comparisons), though 4 did result in approximately 20% inhibition at this concentration (Figure 13a). Fragments were also tested for their ability to affect the thermal stability of RGS17-RH using a thermal shift assay. This is used to determine the proteins melting temperature in the presence and absence of fragments. Again, only 3 resulted in statistically significant changes in the melting temperature (Figure 13b). Treatment of RGS17-RH with 4 resulted in a curve shape for which a melting temperature could not accurately be determined, so it was excluded from analysis.

Figure 13. GAP Activity and Thermal Stability in Presence of Hits
a) Malachite green GAP analysis of 4.5 μM RGS17 in the absence and presence of 1 mM hit fragment. b) Differential scanning fluorimetry using Sypro Orange to determine melting temperature of 10 μM RGS17 in the presence of 1 mM indicated fragment. Data represent mean of n=3 ± S.E.M. **** p \leq 0.0001, one-way ANOVA with multiple comparisons.
Identification of a Small-molecule Binding Site on RGS17

The use of protein-detected NMR as a screening technology and the assignment of RGS17-RH $^1$H-$^{15}$N HSQC backbone resonances allowed the determination of fragment binding sites. Mapping the residues for which fragment-induced chemical shifts are observed to the previously solved RGS17-RH crystal structure (1ZV4) shows that most fragments are binding to a site distant from the Gα-interaction face. These fragments are making contacts with an allosteric region near the loop between α6 and α7. Interestingly, all compounds seem to bind in this general region, and no appreciable chemical shifts are observed within the terminal subdomain.

Crystallography Studies Identify Metal Binding Site on RGS17

In order to determine binding orientation of hit fragments and determine the key interactions formed with RGS17-RH with atomic resolution, a crystallography campaign was begun in collaboration with the laboratory of Dr. Angeline M. Lyon. Successful co-
crystal structure determination would also facilitate future *in silico* efforts to dock larger, more complex molecules into fragment binding sites. Preliminary studies focused on identifying new conditions in which RGS17-RH crystals form, with the hope of soaking crystals in solution with high concentrations of fragment to obtain fragment-bound RGS17-RH. Interestingly, during these studies it was noted that a Ca$^{2+}$ ion was bound near the Ga interacting portion of α4 on RGS17-RH, and this ion was present in both RGS17-RH molecules within the asymmetric unit. NMR was then used to determine if this binding event occurred in solution or was an artifact of crystallization conditions. Incubating RGS17-RH with a 50-fold molar excess of Ca$^{2+}$ resulted in a number of chemical shifts (Figure 15a). Plotting the residual chemical shifts and mapping these to the crystal structure showed that residues near the Ca$^{2+}$ ion in the crystal structure showed CSPs, which confirmed Ca$^{2+}$ binding in solution (Figure 15b,c).

![Figure 15. Ca$^{2+}$ Binding Site on RGS17](image)

Figure 15. Ca$^{2+}$ Binding Site on RGS17 a) $^1$H-$^15$N HSQC in the absence (black) and presence (red) of 50-fold molar excess Ca$^{2+}$. b) Plot of residual CSP in the presence of Ca$^{2+}$. c) Newly solved crystal structure of RGS17 with bound Ca$^{2+}$ shown in blue. Residues exhibiting CSP > 0.2 in the presence of Ca$^{2+}$ are shown as red spheres.
Due to the Ca\textsuperscript{2+} binding site being located on the G\alpha interface, the consequence of excess Ca\textsuperscript{2+} on RGS/G\alpha PPI formation was evaluated using a previously described AlphaScreen assay [84]. Complexation of RGS17-RH with increasing concentrations of G\alpha\textsubscript{o} was monitored in the presence of excess Ca\textsuperscript{2+}, the non-specific chelating agent EDTA, and the more Ca\textsuperscript{2+}-specific chelator EGTA. While these treatments had little to no effect on the rate of association (K\textsubscript{D}), there were significant differences observed in the maximal binding (Figure 16). At a concentration of 12.5 nM G\alpha\textsubscript{o}, inclusion of 10 mM EDTA resulted in a statistically significant decrease in complex formation (p =0.014). Alternatively, addition of 5 mM CaCl\textsubscript{2} resulted in a significant (p =0.014) increase in the maximal signal observed (approximately 25%).

![Graph](image.png)

**Figure 16. Ca\textsuperscript{2+} Binding Increases RGS17/G\alpha\textsubscript{o} Binding** a) Binding isotherm of 100 nM RGS17 with increasing concentrations of G\alpha\textsubscript{o} using AlphaScreen in the presence of indicated buffer additives. b) AlphaScreen binding observed at 12.5 nM G\alpha\textsubscript{o}. * p=0.014, student’s t-test. Data represents mean of n=3 ± S.E.M.

As it is common for metal binding sites to show some degree of promiscuity, it was tested whether Mg\textsuperscript{2+} was capable of binding to this same site on RGS17-RH. Again, using protein detected NMR, it was shown that Mg\textsuperscript{2+} induced chemical shifts in the same residues as Ca\textsuperscript{2+} (Figure 17). Additionally, there may be a second site interacting with Mg\textsuperscript{2+} near residues in the \(\alpha5-\alpha6\) loop, but several residues in this loop have not been
assigned in the RGS17-RH NMR spectra, so further experimentation may be needed to confirm binding at this site. In one of the RGS17-RH molecules within the asymmetric unit of the newly discovered crystal form, a Ca\textsuperscript{2+} ion is observed near this region, lending credence to the possibility of a second metal binding site on RGS17-RH’s G\textsubscript{α} interacting face.

![Figure 17. Mg\textsuperscript{2+} binding to RGS17](image)

**Figure 17. Mg\textsuperscript{2+} binding to RGS17** a) \textsuperscript{1}H-\textsuperscript{15}N HSQC in the absence (black) and presence (red) of 50-fold molar excess Mg\textsuperscript{2+}. b) Plot of residual CSP in the presence of Mg\textsuperscript{2+}. c) Newly solved crystal structure of RGS17 with bound Ca\textsuperscript{2+} shown in blue. Residues exhibiting CSP > 0.1 in presence of Mg\textsuperscript{2+} are shown as red spheres.

### 2.3 Discussion

As HTS has been of limited use in identifying bona fide RGS17-RH inhibitors, an alternative screening paradigm was pursued, namely FBLD. Triple resonance experiments and a \textsuperscript{13}C-\textsuperscript{15}N-\textsuperscript{1}H labeled RGS17 sample allowed the assignment of over 93% of the backbone amide resonances. This allowed us to select an information rich screening paradigm, protein-detected NMR. Collecting \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra allowed the identification RGS17-RH binding fragments and determination of binding site...
information in a single experiment. Using this method, a library of 1,000 fragments was screened against RGS17-RH. Follow up and pool deconvolution to identify the RGS17 binding fragment in each pool resulted in an additional 102 samples. Due to RGS17-RH’s stability in up to 10% DMSO (Figure 7), a total of 272 samples was required to screen RGS17 binding fragments, significantly less than the 1,000 samples that would have been required to screen fragments individually. This resulted in significant savings of $^{15}$N-labeled RGS17-RH protein, consumables (such as NMR tubes), and instrumentation costs, while increasing assay throughput. This ultimately led to the identification of seven fragments that are capable of binding to RGS17-RH, yielding a hit rate of 0.7% (Figure 8, Table 2). This is promising, as the hit rate from a fragment screen can serve to approximate the druggability of a protein of interest. Hit rates greater than 0.1% indicate that a target is more likely to be able to potently bind molecules derived from fragment structures [93]. It is important to note that the 0.7% hit rate refers to RGS17 binding only, if RGS17-RH inhibition (as determined via GAP activity) is used for hit criteria, the rate is lowered to 0.2% (Fragments 3 and 4), which is still above the 0.1% threshold.

By titrating increasing concentrations of fragment at a fixed concentration of RGS17-RH and monitoring CSP using $^1$H-$^{15}$N HSQC, six of these compounds were found to have $K_D$ values ranging from 0.5 to 5.5 mM (Figure 10, Table 2). Using the chemical structures of the compounds and these NMR-derived $K_D$ values, LE for each fragment was calculated. The LE values obtained ranged from 0.18 to 0.31 (Table 2). While $K_D$ values observed are in the approximate range of what is to be expected [94], the LE values are rather low, as a threshold of 0.3 is generally considered acceptable for an initial fragment screening hit worth pursuing in FBLD [95]. In fact, the compound
with the highest LE value, 4, was later found to induce structural changes in RGS17 causing instability at the highest concentrations tested, indicated by a loss of signal in $^{1}H$-$^{15}N$ HSQC RGS17-RH spectra. Moreover, these changes were also observed against RGS17-RH C117S and RGS4. This showed that the loss of structural integrity observed was not occurring through a cysteine-dependent mechanism, which is promising, but it was not specific to RGS17 (Figure 12). 3 was found to interact with RGS17-RH’s C117 residues in a covalent manner. Titration of this compound induced concentration-independent perturbation of the RGS17-RH HSQC spectrum, whereas no significant changes were observed in the RGS17-RH C117S spectrum. This compound also caused structural instability in RGS4-RH, an RGS family member that is known to be sensitive to thiol modification [37, 40, 41].

Having confirmed that these seven fragments are capable of binding to RGS17-RH, it was determined if these compounds were capable of inhibiting RGS17-RH’s GAP activity towards G$\alpha_{i1}$ (R178M, A326S) using a colorimetric assay to measure free phosphate previously developed by the Roman lab [42]. Inorganic phosphate is released during GTP hydrolysis due to G$\alpha_{i1}$ GTPase activity, which is accelerated in the presence of RGS proteins. The R178M mutation reduces the intrinsic GTPase of G$\alpha_{i}$, making phosphate release more RGS-dependent [96]. A326S mutation increases the rate of GDP release from G$\alpha_{i1}$ and subsequent GTP binding, the rate limiting step in G$\alpha$ cycling, so that the system does not require the GEF activity of a GPCR [97]. The only compounds found to reduce the GAP activity of RGS17-RH were 3 and 4, though only the reduction caused by 3 was found to be statistically significant (Figure 13a). This result was to be expected, as these two fragments were found to grossly affect the structural stability of
RGS17-RH in solution. Further, the lack of detectable inhibition by the other fragments may have been predicted due to low affinity of these fragments and the modest structural changes detected via NMR. Future work to improve the affinity of these fragments for RGS17 will be crucial for determining if these fragments can ultimately lead to RGS17-RH inhibition.

One of the ultimate goals of FBLD is to determine the structure of the target protein complexes with fragments. This allows an iterative process in which fragments are tested for binding, models are generated of fragment-bound target (experimentally or computationally), and new, larger compounds with potentially higher affinity are synthesized and tested, and the process repeats. In order to determine the structure of these complexes with atomic resolution, crystallography was pursued. In order to evaluate which fragments may be most likely to produce success in future crystallography efforts, differential scanning fluorimetry was also performed. This assay measures the fluorescence of a dye that exhibits drastically increased fluorescence when bound to unfolded protein over a range of temperatures, allowing the determination of protein melting temperatures (T_{m}) [98]. Previous work has identified that compounds that increase the T_{m} are more likely to yield high-resolution structures than compounds that decrease T_{m} [99]. Similar to results obtained in GAP activity assays, only 3 and 4 were found to affect the thermal stability of RGS17-RH. 3 reduced the T_{m} of RGS17-RH by approximately 11 °C, and a T_{m} change in the presence of 4 could not be calculated due to a melting curve without an appreciable inflection point, which indicates the T_{m}. Every other fragment tested resulted in very slight decreases in protein stability, though none of these reductions were statistically significant (Figure 13b).
Analysis of the identity and location of the residues that underwent fragment concentration-dependent CSP was performed to identify putative fragment binding sites. Interestingly, no fragments were found that perturbed the terminal bundle. Each fragment was found to cause changes in the region of the helical domain distal to the G\(\alpha\) interface. Interestingly, this region has previously been identified as a putative small molecule binding site in RGS4. In RGS4, residues on \(\alpha 5\) near this location mediate interaction with phosphatidylinositol 3, 4, 5-triphosphate (PIP\(_3\)) and CaM. This work found that PIP\(_3\) can inhibit the GAP activity of RGS4, and binding of CaM to RGS4 can prevent this inhibition [100]. As all the fragments tested except for 5 bind RGS17-RH and RGS4-RH, it is possible that this small molecule-binding site is conserved between these two family members. Moreover, it raises the hope that small molecules that bind this site may be capable of inhibiting RGS activity if larger fragment analogs with higher affinities are identified. Additionally, it is possible that 5 is capable of binding RGS4-RH but with lower affinity than it has for RGS17-RH, so that the fragment’s limit of solubility prevents detection of interaction with RGS4-RH in this protein NMR paradigm.

As fragments 1, 2, 5, 6, and 7 only cause slight reductions in the \(T_m\) of RGS17-RH at high concentrations, these fragments were selected for investigation using crystallography. Because these compounds only moderately change the stability of the protein, efforts to generate RGS17/fragment co-crystals are more likely to succeed. Though fragments 3 and 4 inhibit RGS17-RH, the drastic decrease in protein stability caused by these fragments is likely to hamper future structure-based drug design efforts. Efforts to reproduce previously reported crystallography conditions resulted in a structure that is higher resolution and more complete (electron density for more side chains and
α5-α6 loop) than the previously reported structure [91]. Interestingly, this structure also showed binding of Ca$^{2+}$ to RGS17 in several locations. Each monomer with the asymmetric unit showed Ca$^{2+}$ binding near Ser107 and Glu108, near the loop between α3 and α4. Additionally, one RGS17-RH monomer showed another Ca$^{2+}$ ion near Ser145 in the α5-α6 loop. NMR experiments verified that the α3-α4 site binds Ca$^{2+}$ and Mg$^{2+}$ in solution. It is also possible that the α5-α6 loop site binds Mg$^{2+}$ (and potentially Ca$^{2+}$), but difficulty in assigning residues in this region in the RGS17-RH HSQC spectrum precludes definitive confirmation of this second binding site. It was also found that excess Ca$^{2+}$ results in higher association of RGS17-RH with Gαo. This is especially interesting given that increased intracellular Ca$^{2+}$ can occur following a number of G protein-mediated signaling events. For example, Gαq activation (which RGS17 has GAP activity towards), results in increased Ca$^{2+}$ release from the endoplasmic reticulum through a phospholipase C (PLC) and IP$_3$ signaling mechanism [101]. Additionally, some isoforms of Gβγ are able to activate PLC and trigger Ca$^{2+}$ signaling [102]. Since increased intracellular Ca$^{2+}$ increases occur downstream of Gα signaling, it is possible that increased Gα-binding by Ca$^{2+}$-bound RGS proteins serves as a negative feedback mechanism to terminate G protein signaling following GPCR stimulation, but further studies will be required to probe this hypothesis. Determination of the effect of Mg$^{2+}$ binding on RGS function may prove more difficult. Gα subunits require Mg$^{2+}$ for nucleotide binding, so delineation of the effects of Mg$^{2+}$ on Gα nucleotide binding versus RGS binding will require carefully designed experimentation.
Chapter 3. RGS Inhibitor Selectivity and Cysteine Dependence

 Portions of this chapter have been submitted for publication and are undergoing peer review at Molecular Pharmacology as Hayes, M.P., Bodle, C.R., and Roman, D.L. Evaluation of the Selectivity and Cysteine-Dependence of Inhibitors Across the Regulator of G Protein Signaling Family, 2017.

3.1 Introduction

RGS protein family members each contain a 120 amino acid region called the RGS Homology (RH) domain, which directly binds GTP-bound Gα. Due to their ability to modulate signaling cascades occurring through a myriad of GPCRs, RGS proteins have been touted as attractive, but challenging, drug targets. This has led to a number of previous efforts to identify RGS inhibitors, with particular attention paid to RGS4. Initial efforts focused on design of inhibitory peptides, but attention soon turned to small molecule inhibitor discovery using HTS [35, 36, 39]. The first small molecule inhibitor of RGS4 identified was CCG-4986, which was quickly found to inhibit RGS4 through covalent, cysteine modification at an allosteric site opposite the Gα- binding interface [37-39]. Subsequent efforts identified other RGS4 inhibitors, such as CCG-63802, CCG-63808, CCG-50014, CCG-55919, UI-5 and UI-1590, all of which were found to directly interact with these allosteric cysteine residues and/or show cysteine-dependent inhibition [40-42, 44]. Chemical structures of these small molecules are shown in Figure 4.

The RH domain is nine α-helices arranged into two sub-domains, containing between zero (RGS6 and RGS7) and four (RGS2 and RGS4) cysteine residues. A sequence alignment of the RGS RH domains pertinent to this chapter is shown in Figure 18a, where cysteine residues are highlighted in yellow. Some of these cysteine residues are highly conserved, such as C117 (RGS17 numbering, red in Figure 18b), which is found in every RGS domain tested here, except for RGS6 and RGS7. Other cysteine
residues are unique to individual RGS isoforms (blue in Figure 18b). The cysteine located at RGS4 position 148 (purple, Figure 18b) is somewhat conserved, especially within the R4 family, and is present in RGS1, RGS2, RGS4, RGS8, and RGS16.

As RGS proteins are differentially involved in various signaling mechanisms, and therefore different physiological processes and disease states, we set out to explore the molecular pharmacology of RH domain-inhibitor interactions. This was achieved by comparing inhibitor selectivity to the intrinsic reactivity of RH domain cysteine residues of different family members. We also tested each compound against a cysteine-null (Cys to Ala) mutant for every RGS tested to confirm inhibitor cysteine-dependent activity.
3.2 Results

Purification of Wild Type and Cys-null RGS RH Domains

In order to evaluate the molecular pharmacology of RGS inhibitors, the performance of a set of RGS PPI and GAP experiments under uniform conditions was required. Previous work by the Roman lab developed several biochemical assays that detect either the formation of the RGS/Gα complex or measure RGS GAP activity towards Gα [42, 84]. As both of these paradigms required purified Gα subunits and RGS RH domains, the first step was the purification of these recombinant proteins. The Structural Genomics Consortium had previously optimized bacterial protein expression constructs of many of the RGS family members in order to facilitate crystallography efforts, while the Roman lab already had access to a number of additional RH domains [91]. Taken together, RH domains for the following family members were obtained for use in this study: RGS 1, 2, 4, 5, 6, 7, 8, 10, 14, 16, 17, and 18. All RH domains were cloned into bacterial protein expression vectors so that the resulting construct contained a tobacco etch virus (TEV) protease cleavable N-terminal 6X histidine tag. This was selected to allow the rapid purification of these proteins using a single chromatography column, namely a Ni\textsuperscript{2+} immobilized metal affinity chromatographic (IMAC) column. Using this strategy, 6X his-tagged RGS protein was separated from crude, bacterial lysate during the first IMAC run and the 6X his tag was cleaved using a 6X his-tagged TEV protease during simultaneous dialysis to remove excess imidazole from first IMAC run. Subsequently, the sample is subjected to a second round of IMAC to separate now untagged RGS protein from 6X his-tagged TEV. An example of this protein purification strategy is presented in Figure 19.
In addition to the wild type (WT) RH domains obtained for this study, cysteine-null mutants of the RGS domains that contain cysteine residues were also generated. Cysteine-null variants of the following RGS proteins were cloned into bacterial expression vectors and purified alongside their wild type counterparts: RGS 1, 2, 4, 5, 8, 10, 14, 16, 17, and 18. Yields for all WT and cysteine-null RH domains are presented in Table 3 and SDS-PAGE analysis of these proteins is shown in Figure 20. Yields for WT RGS domains ranged from 160 mg (RGS17) to 29 mg (RGS10) per L of bacterial culture, and cysteine-null mutants ranged from 120 mg (RGS17) to 16 mg (RGS5). Interestingly, mutation of the only cysteine residue in RGS5 resulted in drastically reduced yield, as did
mutation of the four cysteine residues found in RGS2. The relative yield of RGS10 was rather low independent of the presence of cysteine residues. Preparation and purification of RGS6 was not required, as a 95+% pure sample was available from previous efforts by the Roman lab.

<table>
<thead>
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<th>mg/L culture</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>WT 128</td>
</tr>
<tr>
<td></td>
<td>-Cys* 80</td>
</tr>
<tr>
<td>2</td>
<td>WT 117</td>
</tr>
<tr>
<td></td>
<td>-Cys* 23</td>
</tr>
<tr>
<td>4</td>
<td>WT 117</td>
</tr>
<tr>
<td></td>
<td>-Cys 104</td>
</tr>
<tr>
<td>5</td>
<td>WT 136</td>
</tr>
<tr>
<td></td>
<td>-Cys* 16</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
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<tr>
<td>7</td>
<td>WT 80</td>
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<td>WT 84</td>
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<td>WT 29</td>
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<td>-Cys* 120</td>
</tr>
<tr>
<td>18</td>
<td>WT 87</td>
</tr>
<tr>
<td></td>
<td>-Cys* 46</td>
</tr>
</tbody>
</table>

* Protein constructs unique to this study

**Table 3. RGS Protein Yield from E. coli**

WT and Cys-null RGS RH domains have GAP activity

The GAP activity against $\alpha_{i1}$ of recombinant RGS proteins was determined using the previously described malachite green colorimetric assay (Figure 21). For the WT RH domains tested, each resulted in GAP activity appreciably above baseline ($\alpha_{i1}$ alone). Unsurprisingly, RGS2 only reached 21% of the maximum GAP activity observed (100% normalized to GAP activity of 1 µM RGS1), as it has been found to be relatively
specific for $\Gamma_\alpha_q$ in most biochemical assays. RGS6 only resulted in 46% maximal GAP activity. Protein concentrations higher than 3.16 µM were not pursued, as higher

\[ \text{Figure 20. SDS-PAGE of Recombinant RGS Proteins} \]

A) IMAC purified wild type RGS protein RH domains. B) IMAC purified cysteine-null RGS protein RH domains. RGS isoform is indicated above well. Each gel contains 25 µg protein stained with coomassie blue.

concentrations can lead to increased protein precipitation following assay quenching, negatively affecting absorbance readings and assay results. Higher potency was observed for the RGS2 cysteine-null mutant, as WT had an EC$_{50}$ of 1100 nM, whereas the mutant’s EC$_{50}$ was 390 nM. The mutant also exhibited higher maximal GAP activity (47%) than
wild type (21%). Mutation of the cysteine residues in RGS1, RGS4, RGS16, RGS17, and RGS18 had limited effect of GAP activity, as for each of these isoforms the WT and cysteine-null exhibited overlapping EC$_{50}$ values at the 95% confidence interval. Mutation of the cysteine residues in RGS8 and RGS10 had slight effects on the observed potency, as mutation increased the EC$_{50}$ from 40 nM to 95 nM (RGS8) and 220 nM to 560 nM (RGS10). RGS5 and RGS14 mutants had 18% and 29% lower maximal GAP activity, respectively, as compared to WT, without significant effects observed on EC$_{50}$.

Figure 21. GAP Activity of Purified RGS Proteins. A-D) Confirmation of GAP activity by WT and cysteine-null RGS RH domains used in this study. Data normalized to absence of RGS protein (0%) and activity of 1 µM RGS1 (100%). Data represent n=3 experiments ± S.E.M.

Inhibition of Biochemical WT RGS/$\alpha$$_{i}$ PPI

AlphaScreen PPI assays were developed for each of the purified RH domains (except WT and cysteine-null RGS2), and the selectivity of a panel of previously described RGS4 inhibitors were tested. Structures of compounds tested are shown in Figure 4 and AlphaScreen PPI inhibition results are presented in Table 4. Interestingly, no appreciable inhibition was observed for RGS6 or RGS7 at compound concentrations
up to 100 µM. As all inhibitor IC₅₀ values against these proteins were well above 100 µM (the highest concentration tested), these results are not included in Table 4. Only UI-1590 showed inhibition of RGS18, though it was not potent, with an IC₅₀ of 95 µM. While this may not be a surprising result for RGS6 and RGS7, as they lack cysteine residues, RGS18 contains two cysteine residues.

CCG-4986 was found to be the most potent against RGS4 with an IC₅₀ of 4.8 µM, and double-digit µM IC₅₀ values were obtained for RGS14, RGS1, RGS16, RGS5, RGS17, and RGS8. CCG-50014, which was previously thought to be selective for RGS4, exhibited 5-fold selectivity for RGS14 over RGS4. This compound resulted in sub-µM IC₅₀ values for RGS14, RGS4, RGS1, and RGS16, and single-digit µM IC₅₀ values for RGS5, RGS17, and RGS8. CCG-55919 exhibited IC₅₀ values overlapping at the 95% CI for RGS4 and RGS14, whereas CCG-63802, CCG-63808, UI-5, and UI-1590 were more selective for RGS14 over RGS4. CCG-63802, CCG-63808, and UI-5 showed little to no inhibition against RGS5, RGS10, and RGS17. These same compounds showed double-digit µM IC₅₀ values for RGS1, RGS8, and RGS16. UI-5 was the most selective compound tested, as it was over 13-fold selective for RGS14 over RGS1.

Inhibition of Biochemical Cysteine-Null RGS/Gαₒ PPI

No appreciable inhibition of PPI formation between the cysteine-null RGS proteins purified above and Gαₒ was detectable with any of the tested inhibitors, except CCG-55919 and CCG-50014 against cysteine-null RGS14. The observed inhibition by these compounds was not potent, as CCG-55919 and CCG-50014 had IC₅₀ (95% CI) values of 77 (51->100) and 40 (20-80) µM, respectively. These values are 27.5- and
40,000-fold lower than the ICs0 values observed against WT RGS14 for CCG-55919 and CCG-50014, respectively.

<table>
<thead>
<tr>
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<th>ICs0, µM (95% CI)</th>
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<tr>
<td>RGS</td>
<td>CCG-4986</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>(27-47)</td>
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<tr>
<td>4</td>
<td>4.8</td>
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<td>(3.2-7.3)</td>
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<td>5</td>
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<td>(39-72)</td>
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<td>8</td>
<td>64</td>
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<td>(49-84)</td>
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<tr>
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<td>(42-87)</td>
</tr>
<tr>
<td>18</td>
<td>&gt;100</td>
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</table>

Table 4. Inhibition of WT-RGS/Gα PPI Summary of biochemical PPI inhibition by RGS inhibitors using AlphaScreen assay. Data are ICs0 values calculated from mean of n=3 experiments with 95% CI given in parentheses. *Data reported in [43].

Inhibition of RH Domain GAP Activity

Next, it was tested how these same inhibitors were able to affect the function of RGS proteins by determining GAP activity, as opposed to inhibition of PPI formation as tested above (Table 5). This was achieved with the malachite green colorimetric phosphate detection assay. Each RGS protein was tested at a concentration that corresponded to its respective EC80, as experimentally determined. RGS proteins for which an EC80 could not be reliably calculated from these data were excluded from analysis (RGS14). Also excluded from analysis were RGS proteins for which no to
minimal inhibition was observed in the AlphaScreen paradigm (RGS6, RGS7, and RGS18).

Similar to the AlphaScreen result, CCG-4986 was found to be the most potent against RGS4 with an IC$_{50}$ of 3.1 µM and 20-fold selectivity over the only other isoform for which inhibition was detectable, RGS1. CCG-50014 was found to inhibit each RGS tested. CCG-50014 was the most potent against RGS1 and RGS4 for which it was over 30-fold more selective over RGS16. Similar to results for CCG-4986, CCG-55919 was 4-fold more potent against RGS4 than RGS1, the only other isoform inhibited. CCG-63802 was 3-fold selective for RGS4 over RSG1, and less potent against RGS5, RGS8, RGS16, and RGS17. CCG-63808 and UI-1590 were most potent against RGS1 and RGS4, with overlapping IC$_{50}$ at the 95% CI, followed by double-digit µM IC$_{50}$ for RGS5, RGS8, RGS16, and RGS17.

<table>
<thead>
<tr>
<th>RGS</th>
<th>CCG-4986</th>
<th>CCG-50014</th>
<th>CCG-55919</th>
<th>CCG-63802</th>
<th>CCG-63808</th>
<th>UI-1590</th>
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<tr>
<td>4</td>
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<tr>
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<tr>
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<td>(43-100)</td>
<td>(33-74)</td>
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</table>

Table 5. WT-RGS GAP Activity Inhibition by Small Molecules Disruption of GAP activity as determined using malachite green PO$_4$ detection assay. Data are IC$_{50}$ values calculated from mean of n=3 experiments with 95% CI given in parentheses. NC indicates a value was not calculable due to lack of inhibition.

Interestingly, the IC$_{50}$ values of CCG-63808 and CCG-50014 against RGS1 and RGS4 overlapped when measuring GAP activity but not PPI formation. CCG-50014 and CCG-5919 were much less potent against RGS5, RGS8, RGS16, and RGS17 in the GAP.
assay as compared to the PPI assay results. This could mean that disruption of the PPI is not sufficient to effectively reduce GAP activity in vitro or it could be due to the increased concentrations of protein required in the GAP assay versus PPI. Additionally, this could be attributed to different Ga subunits used between the assays, as PPI employs rat Ga_o and the GAP assay uses human Ga_i1 (R178M, A326S).

*RGS RH Domain Cysteine Reactivity Determined Using DACM*

Every RGS inhibitor tested in this study exhibited drastically reduced activity against the cysteine-null RH domain mutants, pointing to cysteine-dependent mechanisms of action. It was then tested if the relative potency differences of these inhibitors were simply a function of the intrinsic accessibility and/or reactivity of these cysteine residues. This was assessed using DACM (structure shown in Figure 4), which labels cysteine residues due to its maleimide moiety and undergoes a dramatic increase in fluorescence when bound to protein cysteine residues. Reactivity was tested at multiple pH values, as reaction of a maleimide with cysteine should increase with increasing pH up to the pK_a value of the residue, approximately 8-9 [103]. Additionally, at higher pH values maleimides are less selective for cysteine and can react with other protein residues, so the highest pH tested was 7.5.

Treatment of WT-RH proteins with a molar equivalent of DACM produced dramatically different rates of DACM reaction among RGS isoforms (Table 6, Figure 22). The proteins tested could be grossly categorized into three groups: 1) readily DACM reactive 2) moderately reactive 3) unreactive. Reaction of RGS1, RGS2, RGS4, RGS16, and RGS18 with DACM was appreciably higher than either buffer alone or the cysteine devoid RGS6 and RGS7 at each pH tested. An intermediate level of reactivity was
observed for RGS14 at each pH tested and RGS10 at pH 7.0 and 6.5, but not 7.5. RGS5, RGS8, and RGS17 were labeled with DACM minimally at each pH. Reactivity was quantified from kinetic reaction traces by determining the time required to reach half maximal fluorescence ($t_{0.5}$).

Interaction with DACM was also somewhat independent of the number of cysteines present in each RGS protein. RGS18 exhibited the shortest $t_{0.5}$ at all the pHs analyzed, though it did not exhibit the highest maximal fluorescence signal observed, and only contains two cysteine residues. The next lowest $t_{0.5}$ values measured at each pH were for RGS4, quickly followed by RGS1, which contain four and three cysteines, respectively. RGS4 exhibited higher maximal fluorescence than RGS1 in every case, possibly due to containing an additional cysteine. RGS16 was the next most reactive RH domain, with an approximately four times higher $t_{0.5}$ value compared to RGS4 at pH 7.5 and 7.0. The $t_{0.5}$ of RGS2 and RGS16 were similar at pH 7.5, but RGS2 was less reactive than RGS16 at pH 7.0. The reaction of RGS14’s two cysteine residues was slow, displaying an approximately linear rate, so $t_{0.5}$ values were not calculable, but appreciable fluorescence was present at every pH analyzed. Similar results were obtained for RGS10 at pH 6.5 and 7.0, though a $t_{0.5}$ value was only calculable at pH 7.0. Alternatively, RGS5, RGS8, and RGS17 only showed minimal interaction with DACM. RGS5 (one cys) reacted slower than RGS8 (two cys), though, paradoxically, the $t_{0.5}$ for both proteins did not decrease with increasing pH, as would be predicted. RGS10 and RGS17 both only have calculable $t_{0.5}$ values at pH 7.5 due to kinetic reaction curve shape, though the magnitude of these reactions (fluorescence intensity) is so low, that they may be of limited relevance (Table 6, Figure 22).
Investigation of RGS/Go PPI in Living Cells

A split NanoLuciferase (NanoLuc) system was used to assess the formation of RGS/Go11 PPI in cells. In this system, NanoLuc is divided into a large, 18 kD segment (LgBiT) and a small, 1.3 kD segment (SmBiT), each of which can be fused to either the N- or C-terminus of either interaction partner of a PPI. When a PPI is formed, a catalytically active NanoLuc is formed, and luminescence is measured in the presence of NanoLuc substrate. Initial efforts to characterize this assay had difficulties in differentiating PPI-specific luminescence from background, but it was discovered that addition of AlF4− to cell media allowed measurement of RGS/Go-specific luminescence (Figure 23). Addition of AlF4− allowed for formation of Go -GDP- AlF4− in cells, the transition state mimic for which RGS proteins have high affinity [1]. Comparing luminescence signal of wells treated with either vehicle or AlF4− allowed measurement of RGS/Go PPI, which was further explored here in the presence of small molecule RGS inhibitors.

RGS1, RGS4, RGS8, RGS14, RGS16, and cys-null RGS4 in the presence of CCG-4986, CCG-50014, and CCG-63802 were tested in this system with Go11. RGS2 was tested with Goq. These compounds were chosen as each was a potent RGS inhibitor in both PPI and GAP assays and has been previously used for in vivo analysis of RGS effects [104-106]. CCG-4986 and CCG-50014 inhibited several RGS proteins in this paradigm, but these compounds also inhibited the control PPI provided with the assay, so no definitive conclusions about these compounds can be drawn. Compound CCG-63802 reduced the signal of RGS1, RGS4, RGS2, and RGS14, as compared to control. The most
robust inhibition was observed for RGS4, followed by RGS2 and RGS14, and then RGS1 (data not shown).

Figure 22. DACM Reactivity of WT-RGS Proteins Fluorescent traces of 1 µM of both DACM and indicated RGS. A-C) Proteins with appreciable DACM reactivity at indicated pH. D-F) Proteins with limited DACM reactivity at indicated pH, expanded in G-I. Data are normalized to buffer alone (0%) and maximal response of RGS16 at pH 7.5 (100%) and represent mean of n=3 experiments ± SEM.

3.3 Discussion

In this chapter, the RGS isoform specificity of six previously identified RGS4 inhibitors was analyzed. Inhibition of the RGS/Gα biochemical PPI, RGS GAP activity, and RGS/Gα cell-based PPI was determined against up to twelve WT RGS family members and ten cysteine-null mutants. Further, DACM was used to evaluate the intrinsic accessibility/reactivity of the cysteine residues within the WT RH domains of these proteins to better understand the differences in inhibitor specificity within the family.
Inhibition of RGS6, RGS7, and RGS18 could not be detected, while inhibition of the RH domains of RGS1, RGS2, RGS5, RGS10, and RGS14 by small molecules was uncovered. Notably, some of the compounds used in this study, namely CCG-50014, CCG-4986, and CCG-63802, have previously been used in animal models of RGS4 activity in neuroprotection and nociception. The data presented here raises the possibility that the in vivo effects of these compounds are due to inhibition of multiple RGS isoforms rather than RGS4 specifically.

<table>
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<tr>
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<tr>
<td></td>
<td></td>
<td>(69-80)</td>
<td>(218-232)</td>
<td>(742-781)</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>74</td>
<td>225</td>
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<tr>
<td>2</td>
<td>4</td>
<td>275 (269-282)</td>
<td>1170 (1140-1200)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>61 (55-68)</td>
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<td>316</td>
</tr>
<tr>
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<td>1</td>
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<td>614 (508-746)</td>
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</tr>
<tr>
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<td>2</td>
<td>531 (355-799)</td>
<td>476 (390-583)</td>
<td>&gt;1000</td>
</tr>
<tr>
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<td>1</td>
<td>n.d.</td>
<td>472 (414-539)</td>
<td>&gt;1000</td>
</tr>
<tr>
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<td>2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>224 (216-233)</td>
<td>623 (605-641)</td>
<td>&gt;1000</td>
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<tr>
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<td>n.d.</td>
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<tr>
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<td>2</td>
<td>10 (9-12)</td>
<td>59 (56-63)</td>
<td>244 (237-251)</td>
</tr>
</tbody>
</table>

Table 6. DACM-Cysteine Reactivity of RGS Proteins Summary of DACM experiments with purified RH domains. Data represent time to half maximal fluorescence of n=3 independent experiments each with duplicate wells and 95% CI in parentheses. Curves indistinguishable from cysteine-lacking RGS6 and RGS7 are indicated as n.d.

The finding that five of the seven compounds tested here were more potent against RGS14 rather than RGS4 was unexpected, as previous reports demonstrated selectivity for RGS4. The most surprising result was for CCG-50014, one of the most
potent RGS4 inhibitors described in the literature (IC50 of 30 nM against RGS4) [41, 44]. Here, CCG-50014 was found to be 5-fold more selective for RGS14 over RGS4, without overlapping IC50 values at the 95% CI. Taken together, these data establish that RGS14 is highly sensitive to inhibition and deserves further investigation as a druggable target. It is also notable that for every compound interrogated, activity against the cysteine-null mutants was abolished (or reduced by multiple orders of magnitude).

Having determined that the compounds tested all showed cysteine-dependent inhibition, it was investigated if the newly established RGS isoform selectivity profile of these compounds was due to the number of RH domain cysteine residues. RGS6 and RGS7 lack cysteine residues and no inhibitors for these proteins were identified. Alternatively, five of the seven compounds tested were more potent against RGS14 (two cysteines) than RGS4 (four cysteines), but RGS18, which also contains two cysteines was refractory to inhibition. Additionally, RGS17 only contains one cysteine and RGS8 contains two, but every compound tested showed similar IC50 values against these proteins. Taken together, this indicates that the number of cysteine residues is not a critical determinant of potential inhibition by small molecules, so cysteine

![Figure 23. RGS/Ga Cell-Based PPI via NanoBiT](image)

a) Typical raw luminescence of time for HEK293T cells transfected with indicated NanoBiT vectors in the presence and absence of AlF4⁻. b) Kinetic luminescence trace with background (no AlF4⁻) subtraction. Data represent n=3 ± S.D. Adopted from [107].
reactivity/accessibility using DACM was explored to better understand inhibitor selectivity profiles.

Results from the DACM experiments shed some light on the inhibitor selectivity observed in biochemical PPI and GAP assays. For example, CCG-4986 and CCG-55919 only show inhibition of the GAP activity of RGS1 and RGS4, which react with DACM with lower $t_{0.5}$ values than RGS5, 8, and 17, which exhibit little DACM activity. Further, every other compound tested is more potent against RGS1 and RGS4 in GAP activity assays than RGS5, 8, and 17. RGS14 shows only intermediate DACM reactivity, but nearly every compound explored here potently inhibits PPI formation with $G_{\alpha_\omega}$.

Alternatively, RGS10, which also shows intermediate DACM reactivity, is only inhibited by three of the tested compounds, and this inhibition is not potent, as all IC$_{50}$ values are greater than 20 µM.

Taken together, this indicates that RGS inhibition by cysteine-reactive small molecules is a complex process that is regulated by more than the simple number of cysteine residues present in the RH domain or the global reactivity/accessibility of these residues. Additionally, it is possible that other reactive residues play some role, but these were not explored in this work. It is possible that the selectivity profile of these inhibitors is actually determined by covalent modification of a specific cysteine residue or set of cysteine residues with this domain. Therefore, identification and assessment of accessibility/reactivity of the critical cysteine residue(s) needed for small molecule inhibition may yield more interesting results. For example, RGS10 and RGS14 are closely related, as both reside in the R12 family and share >52% identity within the RH domain. RGS14 contains an additional cysteine residue near its C-terminus in $\alpha_9$ and is
readily inhibited by many of the compounds tested, whereas RGS10 lacks this residue and is only weakly inhibited by three of the compounds tested. This provides some preliminary evidence that this C-terminal cysteine residue may be an important determinant for inhibitor specificity. It also remains possible that protein dynamics and/or altered cysteine pK_a values contribute to inhibitor specificity across the RGS family.

This study was also confined to examination of the RH domains of these RGS proteins, but some RGS families, like the R12 and R7, contain accessory domains that serve functions independent of Gα GAP activity. Future efforts utilizing full-length RGS protein constructs could shed light on the selectivity and inhibitory activity of these inhibitors in a more physiologically relevant context. This is especially true for RGS14, which contains a GoLoco domain with guanine-nucleotide dissociation inhibitor activity that prevents dissociation of GDP from the Gα subunit, altering Gα signaling in a GAP-independent manner. Thus, physiological consequences of RGS14 GAP inhibition on the total regulation of Gα signaling by RGS14 demands further attention.

In conclusion, we have further developed the selectivity profile of several previously identified RGS inhibitors against 12 WT RGS proteins, about 60% of the family, and 10 cysteine-null mutants. This revealed that several compounds identified as RGS4 inhibitors display increased potency against RGS14.
Chapter 4. AC8/CaM PPI HTS Assay Development and Pilot Screen

Portions of this chapter have been reprinted/adapted from Hayes, M.P., Soto-Velasquez, M., Fowler, C.A., Watts, V.J., and Roman, D.L. Identification of FDA-approved small molecules capable of disrupting the calmodulin-adenyl cyclase 8 interaction through direct binding to calmodulin, ACS Chemical Neuroscience, 2017. Copyright 2017 American Chemical Society

4.1 Introduction

Adenylyl cyclases (AC) form cyclic AMP (cAMP) from ATP during signal transduction through multiple pathways, the most notable of which is downstream of GPCR activation, making them an attractive target for drug discovery. As intracellular cAMP levels are tightly controlled, AC activity is regulated by numerous signaling proteins through a number of mechanisms. These regulators include Gβγ, Gαi, Gαs, protein kinases A and C, and Ca2+/CaM. Importantly, CaM only activates AC1, AC3, and AC8, but robust increases in cAMP formation following Ca2+/CaM binding is unique to AC1 and AC8 [71-73]. AC stimulation by CaM occurs through direct interaction between CaM and intracellular AC regions, so inhibition of these PPIs could act to diminish AC-mediated cAMP signaling [71, 72].

AC1 and AC8 interact with CaM in different manners. As described above, the intracellular regions of ACs are separated into five regions: N-terminus (Nt), two catalytic regions (C1a and C2a) and two regulatory regions (C1b and C2b). While AC1’s first cytosolic regulatory (C1b) and second catalytic (C2a) domains bind CaM, CaM interacts with the (Nt) and second cytosolic regulatory domain (C2b) of AC8 (Figure 24a) [74, 75]. Furthermore, on AC8, the Nt and C2b regions interact with CaM in different manners. The AC8-Nt contains a 1-5-8-14 motif and Nt binding to CaM only proceeds if the C-terminal lobe is Ca2+ bound, while the AC8-C2b CaM binding region
harbors an IQ-like motif, and its interaction with CaM is dependent upon Ca$^{2+}$ binding to the N-terminal lobe of CaM [74, 82]. As the CaM-interacting intracellular regions of AC1 and AC8 are divergent, there is hope that small molecule PPI inhibitors may be able to selectively inhibit the interaction between CaM and AC8 or AC1.

![Figure 24](image)

**Figure 24. AC1 and AC8 CaM Interactions** a) Structure of AC domains with CaM-binding regions of AC1 shown in red and CaM-binding regions of AC8 shown in green. b) Schematic of biochemical fluorescence polarization assay developed for detection AC/CaM interactions.

Studies with mice lacking AC1, AC8, or both isoforms have identified that selective inhibition of AC1 or AC8 could be useful in a number of pathological states. Inhibition of AC1 could be clinically useful for treatment of chronic inflammatory pain [60]. AC8 inhibitors may be useful in the treatment of stress, stress-induced anxiety, and excessive alcohol consumption [61-63, 108]. These studies also noted that achieving inhibitor selectivity between AC1 and AC8 would be beneficial, as mice lacking both isoforms show memory impairments, an undesirable potential side effect. These memory
deficits are not as pronounced in mice lacking only a single CaM-stimulated AC isoform [67].

Previously identified AC inhibitors generally suffer from lack of selectivity among AC isoforms, as both the catalytic and FSK binding sites are conserved across the family, or a lack of potency, with IC$_{50}$ values in the high µM range. Additionally, some identified inhibitors have adenine-like structures, for which selectivity over other adenine nucleotide-binding proteins is a concern. Finally, some inhibitors are not cell permeable due to the inclusion of phosphate groups. Due to these concerns, alternative mechanisms of achieving inhibition in an AC isoform-specific manner deserve future attention. The mechanism that will be further explored here is the inhibition of AC1 and AC8 by inhibition of their interactions with CaM.

CaM is an intracellular Ca$^{2+}$ sensing protein composed of N-terminal and C-terminal lobes that each contains two EF hands that bind Ca$^{2+}$. When CaM binds Ca$^{2+}$ its conformation changes so that it can form PPIs with various CaM-target proteins. The CaM target proteins relevant to this chapter are AC1 and AC8. The conformational change that CaM undergoes exposes hydrophobic patches that previous groups have identified as capable of binding small molecule inhibitors. These inhibitors generally contain an amine located close to a hydrophobic region, as determined through analysis of structure-activity relationships [109]. Previously identified CaM inhibitors used here are calmidazolium chloride (CDZ), W7, and trifluoperazine (TFP). All three of these compounds have been well studied due to their ability to prevent CaM-dependent activity of other proteins [110-112]. Most importantly, each of these molecules has been reported to inhibit CaM-mediated AC activity, with CDZ being the most potent [113, 114].
Due to the role of AC8 in ethanol consumption, stress, and stress-induced anxiety, HTS was used to identify novel small molecule inhibitors of the AC8/CaM interaction.

Figure 25. Purification of HisGST-CaM Fusion Protein Resulting FPLC chromatograms for recombinant protein purification from E. coli. a) IMAC column to capture 6X his-tagged protein from crude lysate b) Glutathione column to bind GST-tagged protein c) IMAC column to remove CaM from 6X his-tagged GST and TEV protease proteins d) SDS-PAGE analysis of purification steps. Flow1= flow through from first IMAC column, IMAC1= peak from first IMAC column, Flow2= flow through from glutathione column, GST Peak= peak from glutathione column, IMAC 2 Load= post cleavage with TEV protease, IMAC 2 Flow= flow through from second IMAC column (i.e. untagged CaM). Blue traces represent absorbance at 280 nm, and green traces represent %B.

4.2 Results

*Development of a Biochemical Assay to Detect AC8-CaM Interaction*

Towards execution of an HTS campaign to find AC8/CaM inhibitors, a fluorescence polarization (FP) assay was developed. FP was selected because it is a
relatively cheap and robust technology that is amenable to HTS microplate format. The fluorescent probes selected were peptides corresponding to the Nt and C2b CaM-binding domains of AC8. Peptides from these areas were shown to bind CaM in a Ca\(^{2+}\)-dependent manner [74, 82]. These peptides were N-terminally labeled with the fluorescent dye Cy5. Assays were developed for both peptides, as information regarding which region of AC8 is most critical for CaM-stimulated activity was lacking. In general, polarization increases with the difference in size between the fluorescently labeled probe and unlabeled “receptor”. A 6X his-tagged GST (25 kD) was incorporated on the N-terminus of CaM (14 kD) to create a fusion protein to bolster the size difference between the AC8 peptides (~4 kD) and CaM. A schematic of this newly developed FP assay is shown in Figure 24b. This tag also allowed the rapid purification of this protein construct from *E. coli* using affinity chromatography, as shown in Figure 25.

Preliminary experiments were performed to determine the relative concentrations of Cy5-labeled AC8 peptides and GST-CaM that were required for maximum polarization. Generation of concentration response curves using 100 nM labeled peptide in the presence of increasing concentrations of GST-CaM resulted in increased binding as measured by increased polarization for both AC8 peptides. Additionally, this increase in polarization was Ca\(^{2+}\)-dependent, as addition of EGTA (Ca\(^{2+}\)-chelator) decreased the affinity of the peptides for GST-CaM. The AC8-Nt and AC8-C2b peptides exhibited pEC\(_{50}\) values of 6.92 ± 0.07 and 7.27 ± 0.07 for Nt and C2b, respectively, in the absence of EGTA. The addition of EGTA resulted in 8- and 250-fold decreased affinity for AC8-Nt and AC8-C2b peptides, respectively (Figure 26a,b). To show that the increased polarization observed is in fact due to peptide binding, competition experiments with
increasing concentrations of unlabeled AC8-Nt and C2b peptides were performed. Indeed, unlabeled peptides were able to compete off their labeled counterparts, resulting in pIC\textsubscript{50} values of 5.3 ± 0.1 and 4.95 ± 0.04 for AC8-Nt and AC8-C2b, respectively. To further confirm that peptides were binding directly to CaM, \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra were acquired of isotope labeled CaM in the presence of unlabeled peptides (Figure 27). Drastic changes in the backbone chemical shifts were observed. In fact, CSPs were so numerous and drastic that peptide binding site identification was not possible without the need to perform triple resonance experiments to re-assign the protein amide backbone resonances in the presence of peptide.

Having established that this new assay detected AC8 peptide/CaM interactions, determination of assay suitability to HTS was assessed. As preliminary evidence showed that associations of CaM with these peptides was Ca\textsuperscript{2+}-dependent, inclusion of EGTA in the assay buffer was evaluated as a potential positive control. Wells with and without EGTA were evaluated at varying incubation times, ranging from 1 to 4 hours, and concentrations from 0% to 5% DMSO (common HTS library solvent) for both AC8 peptides. Z’ was calculated for each condition tested, and in every case it was well above 0.5, the lower threshold for an assay that is excellently suited for HTS (Table 7, Figure 28) [115].

**Screening of Selleck FDA-Approved Library Against AC8 peptides/CaM**

A chemical library composed of approximately 1000 FDA-approved drugs was subsequently screened using the AC8-Nt and AC8-C2b/CaM interaction assays. An experiment to probe the day-to-day variability was performed. Screening a subset of the
compound library on two different days showed that the same hit compounds were identified (Figure 29). Following this experiment, the entirety of the FDA-approved compound library was screened against both assays. The results of these screens are presented in Figure 30 and summarized in Table 8. Z’ remained robust through screening, with values ranging from 0.74 to 0.83, based on in-plate positive and negative control wells. Using 10 standard deviations from the mean value of negative control wells as a hit cutoff, an initial hit rate below 1% was observed for both screens. 10 SD from the negative control averages correlates to 32% inhibition (relative to EGTA control) for the AC8-Nt assay and 24% inhibition for the C2b screen.

**Figure 26. AC8 peptide/CaM FP Assay Development** FP results for 100 nM Cy5 labeled AC8-Nt (a) or AC8-C2b (b) and indicated concentration of GST-CaM in the presence and absence of 10 mM EGTA. c) Competition of unlabeled AC8-Nt peptide with 100 nM Cy5 AC8-Nt and 316 nM GST-CaM. d) Competition of unlabeled AC8-C2b peptide with 100 nM Cy5 AC8-C2b and 316 nM GST-CaM. Data represent mean of n=3 experiments ± S.E.M.
Figure 27. NMR Confirmation of AC8 Peptide Binding. $^1$H-$^{15}$N HSQC spectra of $^{15}$N labeled CaM in the absence (black) and presence (red) of 3-fold molar excess unlabeled AC8-Nt (a) and AC8-C2b (b) peptides.
Figure 28. HTS Compatibility of AC8/CaM FP Stability in DMSO of CaM FP assay with AC8-Nt (a) and AC8-C2b (b). Stability over time of CaM interaction with AC8-Nt (c) and AC8-C2b (d). In each case, data were generated using 316 nM GST-CaM and 100 nM Cy5-labeled peptide in the presence (open circles) and absence (closed circles) of 10 mM EGTA. Data represent values obtained for \( n \geq 24 \) wells for each condition.

Cy5 was selected as the fluorophore for peptide labeling in this screening paradigm, in part, because red shifted-dyes are less susceptible to fluorescent interference than commonly used shorter wavelength dyes [116]. Regardless, absorbance wavelength scans were acquired for initial hits to identify compounds that were potentially optically interfering with the assay. Two initial hits were identified that exhibited significant absorbance in the regions of the light spectrum used for Cy5 excitation and emission (Figure 31). These compounds were designated as interfering compounds and were not included in future work. Post screening analysis also identified that one initial hit was the large, cyclic peptide caspofungin. This hit was also excluded from future work due to its unfavorable structure.
Ultimately, AC8-Nt/CaM primary screening yielded six hits that inhibit the FP assay. Two of these compounds were capable of inhibiting the AC8-C2b/CaM, as well. The structures of these hit compounds are presented in Figure 32, along with the structures of CaM inhibitors CDZ, W7, and TFP. Interestingly, TFP was contained within the FDA-approved library but did not inhibit either assay to the 10 SD cutoff that was selected.

**FP Characterization of Screening Hits**

Concentration response curve analysis was performed to confirm AC8 peptide/CaM inhibition and prioritize compounds based on potency. CDZ, W7, and TFP were also tested for concentration-dependent inhibition. Surprisingly, neither TFP or W7 showed inhibitory activity against CaM/AC8-C2b. Against the CaM/AC8-Nt assay, TFP displayed inhibition but not until the highest concentration tested (100 µM) with a pIC$_{50}$ of 4.4 ± 0.6, while W7 showed no inhibition (Figure 33). CDZ showed potent (pIC$_{50}$= 5.56 ± 0.01) and complete (relative to EGTA control) inhibition of CaM/AC8-Nt and inhibited the CaM/AC8-C2b interaction by approximately 50%. Hits identified in the CaM/AC8-Nt screen had more than an order of magnitude difference between the most potent (1, pIC$_{50}$= 5.67 ± 0.02) and least potent hits (2, pIC$_{50}$= 4.57 ± 0.02). The AC8-C2b assay was only inhibited approximately 50% by 1 and 4 (Figure 34).

### Table 7. FP Assay Stability for HTS

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<td>Z'</td>
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<td>Z'</td>
<td>Time (hr)</td>
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<th>AC8-C2b</th>
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The AC8-C2b assay was only inhibited approximately 50% by 1 and 4 (Figure 34).
Figure 29. Day-to-Day AC8/CaM FP Reproducibility FP signal versus well position for AC8-Nt/CaM using FDA library plate 3 on day one (a) and day two (c). Plate position heat map on day one (b) and day two (d).

<table>
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<th>AC8-C2b</th>
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<tr>
<td><strong>Initial Hits (10 S.D. cutoff)</strong></td>
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<tr>
<td><strong>Interfering Compounds and unfavorable structures</strong></td>
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<tr>
<td><strong>Hits</strong></td>
<td>6 (0.59%)</td>
<td>2 (0.20%)</td>
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Table 8. AC8 Peptide/CaM Screening Summary
Figure 30. Screening Results AC8-Nt (a) and AC8-C2b (b) peptide interactions with CaM were screened against 1,018 FDA-approved compounds. Numbers correspond to structures shown in Figure 32. 10 standard deviation cutoff and average EGTA containing control values indicated with dashed lines. TFP is data point corresponding to TFP position in library. Compounds optically interfering with assay or with unfavorable structures are shown as grey.

Detergent Sensitivity of AC8/CaM Screening Hits

During concentration-response curve analyses it was noted that each compound tested exhibited steep Hill slopes that deviated rather strikingly from -1, ranging from
-2.7 ± 0.2 to 1 to -3.5 ± 0.2 for 3 and that logP values were greater than 3. Previous reports in the literature indicated that these observations are often found with compounds that inhibit assays through formation of large, soluble aggregates [117]. To determine if small molecule aggregation was occurring, concentration response analysis was performed with the inclusion of 0.01% Triton X-100 in the assay buffer. Inclusion of detergent has been found to solubilize some aggregates, such that if a molecule is forming inhibitory aggregates its potency will be drastically different in the presence of Triton [118, 119].

**Figure 31. Optically Interfering Hits** 100 μM hit fragments in assay buffer with absorbance monitored from 350 to 700 nm. Hits without absorbance are indistinguishable from background. Hits crystal violet (red) and mitoxantron (green) show appreciable absorbance in spectral region used for Cy5 excitation and emission (grey).

AC8-Nt hits identified showed slight changes in potency following inclusion of detergent, but inhibition was still detectable in every case. The IC$_{50}$ observed for 1 decreased from 2.1 μM to 1.4 μM in the presence of detergent, whereas the IC$_{50}$ for 2 saw a moderate increase from 27 μM to 50 μM. IC$_{50}$ values for 3 and 4 approximately doubled from 17 μM and 5.4 μM to 33 μM and 9.1 μM, respectively. No appreciable change was observed for 5 or 6. For AC8-C2b/CaM, inclusion of detergent had minimal
effects of the potency of 1 or 4, as the pIC$_{50}$ values were nearly identical, but CDZ was approximately half an order of magnitude less potent in the presence of Triton. Increased absolute inhibition relative to EGTA control was observed for all compounds in the presence of detergent in the AC8-C2b/CaM assay.

Figure 32. Chemical Structures of CaM Inhibitors and FDA Hits a) Structures of previous identified CaM inhibitors used in this study b) Screening hits identified from FDA library. Common names: 1, alexidine; 2, benzethonium; 3, otilonium; 4, thonzonium; 5, cetrimonium; 6, domiphen.

*CaM-AC8 NanoBiT Assay Development*

Testing of these newly identified hits against the AC8/CaM PPI in cells required the development of a novel assay, so the NanoBiT split luciferase system was chosen. As described in Chapter 3, this system uses a split NanoLuc to measure luminescence due to PPI formation in living cells. As either protein can be tagged at either terminus with either Lg- or Sm-BiT luciferase fragments, 2$^3$ possible NanoBiT vector combinations exist for a given PPI. These vectors were created and Ca$^{2+}$-stimulated AC8/CaM
complementation was assessed in transiently transfected HEK293T cells. Additional experiments showed that tagging AC8 with the NanoBiT fragments did not abolish catalytic activity, as increased cAMP accumulation was observed in response to A23187 and/or FSK in HEK293T cells transiently transfected with the AC8 NanoBiT vectors generated (data not shown).

**Figure 33. W7 and TFP Do Not Potently Inhibit AC8/CaM FP**
Concentration response analysis of classical CaM inhibitors W7 and TFP was performed using newly developed AC8-Nt/CaM (a) and AC8-C2b (b) polarization assays. Data represent mean of n=3 experiments ± S.E.M.

NanoBiT response following addition of intracellular Ca$^{2+}$ concentration altering compounds was determined. The conditions tested were buffer alone, thapsigargin (releases intracellular Ca$^{2+}$) with and without BAPTA-AM (cell permeable Ca$^{2+}$ chelator) or A23187 (cell permeable Ca$^{2+}$ ionophore), as the specific luminescence due to CaM/AC8 interaction should be Ca$^{2+}$-dependent. Baseline luminescence was recorded for 7.5 min, followed by addition of Ca$^{2+}$-mobilizing agent. Luminescence was recorded for an additional hour, but NanoBiT signal typically returned to baseline levels 22.5 post-Ca$^{2+}$ agent addition. Area under the curve analysis was then performed to quantify luminescence over this 22.5 minute span and plotted in Figure 35. Also included was a negative control Sm-BiT vector, which should not form a PPI with either the AC8 or CaM LgBiT-tagged constructs, to ensure observed luminescence is not due to the low
level of LgBiT auto-luminescence [120]. The resulting curve of a typical kinetic
luminescence experiment is presented Figure 35.

Testing of all possible NanoBiT combinations identified that the highest levels of
luminescence required CaM containing the Lg-BiT tag. This resulted in a 3-fold increase
in Ca$^{2+}$-dependent luminescence over the highest luminescence observed for conditions
in which AC8 was tagged with the Lg-BiT. Further, the greatest luminescence
measurements were obtained when the N-terminus of AC8 is Sm-BiT labeled, which is
interesting given that the AC8-Nt region binds CaM. When AC8 was C-terminally tagged
with Sm-BiT, limited luminescence was observed. This could be due to the tag being too
distant from labeled CaM in the AC8/CaM complex to form a complete NanoLuc, or it
could mean that even this small tag is sufficient to occlude CaM binding.

Another key characteristic that was desirable in a cell-based AC8/CaM PPI assay
was sensitivity to Ca$^{2+}$. Both conditions using N-terminal Sm-BiT-tagged AC8 showed
robust increases in luminescence in the presence of either A23187 or thapsigargin relative
to buffer treated control. Further, for both these conditions, pre-treatment with cell-
permeable Ca$^{2+}$ chelator BAPTA-AM reduced the signal observed upon subsequent
addition of thapsigargin. CaM N-terminally tagged with Lg-BiT and AC8 N-terminally
tagged Sm-BiT was the condition selected for all subsequent experiments, as it showed a
robust luminescence increase in response to A23187 and thapsigargin, and it showed the
most dramatic BAPTA-AM-dependent decrease in luminescence.

**FP Hit Inhibition of CaM-AC8 PPI in Cells**

The CaM inhibitors and AC8/CaM FP hits described above were assessed for
their ability to disrupt this PPI in living cells. Additionally, cytotoxicity of all compounds
used were tested to address if any apparent PPI inhibition observed was an artifact of increased cell death or loss of plasma membrane integrity.

Figure 34. Concentration Response Curves and Detergent Sensitivity of FDA Hits Polarization in the presence of increasing concentrations of CDZ and FP screening hits against AC8-Nt/CaM assay (a) and AC8-C2b assay (b) in the presence and absence of 0.01% Triton X-100. Data represent mean of n=3 experiments ± S.E.M.
Table 9. Polarization Assay Concentration Response Summary  

<table>
<thead>
<tr>
<th></th>
<th>AC8-Nt, pIC$_{50}$</th>
<th>AC8-C2b, pIC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cLogP$^a$ 0% Triton 0.01% Triton</td>
<td>0% Triton 0.01% Triton</td>
</tr>
<tr>
<td>CDZ</td>
<td>10.3 5.55 ± 0.01 5.54 ± 0.07</td>
<td>5.5 ± n.c. 5.01 ± 0.05</td>
</tr>
<tr>
<td>TFP</td>
<td>4.9 4.4 ± 0.6 n.d. -</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>4.3 5.67 ± 0.01 5.85 ± 0.03</td>
<td>5.23 ± 0.07 5.23 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>6.1 4.57 ± 0.02 4.3 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>6.3 4.78 ± 0.01 4.48 ± 0.05</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>8.1 5.27 ± 0.02 5.04 ± 0.06</td>
<td>5.0 ± n.c. 4.9 ± n.c.</td>
</tr>
<tr>
<td>5</td>
<td>6.2 4.94 ± 0.02 4.94 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>6.1 4.84 ± 0.02 4.81 ± 0.02</td>
<td>-</td>
</tr>
</tbody>
</table>

Data represent mean of n=3 experiments ± S.E.M.  
$^a$ Value retrieved from ZINC15 [121].

CDZ was the most potent CaM inhibitor tested and led to a dramatic loss in cell viability at 31.6 $\mu$M (Figure 36). W7 and TFP were both marginally more potent against the cell-based PPI than the biochemical FP assay. TFP resulted in complete inhibition (relative to buffer only treated control) at 31.6 $\mu$M, whereas W7 resulted in approximately 80% inhibition at this concentration. Neither of these compounds reduced cell viability. These degrees of inhibition were higher than those observed in either FP assay at equal concentrations.

In the cell-based PPI assay, 1 was the most potent hit with a pIC$_{50}$ of 5.91 ± 0.07, a value comparable to that observed in the FP biochemical assay. Similar to CDZ, 1 showed reduced cell viability at concentrations above 10 $\mu$M. There was more than a 10-fold difference between the PPI inhibition and cell viability curves. For example, at 3.16
µM, 1 shows approximately 85% PPI inhibition with no effect on cell viability. The next most potent hits in vitro were 2 and 3, which had pIC50 values of 5.4 ± 0.1 and 5.3 ± 0.5, respectively. 31.6 µM 3 reduced cell viability to 77 ± 6%, but 2 showed no effect at this concentration.

Approximately equal potencies were observed for 4 and 5, with the curve shapes obtained very similar to that of CDZ. At 31.6 µM, 5 reduced viability to 89 ± 13%, but 4 did not show appreciable toxicity. With a pIC50 of 5.2 ± 0.1, 6 inhibited the CaM/AC8 PPI in cells with minimal cytotoxic effects (Figure 36).

**Figure 35. AC8/CaM Cell-Based PPI NanoBiT** a) Area under curve (AUC) analysis over first 22.5 minutes following treatment with indicated compound of AC8 and CaM labeled with NanoBiT tags as indicated. Data normalized to baseline luminescence before compound addition (0%) and AUC of CaM-LgBiT/SmBiT-AC8 in presence of thapsigargin (100%). Data represent mean of n=3 experiments ± S.E.M. b) Representative kinetic trace of luminescence normalized to baseline.
After evaluating ability of FDA hits to inhibit AC8/CaM PPI, both biochemically and in living cells, it was critical to determine if this disruption could translate to decreased cAMP production by AC8. Also assessed was their selectivity for AC8 over CaM-stimulated AC1. CDZ and I were the most potent compounds tested against AC8.
with pIC$_{50}$ values of 5.33 ± 0.11 and 5.31 ± 0.06, respectively. Both compounds completely inhibited CaM-stimulated AC activity. All other hits tested against AC8 had similar potencies with pIC$_{50}$ values ranging from 4.76 ± 0.04 for 6 to 4.41 ± 0.04 for 4 data not shown. Hits 1, 3 and 6 were not selective, as they inhibited AC1 and AC8 with similar potencies. Alternatively, 2, 4, and 5 were marginally more potent against AC8 than AC1, though the most selective hit identified, 5, was only 1.8-fold selective for AC8 over AC1, with an IC$_{50}$ of 22 µM against AC8 and 40 µM against AC1. Interestingly, each hit except 4 inhibited AC8 activity to the same degree as CDZ (data not shown).

**FDA Hits Directly Bind CaM**

Post-hoc analysis of the chemical structures of the FDA-approved hits identified during screening indicated that their mechanism of action might involve directly binding CaM. The hits adhered to the basic CaM-binding pharmacophore as each has a basic nitrogen atom connected to large, hydrophobic region. That most of these compounds inhibited AC8 and AC1 with similar potencies *in vitro* further supported this hypothesis. $^1$H-$^{15}$N HSQC spectra of CaM in the presence of 5-fold molar excess hit compounds 1, 2, 4, and 6 were acquired. Each hit analyzed bound and changed the conformation of CaM (Figure 37). CSP observed were so dramatic that accurate binding site identification could not be determined.

### 4.3 Discussion

AC8 is an attractive potential drug target for the treatment of stress, anxiety and excessive alcohol consumption. AC8 inhibitors would result in decreased cAMP production, but identification of AC inhibitors with favorable molecular characteristics
that bind either the ATP or FSK sites has been difficult for numerous reasons. The efforts described here provide evidence that AC8/CaM PPI inhibition is a viable method to reduce AC8 activity. Development of a fluorescence polarization assay that measured interaction between CaM and peptides derived from the regions of AC8 that mediate binding allowed the screening of a small chemical library of FDA-approved small molecules. Experiments to analyze the Ca\textsuperscript{2+} dependence and reversibility (with unlabeled peptide) of complex formation further validated the assay, and peptide binding was confirmed via NMR. Most importantly, Z’ for screening was excellent and stable over time and in DMSO. Screening identified nine compounds that were able to disrupt the
AC8 peptides’ ability to bind CaM, but three of these compounds were excluded due to interference with assay technology or unfavorable chemical structures, ultimately, yielding six hits.

The most potent hit in each assay performed was 1, and it was identified as an inhibitor of both AC8 polarization assays, though it was slightly more potent against AC8-Nt (IC$_{50}$= 2.1 µM) than AC8-C2b (IC$_{50}$= 5.9 µM). 1 was more potent than even CDZ against AC8-Nt/CaM, which is noteworthy given that CDZ was the only CaM inhibitor with significant effects on AC1 activity of 39 tested [114]. Furthermore, 1 was more potent than any other compound tested, including CDZ, in disruption of AC8/CaM in living cells. Both CDZ and 1 reduced cell viability at high concentrations, but in the case of 1, this only occurred at concentrations beyond those required to fully inhibit the NanoBiT signal. Also, 1 was the most potent hit tested in AC activity inhibition for both AC1 and AC8, though no selectivity was observed, as pIC$_{50}$ values were overlapping with experimental error taken into account. Interestingly, 1 had a single-digit IC$_{50}$ in every paradigm tested here, with the largest inter-assay difference occurring between NanoBiT (IC$_{50}$= 1.2 µM) and AC8 cAMP accumulation (IC$_{50}$= 4.9 µM). 1 (alexidine) is an antiseptic with a bisbiguanide structure that is bactericidal and can be apoptosis-inducing when analyzed using in vitro and in vivo cancer models [122, 123].

Hit 4 (thonzonium) was the only other compound found to inhibit both AC8 polarization assays, and similar to 1, was more potent against the Nt peptide (IC$_{50}$= 5.4 µM) than the C2b (IC$_{50}$= 10 µM). 4 showed greater than 50% inhibition of the NanoBiT assay at 10 µM, but in AC8 functional assays, it inhibited cAMP formation with an IC$_{50}$
of 39 µM. This compound is used as a surfactant and previous work has identified both compounds 1 and 4 can inhibit vacuolar ATPase proton transport [124].

Hits 5 (cetrimonium) and 6 (domiphen) share structural elements with 4. Each of these compounds contains a long, aliphatic chain connected to a quaternary amine, which is also bound to two methyl groups. While 4 is more potent in the FP assays, 6 was found to be more potent in both the cell-based PPI assay and AC8 activity assays. 5 is a surfactant that induces apoptosis in cancerous cells, while 6 is an oral antiseptic [125, 126].

Hit 3 (otilonium) binds L-type Ca\(^{2+}\) channels, reducing gut smooth muscle cell contractility, making it useful for treatment of diarrhea [127]. Interestingly, 3 has previously been identified as capable of binding CaM and inhibiting CaM-stimulated cyclic nucleotide phosphodiesterase activity [128]. Here it was most potent in the NanoBiT PPI assay, with an IC\(_{50}\) of 5 µM, whereas in AC8-Nt/CaM FP and AC8 cAMP accumulation IC\(_{50}\) values of 17 µM and 24 µM, respectively, were observed.

NMR was used to confirm binding of several of these molecules to CaM. Similar to results obtained for CaM in the presence of AC8 peptides, the large and numerous CSPs induced precluded binding site identification without additional time intensive multidimensional NMR experiments.

The goal here was to develop technology to identify small molecules capable of inhibiting AC8’s interaction with CaM. Two novel assays were developed, allowing the detection of AC8/CaM binding both biochemically and in living cells. 1018 FDA-approved small molecules were screened and six were identified as inhibitors. Subsequent effort proved that hits identified in the FP screen were in fact able to inhibit
CaM/AC8 association in cells, and, ultimately, AC8 cAMP activity, validating these methods as viable tools for small molecule inhibitor discovery. Although selectivity over AC1 was not achieved, these data indicate that disruption of the AC8/CaM interaction can result in the inhibition of cyclase activity. Further, the hits identified here may be useful preclinical tool compounds to probe the biology of AC1 and AC8 in relevant disease states.
Chapter 5. Conclusions and Future Directions

5.1 Conclusions

The work presented here focused on identifying and better understanding modulators of protein-protein interactions that result in increased intracellular cAMP, following activation of GPCR signaling mechanisms. Excessive RGS protein activity results in decreased inhibitory Gα subunit signaling, and increased AC activity. Binding of Ca^{2+}/CaM to AC1 and AC8 results in robust stimulation of catalytic activity, increasing cAMP formation. Additionally, this work is unified under the common theme of drug discovery using HTS. In Chapter 2, due to the lack of success of previous HTS efforts using ‘drug-like’ chemical libraries, an alternative, medium throughput method of fragment-based ligand discovery was pursued and the first steps towards structure-based drug discovery targeting RGS17 were taken. In Chapter 3, the selectivity and cysteine-dependence of RGS inhibitors previously identified through HTS were analyzed against the largest panel of purified recombinant RGS proteins assembled to date. In Chapter 4, HTS- and microplate-compatible biochemical and cell-based assays were developed, and a pilot screen of FDA-approved small molecules discovered six AC inhibitors that disrupted AC/CaM PPIs.

RGS17 Fragment Screening

In the second chapter of the work described here, a novel fragment screen using protein-detected NMR was performed to identify novel chemotypes capable of binding to the RH domain of RGS17, as small molecule inhibitors of RGS17 could be clinically useful as treatments for multiple types of cancer. FBLD was pursued as HTS against RGS17 has provided results with limited potential utility. This FBLD screen identified
seven fragments that bind RGS17. One of these compounds was found to bind RGS17 in a cysteine-dependent manner and was nonspecific for RGS17, as it could also bind RGS4. Another fragment induced structural instability in RGS17 non-specifically and independent of cysteine residues, as similar results were observed against RGS17 C117S and RGS4. Interestingly only these two fragments were able to affect either the melting protein temperature or RGS GAP activity, as determined using DSF and malachite green assays, respectively. No other hit fragments caused significant changes in either of these paradigms. Additional work to identify RGS17 crystallization conditions in collaboration with the Lyon lab identified a divalent cation binding site on RGS17. This site located in the α3-α4 loop bound either Ca^{2+} or Mg^{2+} in solution, as determined using protein-detected NMR. The existence of a second cation binding site at the end of α5 remains possible. Further, it was found that excess Ca^{2+} was sufficient to cause increased maximal association of RGS17 with Ga\textsubscript{o} in a biochemical PPI assay.

**RGS Inhibitor Specificity**

Due to their nature as attractive drug targets, a number of previous efforts using HTS have sought to identify RGS inhibitors. Most of these efforts focused on RGS4 and subsequent medicinal chemistry efforts have yielded a few compounds that were touted as RGS4 specific. In Chapter 3, the selectivity of several RGS inhibitors against 12 RGS family members (~60% of the RGS family) and 10 cysteine-null mutants was assessed using a biochemical PPI assay. This identified that a number of previously RGS4 specific inhibitors show selectivity for RGS14 in this paradigm, a novel finding. Follow up work using a GAP activity assay found that these molecules also contained high affinity for RGS1 as well as RGS4, though the low intrinsic GAP activity of RGS14 precluded
testing against this isoform. As all compounds were found to exhibit cysteine-dependent activity, as evidenced by the drastically reduced affinity for the cysteine-null mutants in PPI assays, the reactivity/accessibility of the cysteine residues in the wild type RGS RH domains was assessed using DACM. This showed that reactivity was somewhat independent of the number of cysteine residues and that reactivity did not perfectly correlate with inhibitor specificity. Notable RGS14 was readily inhibited but exhibited only intermediate DACM-cysteine reactivity. RGS18 reacted readily with DACM, but was generally refractory to small molecule inhibition. Alternatively, RGS4 and RGS1 were reactive with DACM and readily inhibited, whereas RGS5, 8, and 17 were not reactive with DACM, and inhibitors showed lower potencies against these isoforms. Overall, this identified new inhibitors of some RGS isoforms, such as RGS1 and RGS14, and furthers the understanding of the molecular determinants of cysteine modification by small molecule RGS inhibitors.

AC8/CaM Inhibition

In Chapter 4, novel assay methodologies were developed and a pilot screen was performed to identify novel inhibitors of the AC8/CaM PPI. Inhibitors of AC8 would be useful therapeutics for the treatment of stress, stress-related anxiety, and alcohol consumption. PPI inhibition was explored because previous efforts to identify isoform specific AC inhibitors has proved difficult, and CaM stimulation was only observed in Group I ACs. A novel biochemical FP assay was designed and 1,000 FDA-approved drugs were screened. Six drugs were identified that interrupted CaM association with AC8 peptides. Subsequently, development of a cell-based AC8/CaM PPI NanoBiT assay showed that these same compounds disrupted interaction of full-length AC8 with CaM in
cells. These compounds also inhibited AC8 cAMP formation in cell membrane preparations. Ultimately, these compounds were not selective for AC8 over AC1, but they could still be used in mouse models to evaluate small molecule inhibition of CaM-stimulated AC activity.

5.2 Future Directions

**RGS17 Fragment Screening**

In Chapter 2, a library of 1,000 fragments was screened using protein-detected NMR and identified seven that bind RGS17, one of which shows cysteine-dependent activity. To date, this is the first report of a fragment screen against an RGS protein for which hit compounds were identified. Additionally, this is the first report of RGS17-binding compounds with an identified binding site. Another key finding from this work was the identification of at least one divalent cation binding site on the surface of RGS17 that interacts with GTP-bound Ga.

This is an advancement in the field of RGS17 inhibition because for the first time binding site information is known. This allows for a number of future possibilities. First and foremost, screening of additional fragments needs to be completed. This work represented a pilot screen of 1,000 fragments to prove that fragment screening against an RGS protein using protein-detected NMR is possible and can generate interesting results. There remain another 1,500 compounds from the Maybridge Ro3 library alone, which warrant evaluation for RGS17 binding. As the RGS17 $^{1}$H-$^{15}$N HSQC is now assigned and screening variables, such as the protein’s DMSO tolerance, have been established, screening of these compounds, or even larger fragment libraries, is more accessible than
ever before. Additionally, for each of the fragments identified, many larger, more complex chemical analogs are commercially available. Evaluation of this focused set of compounds, a process often referred to as called ‘Structure-Activity Relationships by Catalog,’ could allow for identification of fragments with higher affinity for RGS17 and/or identification of binding sites adjacent to those occupied by currently identified fragments.

Another avenue worth further exploration is the identification of fragment binding poses with atomic resolution using X-ray crystallography. This can be accomplished through determination of conditions for co-crystallography of fragment/RGS17 complexes and/or soaking of RGS17 crystals in solutions containing high concentrations of hit fragments. Preliminary efforts to this end resulted in determination of a higher resolution, more complete structure than previously available. Importantly, the side chains of residues near the fragment binding sites are more complete in this new model. This could allow for \textit{in silico} prediction of binding poses, and these models could be confirmed or refuted by a combination of site-directed mutagenesis of residues determined to be critical for fragment binding and protein NMR.

Aside from the higher resolution structure, another key finding from preliminary crystallography work was the identification of a divalent cation binding site on RGS17. RGS17 is capable of binding both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} in solution, and excess Ca\textsuperscript{2+} increases the association of RGS17 with G\textsubscript{a0}. Determination of the effect of this on RGS activity may require the development of new methodology, as most RGS GAP activity assays measure PO\textsubscript{4} release, which in the presence of excess Ca\textsuperscript{2+} can lead to formation of insoluble precipitates. One method which may be useful is an amalgamation of techniques
generated in Chapters 3 and 4. NanoBiT tagged RGS17 and Gαi1 could be used to monitor AlF4−-dependent RGS17/Gα PPI in cells following treatment with thapsigargin or A23187 in the presence and absence of BAPTA-AM to alter intracellular Ca2+ levels. Additionally, either HSQC titration experiments or isothermal titration calorimetry with various physiologically relevant divalent cations, such as Ca2+, Mg2+, Zn2+, Cu2+, and Mn2+, could be performed to determine the affinity and selectivity of RGS17 for these ions. Further, of the RGS family members purified in Chapter 3, the residues in RGS17 that bind Ca2+ (Ser107 to Glu109) are conserved in RGS1, RGS4, RGS5, RGS8, RGS10, RGS16, and RGS18. Evaluation of these proteins for metal binding deserves further attention.

RGS Inhibitor Specificity

In Chapter 3, a panel of 12 WT RGS proteins and 10 cysteine-null mutants were tested against a number of small molecule RGS inhibitors. The cysteine accessibility/reactivity of the WT RGS proteins was also determined using the environmentally-sensitive thiol probe DACM. This work determined that several inhibitors touted as being RGS4 selective, due to their selectivity for RGS4 over the closely related RGS8, are in fact more potent inhibitors of RGS14 in a biochemical, AlphaScreen PPI assay. Interestingly, these same compounds were orders of magnitude less potent against RGS10, which is closely related to RGS14. This is important to the field of RGS drug discovery as it uncovers that simple analysis of 1° structure is not sufficient to predict relative order of RGS inhibition by small molecules. This highlights the need for comprehensive experimental evidence before making claims of inhibitor specificity for one RGS family member over others. Furthermore this work highlights that while the number and reactivity of cysteine residues is not the sole determinant for
inhibition, it is one contributing factor, as no inhibitor activity could be observed against RGS6 and RGS7, which lack RH domain cysteine residues. DACM analysis identified that RH domains could be roughly divided into those with readily reactive cysteine residues, those with moderate reactivity, and those without appreciably reactive cysteines. In general, inhibitors were found to be less potent against RGS proteins without appreciable reactivity.

Future work to characterize RGS RH domain interactions with small molecule inhibitors will further the understanding of selectivity across the family. As all the compounds tested showed cysteine-dependent activity, future experiments are needed to determine which cysteine residues are critical for inhibition. One can envision this being achieved through PPI and GAP assays using single cysteine RGS mutants. As the cysteine at position 117 in RGS17 is the most conserved across the family, analysis of compound potencies against mutants containing only this cysteine would be of the greatest interest. This would also allow for a more robust quantitative analysis of the kinetics of cysteine reactivity and accessibility using DACM, as only a single labeling site would exist on each RGS molecule. Additionally, creation of RGS chimeras by swapping whole helices between closely related RGS family members, like RGS10 and RSG14, could provide potential insights into inhibitor specificity. To compliment this, mass spectrometry of WT RH domains following incubation with cysteine-reactive inhibitors could be used to identify the cysteine residues that are covalently modified. Alternatively, some groups have begun to focus on RGS protein dynamics with interesting results, so it is possible that inhibitor specificity is due to concerted protein movements [129, 130]. Hydrogen-deuterium exchange experiments using either NMR or mass spectrometry could shed light
on which RGS proteins are most dynamic to determine if this better correlates with the inhibitor specificity data described in this work.

<table>
<thead>
<tr>
<th>% DMSO</th>
<th>AC1-C1b Fluorescence Polarization</th>
<th>AC1-C1b, AlphaScreen</th>
</tr>
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<tr>
<td></td>
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</table>

Table 10. AC1 HTS Assay Preliminary Z’

AC/CaM Inhibitors

In Chapter 4, inhibitors of AC8 association with CaM were sought, as these could have therapeutic utility for stress, anxiety, and ethanol consumption. A novel HTS-compatible FP assay to detect AC8 peptide/CaM interactions was developed, as was a cell-based NanoBiT PPI system using full-length AC8 and CaM. These experiments, combined with AC8 functional assays, allowed the identification of six inhibitors of AC8 that act by disrupting CaM binding. NMR experiments verified that these compounds directly bound and changed the structure of CaM. This represents an important finding to the field of AC inhibition, as it validates alternative methods of AC inhibition, such as disruption of PPIs. Also, as the compounds identified are FDA-approved, it remains possible that inhibition of CaM is involved in their mechanisms of action.

In this work, only approximately 1,000 compounds were screened, but six compounds with inhibitory activity were identified. Screening larger, more complex
chemical libraries could identify compounds with increased potency and selectivity for AC8/CaM over AC1. As the Z’ observed for AC8 FP assays were well above 0.5, miniaturization from 384-well to 1536-well format may be possible, allowing more rapid screening in the future. The NanoBiT assay may also be amendable to HTS, allowing for cell-based detection of PPI inhibitors, but Z’ for this assay has yet to be assessed.

The compounds identified here each had activity in every assay employed with single- and double-digit IC₅₀ values, such that they may deserve further attention as dual AC1/AC8 inhibitors. Previous work using AC1/8 double knockout mice showed memory issues due to complete lack of these ACs, but these small molecules can be used at varying concentrations in vivo to achieve varying levels of AC inhibition. This would provide insight into how critical AC1/AC8 selectivity is to achieve positive therapeutic outcomes without negative side effects. These compounds could be explored in mouse models AC8 related pathologies, like stress, anxiety, and ethanol consumptions, as well as chronic pain, which is AC1-mediated.
Figure 39. AC1-C1b/CaM AlphaScreen Development a) Diagram of AC1/CaM Alpha Screen assay using biotin-labeled CaM and FLAG-tagged AC1 peptide. b) Sensitivity of AC1 AlphaScreen to various concentrations of Ca\(^{2+}\) chelator EGTA. c) Assay stability over time. Z’ shown in Table 10.

Future AC8/CaM screening should seek to eliminate hits that are not selective for AC8 over AC1 earlier in the screening process. To this end, preliminary efforts have identified two screening paradigms using a peptide derived from the first intracellular regulatory region of AC1 (AC1-C1b), which mediates CaM binding (Table 10). FP, similar to that used for AC8, produced Z’ that are suitable for HTS for up to 6 hours and 2.5% DMSO (Figure 38). Also, AlphaScreen was developed as a second methodology, and acceptable Z’ was observed at each time point measured (Figure 39). These assays can serve as a counter screen to quickly triage AC8 hits that are not selective. These assays could also be used for future HTS campaigns to identify AC1/CaM inhibitors that could be useful in the treatment of chronic pain. Future work will screen the same FDA-
approved library against one or both of these AC1 assays and coupled with prior results of AC8 screening, this could be used to identify AC1-specific hits. Future work will also need to develop an AC1/CaM NanoBiT assay, similar to the one produced here for AC8, but preliminary results indicate that alternative DNA constructs may need to be generated, as very low levels of Ca\textsuperscript{2+}-induced luminescence are observed.
Chapter 6. Materials and Methods

6.1 RGS Methods

Cloning and Molecular Biology

Wild type RGS protein constructs were obtained from Addgene and were a gift from Nicola Burgess-Brown [Addgene plasmid # 39143 (RGS1), 38812 (RGS2), 38932 (RGS6) 38813 (RGS7), 38805 (RGS8), 39138 (RGS10), 39139 (RGS14), 39140 (RGS16), 39141 (RGS17), 39142 (RGS18)]. Human RGS5 residues 52-185 and rat RGS4 residues 51-179 were cloned into pNIC-Bsa4, which was a gift from Opher Gileadi (Addgene #26103), using previously described ligation independent cloning methods [131]. Cysteine-null mutants and RGS17 C117S vectors were constructed by purchasing G Blocks (Integrated DNA Technologies, Coralville, IA) containing RGS coding sequences with all cysteine mutated to Ala (GCG codon) or for RGS17 C117S to serine (TCT codon). These sequences were amplified using PCR and cloned into pNIC-Bsa4 (Addgene #26103), using ligation independent cloning as previously described [131]. All RGS constructs were designed to code for the RGS RH domain with an N-terminal TEV protease-cleavable 6X-his tag.

Protein Purification

RGS protein-coding plasmids were transformed into BL21-CodonPlus(DE3)-RIPL cells and grown overnight at 37 °C on LB agar (RPMI) plates supplemented with selection marker encoded by plasmid (kanamycin or ampicillin), as well as chloramphenicol and spectinomycin. The next day, colonies were selected, seeded into Terrific Broth supplemented with appropriate antibiotic and chloramphenicol, and grown at 37°C and 275 -300 rpm until an OD_{600} of 2.0 was reached, as measured using a
Biotech Synergy 2 plate reader. Protein production was then induced with 0.5 -1 mM IPTG (RPMI), and culture was incubated for an additional 16 hours at 18 °C while shaking at 275-300 rpm. Bacterial cells were then pelleted at 3,500 g at 4 °C, re-suspended in 50 mm HEPES, 500 mM NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole at pH 8 (Buffer A) at 4 °C, lysed with lysozyme (1 mg per mL culture), and DNAse I (approximately 2 mg) was added to cleave DNA in sample and reduced lysate viscosity. Lysate was then subjected to multiple freeze-thaw cycles in liquid N2. Soluble and insoluble lysate fractions were separated by centrifugation at 100,000g.

Soluble supernatant was loaded onto immobilized metal affinity chromatography column (IMAC) (Ni Sepharose 6 Fast Flow, GE Healthcare, Chicago, IL). Sample loading was completed with 2 column volumes (CV, 1 CV= 18 mL) of buffer A, followed by column washing with an additional 5 CV of buffer A. A gradient from 0% to 20% buffer B (buffer A supplemented with 400 mM imidazole) over 5 CV removed non-specifically bound proteins from column, followed by the His-RGS protein elution step increasing from 20% to 100% buffer B over 10 CV.

Fractions containing RGS protein were then treated with His-tagged TEV protease at approximately 1:20 TEV:RGS molar ratio and dialyzed overnight at 4°C against 5 L buffer A to cleave 6X-His tag. Samples were again subjected to IMAC, like above, and flow through collected, resulting in >90-95% purity as determined using SDS-PAGE. Rate-altered mutant hGαi1 (R178M, A326S) and rGαo-GST were purified as previously described [42].

Isotope labeled (15N and 13C-15N) RGS17 was purified largely as above, with the notable exception that when culture OD600 reached 1.5, cells were pelleted at 3,500 g at 4
°C for 15 minutes, and re-suspended in an equal volume of M9 minimal media supplemented with 2 g D-Glucose-\(^{13}\)C\(_6\) and 1 g \(^{15}\)NH\(_4\)Cl (Cambridge Isotope Laboratories) per L culture for \(^{13}\)C-\(^{15}\)N sample or 1 g \(^{15}\)NH\(_4\)Cl for 1 g \(^{15}\)N sample. Isotope labeled samples were subjected to size-exclusion chromatography following IMAC to obtain 99+% pure RGS17-RH, as determined by SDS-PAGE, and concentrated to >1 mM in 20 mM potassium phosphate buffer containing 100 mM NaCl, 0.5 mM β-mercaptoethanol, and 2 mM NaN\(_3\), at pH 7.6.

*RGS17 \(^1\)H-\(^{15}\)N HSQC Assignment*

The following triple resonance experiments were performed at 25 °C using a 600 MHz Varian INOVA NMR spectrometer equipped with a triple resonance gradient probe to assign RGS17-RH backbone (and Cβ) chemical shifts: HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO). Data was processed and analyzed using NMR Pipe and CCPNAnalysis, respectively [132, 133]. Sample was > 750 µM in 20 mM potassium phosphate buffer containing 100 mM NaCl, 0.5 mM β-mercaptoethanol, and 2 mM NaN\(_3\), at pH 7.6.

*NMR Screen Liquid Handling*

5 µL of 100 mM fragment stocks of Maybridge Ro3 core set (1000 fragments) in 100% DMSO were combined into pools of six fragments (30 µL total) in 2 mL 96 DeepWell (Nunc) plates already containing 337.5 µL of 22 mM potassium phosphate, 110 mM NaCl, 0.55 mM β-mercaptoethanol, and 2.2 mM NaN\(_3\), at pH 7.6 using a Hamilton Microlab Star liquid handling system. Plates were then sealed and stored at -80 °C.
**RGS17 NMR Fragment Screening**

Immediately before screening, 50 µL D$_2$O and 82.5 µL of 606 µM $^{15}$N-RGS17 were added to each well to achieve 1 mM of each of six fragments and 100 µM RGS17 for each sample. Well contents were manually transferred to 5 mm NMR tubes (Deuterotubes). Samples were then analyzed using a Bruker Avance II 500 MHz spectrometer equipped with triple resonance gradient probe and B-ACS 60 sample changer. DMSO tolerance experiments were performed as above but with final concentrations of DMSO of 0%, 5%, and 10%. Pool deconvolution experiments were performed as above, except that each sample contained 5 µL of a single fragment and 25 µL DMSO. Data were processed and analyzed using NMRPipe and CCPNAnalysis, respectively, and acquired spectra were manually inspected for fragment-induced changes in chemical shifts.

**Fragment Affinity Determination via NMR**

For $K_D$ determination experiments, 100 µM $^{15}$N-RGS17 was incubated with fragment concentrations ranging from 100 µM up to the fragments’ limit of solubility (generally 2-10 mM). CSP was then plotted as function of fragment concentration (in mM) and data was fit to one-site specific binding model using GraphPad Prism. Ligand efficiency is a metric for ranking fragments for further development into lead compounds through additional screening and/or medicinal chemistry. It can be considered the change in free energy per heavy atom in a compound. It is calculated as:

$$LE= (-2.3039 \times R \times T / [HA \times \log(K_D)])$$
DACM Determination of Cysteine Reactivity of Fragment 3

10 µL of 8 µM RGS17 in 20 mM potassium phosphate buffer containing 100 mM NaCl, and 2 mM NaN₃, at pH 7.6 or buffer alone was added to 10 µL buffer, 10 µL of 80 µM fragment 3 or NEM in a black non-binding surface 384-well plate (Corning) and incubated at ambient temperature for 30 minutes. Following this 20 µL of 40 µM DACM (Anaspec) was added, and fluorescence was immediately measured for 1,000 seconds with a Perkin-Elmer Envision using excitation and emission wavelengths of 385 and 440 nm, respectively. This resulted in a final reaction mixture of 2 µM RGS17, 20 µM compound, and 20 µM DACM in phosphate buffer. Reactivity was determined by analyzing slope of DACM fluorescence of all conditions tested, with fluorescence in the absence of protein normalized to 0% and fluorescence of RGS17 with no compound treatment normalized to 100%. Statistical significance was analyzed using one-way ANOVA with multiple comparisons using GraphPad Prism.

RGS17 Fragment Differential Scanning Fluorimetry

DSF experiments were performed using final concentrations of 10 µM RGS17, 1 mM of indicated fragment or DMSO control, and 2X Sypro Orange dye (Thermo Fisher) in a total reaction volume of 20 µL. Fragment was incubated with RGS17 for 30 minutes at ambient temperature in white 384-well PCR plate (Sorenson) prior to addition of Sypro Orange. Fluorescence was then measured from 20 to 80 °C using a Roche 480 Lightcycler II. Tₘ values were calculated using DMAN [134].
**RGS17 Metal-binding Site Identification by NMR**

300 µM RGS17 in 50 mM HEPES, 100 mM NaCl, 0.5 mM β-mercaptoethanol, pH 7.6 in 10% D2O was incubated with or without 15 mM CaCl2 or 15 mM MgCl2 for 1 hour. Subsequently, 1H-15N HSQC spectra were acquired at 25 °C using either a 500 MHz Bruker Avance II (Ca2+ treated sample and control) or a 600 MHz Varian INOVA (Mg2+ treated sample and control) spectrometer each equipped with a triple resonance gradient probe.

**RGS17/Gαo AlphaScreen Binding in the Presence of Ca2+**

RGS17/Gαo binding was assessed as previously described using biotinylated RGS17 [43]. Biotinylated RGS17 was conjugated to streptavidin donor bead in Alpha Buffer (20 mM HEPES, 100 mM NaCl, 1% BSA, 1% Lubrol, pH 8). GST-rGαo was conjugated to anti-GST acceptor beads in Alpha buffer. Gαo mixture was supplemented with a 5 µM AlCl3, 5 mM MgCl2, and 5 mM NaF (AMF), and 2.5 mM GDP. Final concentrations were 100 nM RGS17, indicated concentration of Gαo, and 15 ng/µl of each bead. Assay was incubated for 1 hr at ambient temperature then fluorescence measured using a Perkin Elmer Envision plate reader. Wells lacking AMF represented negative control and were normalized to 0%, and wells containing RGS17 in Alpha buffer alone were normalized to 100%. Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

**Malachite Green GAP Activity Assay**

PO4 detection assay using malachite green was performed as described previously [42]. For RGS17 and fragment samples, final concentrations of 300 µM GTP, 1 mM
fragment, and 4.5 µM RGS17 were used. For assessment of RH domain GAP activities, RGS protein of interest was serially diluted in half logarithmic concentrations covering three orders of magnitude from 3.16 µM to 3.16 nM. Concentration response experiments performed in the presence of RGS inhibitors were performed using each RGS protein’s respective EC$_{80}$ protein concentration, which was determined experimentally following protein purification. Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

*RGS RH Domain/Ga$_o$ AlphaScreen*

AlphaScreen was performed largely as described in previous literature and above [43, 84]. Briefly, RH domains proteins were biotinylated according to manufacturer protocol with EZ-link NHS-Biotin (Thermo Scientific). Final concentrations for assay components are as follows: 10 nM for Ga$_o$, 15 ng/µl acceptor and donor beads, and 10 nM RGS RH domain. Notably, mutant RGS1 and RGS14 required altered RH domain concentrations to achieve appreciable signal (31.6 and 100 nM, respectively). Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

*DACM RH Domain Cysteine Reactivity*

In order to remove residual β-mercaptoethanol which interferes with DACM assay, extensive dialysis of RH domain samples was performed against 50 mM HEPES 500 mM NaCl at the indicated pH. First, 20 µL of 2 µM RH domain of interest was added to a black, low binding surface 384-well plate. This was followed by addition of an equal volume of 2 µM DACM. Then, fluorescence intensity was continuously recorded using a Perkin Elmer Envision plate reader for 1500 seconds. Excitation and emission
wavelengths of 385 and 440 nm, respectively, were used. Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

**NanoBiT RGS/Gαi1 Cell-based PPI Assay**

The NanoBiT cell-based PPI assay was performed largely as previously described [107]. NanoLuc substrate was prepared as a 5X stock and added to wells of a black 96-well half area, optical bottom plate at 10 µl per well. Baseline luminescence was measured for 30 minutes after reagent addition using BioTek Synergy 2, then 10 µl AlF$_4^-$ (8 mM NaF, 100 µM AlCl$_3$ final concentration) was added to wells to form Gα$_{i1}$-GDP-AlF$_4^-$ transition state, and luminescence measurements proceeded for another 30 minutes. Finally, 4X concentrated stock of compound of interest or DMSO control was added, and luminescence was recorded for an additional hour. Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

### 6.2 AC Methods

**Cloning**

Protein coding sequence for human CaM, corresponding to residues 1-149, (Addgene plasmid #47598) was cloned into pET-His6-GST-TEV-LIC (Addgene plasmid #29655) using ligation independent cloning and forward primer 5’-

TACTTCCAATCCAATGCAATGGCTGACCAGCTGA-3’ and reverse primer 5’-

TTATCCACTTCCAATGTTATTATTACTTTTCAGTCATCCTCATCTGTA-3’. This DNA construct yielded a bacterial protein expression vector in which CaM contained an N-terminal His-tagged GST tag with a TEV protease recognition site between the GST and CaM regions to allow for cleavage of the fusion protein (His-GST-TEV-CaM).
For NanoBiT experiments, human CaM residues 1-149 and rat AC8 residues 1-1248 were cloned into NanoBiT PPI TK/BiT MCS vectors, according to manufacturer directions using BglII/XhoI (rAC8) and NheI/XhoI (hCaM) restriction sites (Promega, Madison, WI). CaM vectors with N-terminal NanoLuc fragments used forward primer 5’-AGGAAACTCGAGCGGTATGGCTGACCAGCTGA -3’ and reverse primer 5’-AGGAAAGCTAGCCCTTACTTTGCAGTCATCTCTGTA -3’. CaM vectors with C-terminal NanoLuc fragments used forward primer 5’-AGGAAAGCTAGCATGGCTGA CCAGCTGA -3’ and reverse primer 5’-AGGAAACTCGAGCCCTTTGCAGTCATCATCTCTGTA -3’. AC8 vectors with N-terminal NanoLuc fragments used forward primer 5’-AGGAAACTCGAGCGGTATGGAACTCTCGGATGTG -3’ and reverse primer 5’-AGGAAAGATCTTTATGGCAAATCGGATTT -3’. AC8 vectors with C-terminal NanoLuc fragments used forward primer 5’-AGGAAAGATCTATGGAACTCTCGGATGTG -3’ and reverse primer 5’-AGGAAAGATCTATGGAACTCTCGGATGTG -3’. Sanger sequencing was used to confirm all DNA sequences (Iowa Institute of Human Genetics, Iowa City, IA).

Protein Purification

BL21-CodonPlus (DE3)-RIPL E. coli were transformed and grown while shaking at 37°C and 300 rpm until OD$_{600}$ of 2.0 was reached, at which point protein production was induced with 1.0 mM IPTG (RPMI), and protein production proceeded for 16 hours at 300 rpm and 18 °C. Culture was pelleted at 3,500 g and 4 °C and re-suspended in 50 mL of 50 mM Tris pH 8, 150 mM NaCl, 10 mM imidazole with protease inhibitor cocktail and flash frozen in liquid N$_2$. Prior to purification, pellets were thawed, 1 mg/ml chicken egg lysozyme (Sigma-Aldrich) was added, sample was agitated for 1 hour, and
sample was subjected to two additional freeze thaw cycles in liquid N\textsubscript{2} to achieve cell lysis. 100 µg DNase (Roche) was added to cleave bacterial DNA and reduce sample viscosity prior to centrifugation. Lysate was clarified by centrifugation at 100,000 g, and the supernatant was purified largely as described previously [135].

Briefly, lysate was loaded on Ni\textsubscript{66}FF resin using an AKTA FPLC (GE Life Sciences) and eluted via a gradient over 20 column volumes of resuspension buffer with 400 mM imidazole added. Fractions from the eluted peak were pooled and subjected to a GST affinity chromatography on Glutathione Sepharose 4FF column (GE Life Sciences) equilibrated with 50mM Tris pH 7.5, 1 mM CaCl\textsubscript{2}. GST-CaM was then eluted via gradient elution over 10 column volumes using 50 mM Tris pH 7.5, 10 mM glutathione, 1 mM CaCl\textsubscript{2}. For GST-CaM, pooled fractions (90+% pure, via SDS-PAGE and coomassie staining) were exhaustively dialyzed against 20 mM HEPES, 100 mM KCl, pH 7.4 at 4 °C to remove Ca\textsuperscript{2+}, flash frozen in liquid N\textsubscript{2}, and stored at -80 °C until needed.

To produce CaM lacking the GST tag, GST-CaM was incubated with 1:20 molar ratio of His-TEV to GST-CaM overnight at 4°C with simultaneous dialysis against 5 L of pH 7.4 20 mM HEPES, 100 mM KCl, 1mM CaCl\textsubscript{2}. Following cleavage and dialysis, sample was subjected to further IMAC to capture cleaved His-GST and His-TEV. The resulting flow through, containing 95+% pure (SDS-PAGE, coomassie staining) cleaved CaM, was collected, concentrated to > 10 mg/mL using a stirred concentration cell (Amicon, EMD Millipore, Billerica, MA), flash frozen in liquid N\textsubscript{2}, and stored at -80°C until needed.

\textsuperscript{15}N-labeled hCaM for NMR experiments was produced as above, with the
exception that RIPL were grown on M9 minimal media supplemented with 1 gram
\(^{15}\text{NH}_4\text{Cl}\) (Cambridge Isotope Laboratories) per L of media.

**Fluorescence Polarization (FP) Assay**

Peptides from human AC8 containing residues 30-54 (AC8-Nt) and residues 1191-
1214 (AC8-C2b), each with an additional N-terminal Cys residue labeled with Cy5 were
purchased from Genscript. AC1-C1b peptide corresponding to human AC1 residues 493-
520 with N-terminus directly labeled with Cy5 was obtained from Genscript. All FP
experiments were performed in 384 well, black polystyrene, non-binding plates (Corning
3575) and polarization was analyzed using a BioTek Synergy 2. This instrument was
equipped with an Excitation 620/40 nm filter, an Emission 680/30 nm filter, and a 660 nm
dichroic mirror with polarizers.

First, 20 µL of FP buffer (20 mM HEPES pH 7.4, 100 mM KCl, 50 µM CaCl\(_2\))
either with or without 3X EGTA (10 mM final) or 3X final DMSO concentration or 3X
concentrated indicated compound was added to each well. For experiments using Triton-
X100, this buffer aliquot also included 3X concentrated Triton. For competition
experiments, unlabeled peptide (Genscript, Piscataway, NJ) was included at 3X
concentration indicated. This was followed by 20 µL of GST-CaM 3X concentrated (316
nM final for all experiments except GST-CaM concentration response). The plate was
then incubated at ambient temperature for 30 minutes. Finally, 20µL Cy5-labeled peptide
at 3X concentration (100 nM final) in assay buffer was added to the plate, which was
further incubated for 2 hours or amount of time indicated during assay stability
experiments. Fluorescence was then measured and polarization (in mP) calculated as
follows:
\[ P = 1000 \times \frac{(I_{\text{parallel}} - I_{\text{perpendicular}})}{(I_{\text{parallel}} + I_{\text{perpendicular}})} \]

where \( P \) is polarization (in mP) and \( I \) is fluorescence intensity in indicated direction.

**FP Screen of FDA-approved Library**

For screening, 1 µL of 800 µM FDA-approved Drug Library (Selleck Chemical) in 100% DMSO was added to each well of 384-well plate that already contained 19 µL of FP buffer using a Hamilton MicroLab Star. Then, GST-CaM and Cy5-labeled peptide were added and polarization was measured as above. Final screening concentration of chemical library was 13.3 µM.

Absorbance wavelength scan was performed by measuring absorbance every 5 nm from 350-700 nm in a clear 96-well plate using a BioTek Synergy 2 to identify optically interfering compounds. Each well contained 100 µM compound dissolved in FP buffer to achieve a total volume of 100 µL.

**AC8/CaM NanoBiT Assay**

All NanoBiT assays were performed using a BioTek Synergy 2 at 37 °C. HEK293T cells were cultured in DMEM with 10% FBS and 1% Pen/Strep at 5% CO\(_2\) and 37 °C at passage number \( \leq 20 \). Cells were plated at 25,000 cells/well in 100 µL culture medium in white, half area 96-well plates (Greiner) that was previously coated with poly-D-lysine. Cells were allowed to grow for 16 hours, after which they were transfected using Lipofectamine 3000 (Life Technologies) and the NanoBiT DNA constructs cloned above. Transfection was performed following manufacturer’s instructions, and cells were then incubated for two days.

Prior to the assay, culture medium was removed and 40µL HBSS (Gibco)
supplemented with 20 mM HEPES pH 7.4 and either 10µL 5X BAPTA-AM (10µM final) (Sigma-Aldrich), 10µL 5X compound, or vehicle was added. The plate was incubated for 30 minutes at 37 °C. Next 12.5µL of 5X NanoGlo Live Cell Substrate (Promega) was prepared and added according to manufacturer instructions. Luminescence was monitored for 30 minutes to establish baseline luminescence. After baseline, 12.5µL of 6X concentrated thapsigargin (1µM final) (Alfa Aesar), A23187 (1µM final) (Sigma-Aldrich), or vehicle, was added and the luminescence was monitored for another 1.5 hours. During data analysis, baseline luminescence was normalized to zero, and Area Under Curve (AUC) analysis was used to quantify thapsigargin- or A23187-induced AC8/CaM association observed over first 20-25 minutes following thapsigargin or A23187 addition. Data represent mean of n=3 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

CytoTox-Fluor Acute Cytotoxicity Assay

CytoTox measurements were performed using a BioTek Synergy 2 at 37 °C with Excitation 485/20 and Emission 528/20 filters. HEK293T cells were cultured and plated as in NanoBiT assay, but in black, half area 96-well plates and cultured for 2 days. On the day of the assay, culture medium was exchanged for 25 µL HBSS supplemented with 20 mM HEPES pH 7.4 and 25 µL 2X compound or vehicle. The plate was then incubated for 30 minutes at 37 °C. 2X concentrated CytoTox-Fluor reagent (Promega) was made as per manufacturer’s instructions, added to the plate, and the plate was incubated in for another 30 minutes. After this time fluorescence was measured. Toxicity was normalized relative to vehicle control (0%) and 30 ug ml⁻¹ digitonin control (100%) (Sigma-Aldrich). For ease of data interpretation, viability was determined as the difference between 100%
(0% toxicity, i.e. DMSO vehicle) and the observed % Toxicity (relative to 100% digitonin control). Data represent mean of n=4 independent experiments ± S.E.M. Data analysis was performed using GraphPad Prism 7.

CaM HSQC NMR

For all NMR samples 100 µM ¹⁵N-CaM was incubated with or without indicated compound at 5X molar excess in the following buffer: 20 mM HEPES pH 7.4, 100 mM KCl, 10 mM CaCl₂, 10% D₂O, 5% DMSO. All spectra were acquired at 25 °C using a 600 MHz Varian INOVA NMR spectrometer equipped with a triple resonance gradient probe. NMR spectra were processed using NMRPipe [132].

AC1-C1b/CaM AlphaScreen Binding Assay

AC1-C1b peptide corresponding to human AC1 residues 493-520 with C-terminal GSSG followed by FLAG tag was obtained from Genscript. 8 µL biotinylated CaM (AG Scientific) concentrated to 5X was incubated with 8 µL of 5X indicated concentration of EGTA in 20 mM HEPES pH 7.4, 100 mM KCl, 1 mM CaCl₂ in a white low volume 384-well plate with nonbinding surface (Corning) at ambient temperature for 30 min. Then, 8 µL of 5X FLAG-tagged AC1 peptide was added and incubated 1 hr further. Next, 8 µL of 5X concentrated Alpha M2 anti-FLAG acceptor bead was added and plate incubated for 1 hr in dark. Finally, 8 µL of 5X concentrated streptavidin donor bead is added, plate was incubated for 30 min in dark, and plate was read using Perkin Elmer Envision, as above. Final concentrations of assay components were 20 µg/ml of each bead, 10 nM CaM, and 100 nM FLAG-AC1.
References


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