DEVELOPMENT OF SMALL MOLECULE RGS INHIBITORS AS A MECHANISM TO MODULATE G-PROTEIN SIGNALING

by

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For My Parents

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List of Abbreviations

1-8ANS: 1-anilinonapthalene-8-sulfonic acid

AchE: Acetylcholinesterase

AD: Alzheimer's Disease

AF488: AlexaFluor 488

AF532: AlexaFluor-532

$AlCl_3$: aluminum chloride

ANOVA: Analysis of variance

APP: Amyloid precursor protein

BSA: Bovine serum albumin

cAMP: 3'-5'-cyclic adenosine monophosphate

CCG: Center for Chemical Genomics, University of Michigan

CNS: Central nervous system

δ receptor: delta opioid receptor

DAX: Domain present in Dishevelled and Axin

DEP: Dishevelled/EGL10/Plextrin homology domain

DH: Dbl homologous domain

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulphoxide

Dox: doxycycline

DRC: Dose-response curve

DTT: dithiothreitol

ELISA: Enzyme-linked immunosorbent assay

FCPIA: flow-cytometry protein interaction assay

FITC casein: fluorescein-conjugated casein

FKBP: FK506 binding protein

GAP: GTPase accelerating protein

GDP: Guanosine-5’ diphosphate

GEF: Guanine nucleotide exchange factor

GFP: Green fluorescent protein

GGL: Gγ-like domain

GIRK: G-protein inwardly rectifying potassium channel

GoLoco: guanine nucleotide-dissocation inhibitor domain

GPCR: G protein coupled receptor

GRK: G protein coupled receptor kinase

GSK: Glycogen synthase kinase

GSIS: glucose-stimulated insulin release

GTP: Guanosine-5’ triphosphate

GTPγS: Guanosine-5’-O-[γthio]triphosphate

HEK: Human embryonic kidney cells

HEPES: 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid

His6: 6x histidine tag

IA: Iodoacetamide

IBMX: 3-isobutyl-1-methylxanthine

IC_{50}: Inhibitory concentration, 50%

KFC: Knowledge–based Fade and Contacts

LARG: Leukemia-associated RhoGEF

LC-MS: Liquid chromatography mass spectrometry

LGA: Lamarckian Genetic Algorithm

MAGI: MAGUK with Inverted orientation protein

MBP: Maltose binding protein

$MgCl_2$: magnesium chloride

Min: minute

mL: milliliter

NA: Not available

NaCl: sodium chloride

NaF: Sodium fluoride

NEM: N-ethyl maleimide

NGF: Nerve growth factor

NHERF1: Na^+/H^+ exchanger regulatory factor 1

NI: No inhibition

NIH: National Institutes of Health

NMR: nuclear magnetic resonance spectroscopy

NT: Not tested

PD: Parkinson's Disease

PDB: Protein Databank

PDEγ: Phosphodiesterase γ

PDZ: PSD95/Dlg/Z0-1 domain

PH domain: Plextrin homology domain

PI3K: Phosphoinositide 3-kinase

PIP_3: (3,4,5)P_3-phosphotidyl inositol

PLCβ: Phospholipase C, β isoform

PPI: Protein-protein interaction

PPII: Protein-protein interaction inhibitors

PTB: phosphotyrosine binding domain

PTEN: Phosphatase and tensin homolog

RBD: Ras binding domain

RGS: Regulator of G Protein Signaling

RGS4c: a mutant RGS4 with no cysteines in the RH domain

RH domain: RGS homology domain

RhoGEF: Rho guanine nucleotide exchange factor

SAR: Structure-activity relationship

SD: Standard deviation

Sec: Second

SEM: standard error of the mean

siRNA: short interfering ribonucleic acid

SMPPII: small molecule protein-protein interaction inhibitors

S/T Kinase: serine/threonine kinase domain

TCEP: tris(2-carboxyethyl) phosphine

TFA: tetrafluoroacetic acid

T_m: melting temperature

TR-FRET: time-resolved fluorescence resonance energy transfer

μ receptor: mu opioid receptor

μL: microliter

WST-1: 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

Abstract

DEVELOPMENT OF SMALL MOLECULE RGS INHIBITORS AS A MECHANISM TO MODULATE G-PROTEIN SIGNALING

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Chair: Richard R. Neubig

Regulator of G-protein Signaling (RGS) proteins are important regulatory molecules in the transduction of G-Protein Coupled Receptor (GPCR) signaling. They function by directly binding to G alpha subunits and accelerating GTP hydrolysis, thus potently inhibiting GPCR signaling. We and others have proposed that small molecule inhibitors of RGS proteins may provide a novel mechanism for therapeutic intervention in diseases stemming from deficiencies in GPCR signaling. This thesis details the identification and characterization of two novel classes of small molecule RGS inhibitors with unique properties. These compounds were identified from a series of high throughput screens performed by myself and others in our laboratory. The CCG-63802 class of molecules includes the first examples of reversible inhibitors of RGS4. These compounds can inhibit the *in vitro* binding and activity of several RGS proteins with IC_{50} values in the 3-100 micromolar

range. They function by binding to RGS4 near a site thought to be important for allosteric regulation by endogenous acidic phospholipids. The second class of molecules, typified by CCG-50014, includes the most potent RGS4 inhibitors identified to date. This compound irreversibly inhibits RGS4 with nanomolar potency (IC_{50} 30±6 nM) by covalently interacting with at least one cysteine on the RGS. In spite of the thiol dependence of these compounds, several members of this class can inhibit RGS binding and activity on G protein alpha subunits in living cells. Future work with these compounds is focused upon testing their activity in a variety of isolated organ and whole-animal studies. It is hoped that these compounds will provide a foundation for the development of new, more active RGS inhibitors with potential clinical and/or research utility.

CHAPTER I:
Introduction

The crux of this thesis is based upon the development of two classes of small molecules that inhibit a therapeutically interesting and academically intriguing family of protein-protein interactions (PPI). Generally speaking, PPIs are a particularly challenging class of drug targets that have the potential for great therapeutic benefit in a number of different diseases. While this thesis focuses primarily upon one specific PPI, the work contained herein provides technical insight and warnings of potential pitfalls that can be expected when performing high-throughput screening for small molecule inhibitors of any PPI. Because of the immense potential of small molecule PPI modulators as therapeutic agents and as research tools, it is hoped that this work will accelerate the development of this important class of compounds. It should be noted that parts of this chapter were compiled into a review article published in Neuropsychopharmacology [1].

Protein-Protein Interactions: What are they & why target them?

Protein-protein interactions are essential components of virtually all cellular processes. The binding of two or more proteins in a cell can have a wide array of effects, including modulating or initiating signal transduction, regulating patterns of gene transcription, providing cytoskeletal stability, and promoting

cellular replication or death. Because the cellular network of PPIs is vast and essential, in theory it should contain many potential sites at which a drug may be targeted. In the past several years, there has been much effort focused towards identifying specific inhibitors of PPIs. Currently, there are a number of clinically relevant therapies that target PPI interfaces. Most currently used PPI inhibitors (PPIIs) in the clinic are based upon humanized monoclonal antibodies. While this class of therapeutics possesses some very desirable drug properties (e.g. high specificity, low toxicity) it also has several drawbacks that make the approach less applicable to the widespread development of PPIIs (e.g. lack of cell/blood-brain barrier permeability, poor oral bioavailability, high cost of manufacture).

While all organ systems contain PPIs that are potential drug targets, the central nervous system (CNS) is, in particular, ripe for targeting of protein-protein interactions. This is due, in part, to the fact that the highly organized nature of CNS signal transduction relies heavily on localization and compartmentalization of signaling functions. Blocking the protein-protein interactions underlying this compartmentalization (e.g. PSD95/Dlg/Z0-1 domain, (PDZ)) domain targets) could provide more subtle tissue-specific therapeutic actions than would blocking the signal pathway itself. Furthermore, highly specific neural transcriptional patterns of regulatory molecules (e.g. Regulator of G Protein Signaling (RGS) proteins, see below) provide great opportunities for cell-type selective modulation of signaling. This burgeoning field is only starting to be developed and entails a large number of unexplored potential drug targets of which some of the best-developed examples will be discussed.

Inhibiting Protein-Protein Interactions outside of the CNS:

Directly targeting PPIs with small molecules has only recently become a feasible approach to drug development. Over the last two decades, significant progress has been made in the development of small, drug-like molecules that are capable of inhibiting the interaction between two proteins. However, this progress has not come easily - PPI interfaces have proven to be particularly difficult drug targets and had been deemed intractable in many instances [2, 3]. The difficulties encountered in targeting a PPI are substantial and it takes a great deal of work to develop useful lead compounds. The most obvious obstacle is the sheer size and geometry of the standard protein interaction interface. These regions are often relatively featureless expanses of protein surface that cover 750-1500 $Å^2$[4] and are devoid of traditional 'pockets' into which a small molecule can dock in an energetically favorable manner. While developing a cell-permeant, bioavailable small molecule that is capable of occluding such a large interaction surface was considered exceedingly difficult by many, recent advances in the field have shown that this conclusion was premature. Numerous families of small molecule protein-protein inhibitors have been developed for a number of targets, the majority of which are directed towards potential application for cancer therapy. For example, much progress has been made in the development of inhibitors of the p53/MDM2 interaction, the Bak/Bcl2 interaction, or the Myc/Max interaction [4, 5]. While the development of these inhibitors is of great academic and clinical interest, they are beyond the scope of this thesis and

as such will not be discussed further. Several good reviews have been published on small molecule PPIIs that function as cancer therapeutics [4-8], so I will focus here on CNS-related targets.

A major breakthrough in the development of small molecule PPIIs was the discovery of 'hot spots' on protein interaction surfaces (**Fig. 1.1**). These small regions of the interaction interface, often identified by alanine scanning mutagenesis [9, 10], are responsible for a disproportionate contribution to the binding energy of the two proteins. An extensive database of single alanine mutations has shown that these hot spots are often enriched in aromatic and positively charged residues [9]. The discovery that many PPIs are primarily governed by a relatively small section of the dimer interface has given renewed life to the idea that large, relatively flat protein interaction interfaces could bind small molecules in a way that occludes protein dimerization. By identifying and targeting these sites, a small molecule has a much greater chance of directly disrupting a PPI. To this end, at least two independent web servers have been developed that analyze PPIs or predict interaction "hot spots [11, 12]." One of these servers, the Knowledge–based Fade and Contacts (KFC) server, has been developed to predict protein interaction "hot spots" based upon the three dimensional structure of the PPI (**Fig. 1.1**). This prediction software functions primarily upon a structure of the PPI complex, but can also capable incorporate information from Robetta's alanine scanning [13], ConSurf sequence conservation [14, 15], the alanine scanning energetics database [16] or the binding interface database [17]. While clearly still just a prediction requiring

experimental confirmation, algorithms such as these may provide a rapid mechanism to determine if a particular PPI contains a well-defined “hot spot” that may be amenable to small molecule targeting.

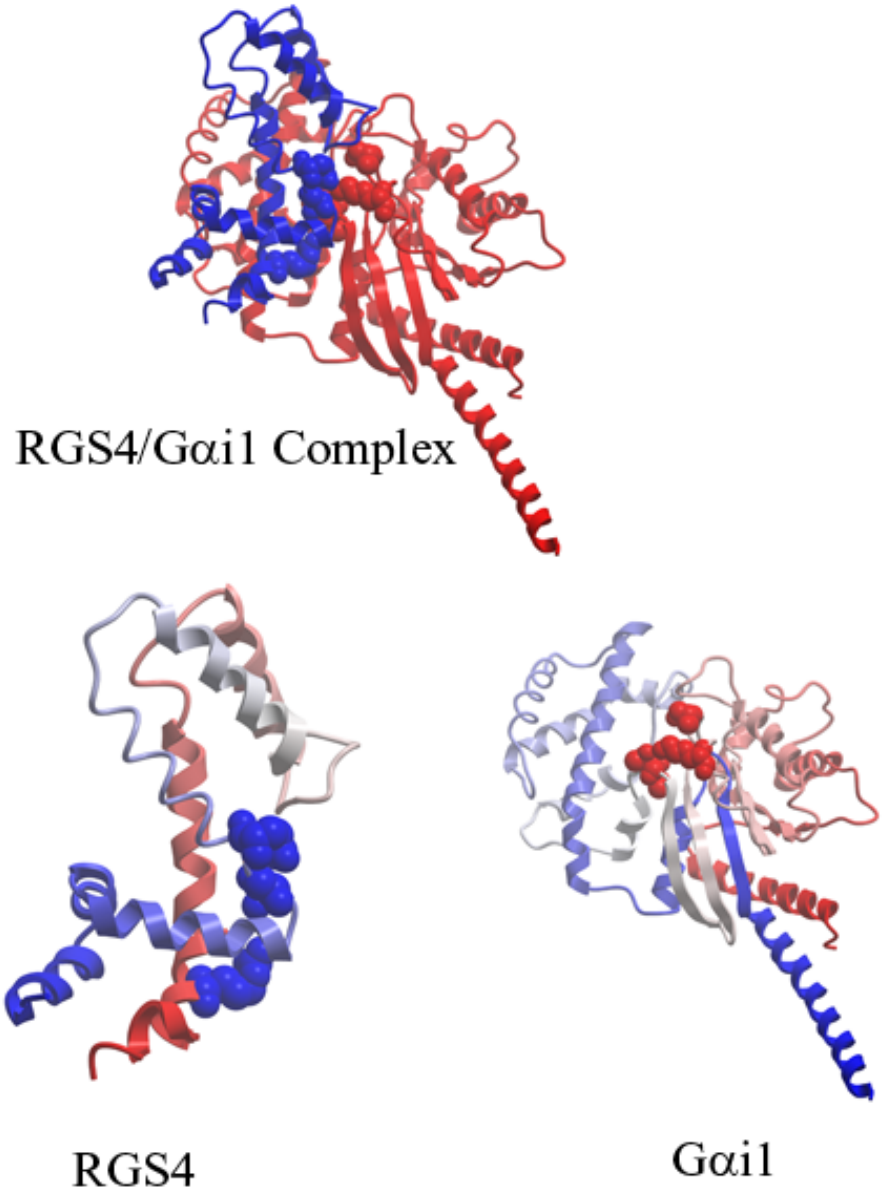

Figure 1.1 Predicted “hot spots” on the Regulator of G protein signaling 4 (RGS4)/Gαi1 protein-protein interaction interface. The highlighted residues on both surfaces (spacefill) are predicted by the KFC Server to be energetically important for the protein-protein interaction [11]. Structure from PDB ID 1AGR [18].

Rationale for targeting Protein-Protein Interactions in the CNS:

The importance of PPIs in proper cellular function is particularly striking in the nervous system. In the CNS, a host of PPIs is required for virtually all cellular processes, including neurite outgrowth, synapse formation and modulation, neurotransmission, signal transduction, and the induction of apoptosis [19-22]. Indeed, the highly specialized structures and discrete localization of signaling molecules in the synapse are dependent on a large network of PPIs. Targeting specific PPIs in the CNS may provide novel mechanisms to modulate neural function downstream of receptor activation or to disrupt localization signals that contribute to the efficiency or specificity of signaling. Furthermore, by targeting these processes, it may be possible to more subtly and specifically tune neural functioning than can be achieved by administering a receptor agonist/antagonist. Most receptor-targeted drugs do not have the ability to selectively act upon receptors in a particular region of the body. For example, μ-opioid receptor (μ) agonists (e.g. morphine, fentanyl) commonly cause constipation due to their effects on μ receptors in the intestine. The benefit of targeting localization signals or downstream members of a signaling pathway is that, in many instances, those factors are expressed in a much more tissue specific manner than are the receptors themselves. By using this approach, it may be possible to provide a measure of tissue specificity in the intrinsic mechanism of a drug. This benefit could be particularly important in the development of centrally acting drugs, as many broadly acting drugs in the CNS tend to have serious side effects limiting their use [23]. Theoretically, this selectivity could be achieved at various points in

the signaling cascade, as there are often several steps in a signal transduction pathway that are dependent on PPIs. Another mechanism that targeting PPIs affords is the potential ability to localize two important signaling molecules with a bifunctional molecule that facilitates the interaction [24]. Such a molecule is comprised of two protein binding moieties joined by a short linker region and functions to localize the two potential binding partners by non-covalently tethering them together. While these bifunctional molecules are more of a PPI facilitator (or agonist) than an inhibitor, they may also provide a mechanism to specifically modulate neural signaling. Overall, targeting a downstream signaling modulator is likely to provide an increase in tissue specificity of the therapeutic effect and may also provide a mechanism to subtly modulate neural firing downstream of natural neurotransmission.

Inhibiting protein aggregation in the CNS:

Amyloid Beta Aggregation:

Alzheimer's disease (AD), Parkinson's disease (PD), and other 'plaqueopathies' are becoming increasingly prevalent in our society and there is growing interest in the mechanism, prevention, and treatment of these protein aggregation diseases. Therapies for these diseases, typified by accumulation of aggregated protein plaques, have largely dealt solely with the symptoms of the disease (i.e. dyskinesias, decline of cognitive abilities). While these treatments can offer some benefit, they offer no real chance of disease reversal nor can they halt its progression. There has been great interest, however, in understanding

the biochemistry and pathophysiology of the plaque development and in discovering methods to inhibit or reverse plaque formation. Emphasis recently has shifted to finding compounds that inhibit the development of the small oligomeric species that both lead to the macroscopic plaques and are believed to be the pathogenic factor in these diseases [25]. Several of these methods rely upon directly inhibiting the aggregation of the protein, while a subset are focused upon modulating the expression levels of the plaque-forming protein or the chaperones that assist it into its native conformation. I will focus on the former.

Identifying compounds that selectively disrupt protein aggregates or that prevent plaque formation by inhibiting protein aggregation could be a viable approach to the treatment of protein aggregation diseases. As such, there has been a push for the discovery and development of compounds that selectively inhibit protein aggregation. Compounds have been identified that inhibit the aggregation of a variety of proteins including, huntingtin [26, 27], amyloid beta [25, 28-31], and tau [32]. Particular attention has been paid to the proteins that form the basis of plaque formation in AD, namely amyloid beta and tau. It has long been known that a variety of dyes bind to and can destabilize or inhibit plaque formation (for an extensive list, see [33]). Histopathological evaluation of brains from AD patients has shown at least two distinct types of plaques form during this disease. In the brain of an AD patient, aggregates of amyloid beta form in the extracellular matrix and neurofilbrillary tangles of aggregated tau protein form intracellularly. Both of these aggregates are correlated with AD, but it has yet to be conclusively shown that these plaques cause the observed

neurodegeneration and are not merely coincident with it or even a result of it. In fact, significant plaque development has been observed in a population of cognitively normal 70-year olds [34]. A current hypothesis states that it is not the mature plaques that are the triggering factor for neurodegeration, but rather the protofibrils – small oligermeric complexes of the protein – that are the basis of (or are at least correlated with) disease progression [35]. Due to the lack of *in vivo* imaging methods for visualizing protofibril formation, this hypothesis has yet to be tested in living human patients. This suggests that by inhibiting the development of protofibrils it might be possible to slow the disease progression. Indeed, several drugs that inhibit amyloid beta fibril formation via distinct mechanisms are currently in or have been tested in clinical trials [25, 28, 36]. One of these drugs, Alzhemed (**Fig. 1.2A**, tramiprosate) is a PPII that functions by sequestering monomeric amyloid beta protein [25, 28, 36]. This drug passed through phase II clinical trials, but failed in phase III clinical trials [37]. While tramiprosate ultimately failed in the clinical trials, it provides a proof of concept that small molecule inhibitors of amyloid beta protofibril formation are capable of reaching late stage development and that analogs with better pharmacokinetic/pharmacodynamic properties may still provide a viable approach to AD treatment.

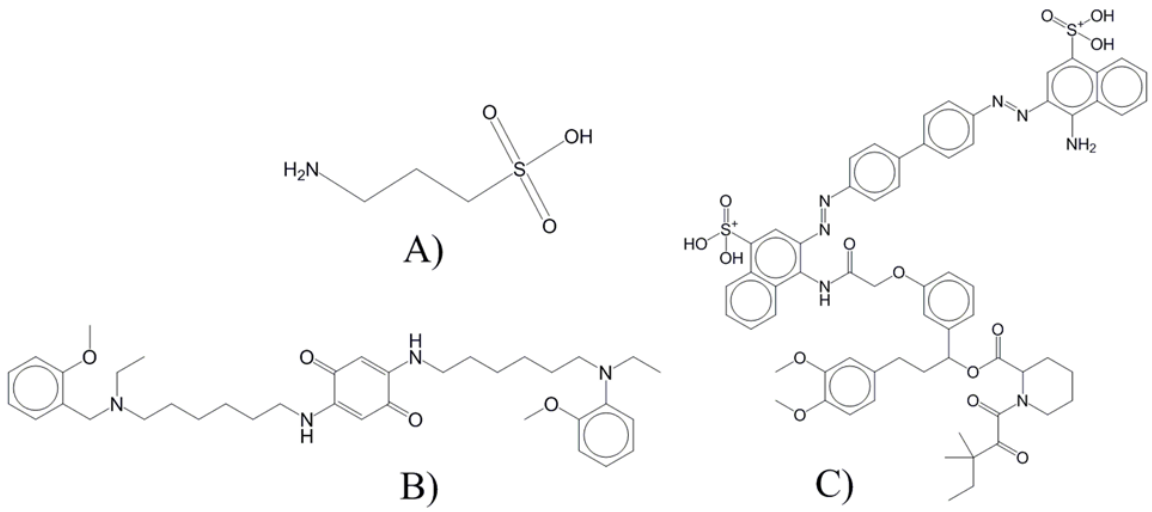

Figure 1.2 Examples of amyloid beta aggregation inhibitors. **A)** Alzhemed (Tramiprosate, homotaurine) [28]. **B)** Memoquin, a multifactorial compound for the treatment of Alzheimer's disease [38-41]. **C)** SLF-CR, a bifunctional molecule that recruits the FKBP family of chaperones to aggregating amyloid beta [31].

Another recent development in amyloid beta aggregation inhibitors was the development of the multifunctional compound memoquin (**Fig. 1.2B**) [38-41]. This compound was rationally designed by incorporating a radical scavenging moiety (the benzoquinone fragment of coenzyme Q10) into a series of cholinesterase inhibitor derivatives [29]. Along with possessing antioxidant activity, coenzyme Q10 and other benzoquinones have been shown to directly inhibit amyloid beta aggregation [42, 43]. It was therefore expected that this compound would be a multifactorial therapy for the treatment of AD, acting as an acetylcholine esterase (AchE) inhibitor, a free-radical scavenger, and an inhibitor of amyloid beta aggregation. Indeed, it was found that memoquin is a potent AchE inhibitor (2.6 nM K_i) and is capable of inhibiting both the AchE-induced and intrinsic aggregation of amyloid beta [29, 30]. This compound is orally bioavailable, crosses the blood brain barrier, and reduces amyloid plaque

accumulation in a mouse model overexpressing human APP (TG2576). It was also shown to prevent AD-like neurodegeneration in anti-NGF transgenic mice (AD11), another model of AD [29, 44, 45]. As of June 2010, there were no clinical data readily available on the efficacy of the compound in human subjects.

While the previous small molecule inhibitors discussed have directly inhibited amyloid beta aggregation, a different approach was taken by Gestwicki, et al. [31]. They developed a bifunctional molecule that recruits the FK506 binding protein (FKBP) family of chaperones to a developing amyloid beta aggregate (**Fig. 1.2C**). This series of molecules was created by using various linkers to join Congo red – a dye known to bind to amyloid beta – and SLF, a synthetic ligand for FKBPs. By recruiting the chaperone, the molecule dramatically increases its steric bulk and becomes capable of inhibiting the aggregation of amyloid beta. The recruitment of FKBP by this molecule is essential for its activity, suggesting that Congo red on its own does not disrupt the amyloid beta interaction energy as much as some of the previously mentioned compounds. Tethering large molecules together with selective bifunctional small molecules may be an important and powerful mechanism to modulate PPIs in the CNS, as many of the current small molecule inhibitors are bulky and may not have good permeability across the blood-brain barrier. Furthermore, this approach allows for the development of not just PPIIs, but also for the development of PPI facilitators. There are instances where it would clearly be desirable to promote PPIs in a cell rather than inhibit them and through this

general schema it may be possible to selectively colocalize different molecules in a single cell by varying one component of the bifunctional molecule.

While the pathophysiological mechanism behind the development of AD has yet to be fully understood, it seems reasonable to hypothesize that amyloid beta protofibril formation plays a significant role in the progression of the disease. Several small molecules have been developed that inhibit the oligomerization of amyloid beta either *in vitro* or *in vivo*. While inhibiting amyloid beta aggregation may provide therapeutic benefit on its own, the development of multifactorial agents such as memoquin has the potential to be much more efficacious in terms of treating the underlying disease.

Alpha Synuclein Aggregation:

Parkinson's disease is the second most common neurodegenerative disorder in most Western countries [46, 47]. This disease is characterized by the loss of dopaminergic neurons in several brain regions, including the substantia nigra pars compacta and other regions important for higher order functioning [48]. Histopathological evaluation of the postmortem brains of Parkinson's patients has revealed the presence of large intraneuronal aggregates termed "Lewy bodies." These aggregates are primarily composed of a 140 amino acid protein, α-synuclein, although they are generally not as homogenous as amyloid beta plaques [48]. It has been shown that overexpression of alpha-synuclein in several model organisms causes the development of Parkinsonian-like symptoms [49-51]. Further study of α-synuclein has shown that the protein

contains a highly amyloidogenic domain that, when misfolded, oligomerizes and forms a series of self-associating β-pleated sheets that spontaneously form Lewy bodies [52, 53]. Like amyloid beta oligomers in AD, it is believed that it is the α-synuclein oligomers and not the fully formed Lewy bodies that are the pathological factor in PD. The current hypothesis states that α-synuclein oligomers are capable of forming membrane pores that disrupt organelle function, leading to cell dysfunction and death. [48]

Several inhibitors of α-synuclein aggregation have been identified [54-58] **(Fig. 1.3)**. An intriguing finding is that catecholamines can inhibit α-synuclein aggregation [54, 55]. This has also been shown in a mouse model of α-synuclein aggregation, where Lewy bodies were dissolved in brain slices by the addition of l-dopa [59]. The oxidation state of the catecholamines are important for this activity, whereby the several oxidation products of dopamine are more potent at inhibiting α-synuclein aggregation than is the parent neurotransmitter [59] (**Fig. 1.3**). The link, if any, between dopaminergic neuron loss and the ability of catecholamines to inhibit α-synuclein aggregation has yet to be fully understood, but remains an intriguing concept in the pathophysiology of Parkinson's disease.

A series of peptide inhibitors of α-synuclein aggregation were identified by developing a library of overlapping heptapeptides that span the α-synuclein sequence. The active peptides were centered around residues 69-72 of α-synuclein, suggesting that this region of the molecule was important for self-association [60, 61]. It appears that short peptide fragments of α-synuclein also occur naturally, as the serine protease neurosin degrades α-synuclein into

fragments that can inhibit α-synuclein polymerization [62]. Current work is focused upon developing peptidomimetics and identifying small molecule inhibitors of α-synuclein using both high-throughput screening (HTS) and rational design from the information obtained in the peptide library study [63].

Other small molecule inhibitors of α-synuclein have been identified. Rifampicin and several of its derivatives can inhibit both α-synuclein [64]and amyloid beta [65, 66] aggregation in a concentration-dependent manner with reasonable potency (< 10 μM IC_{50}). A conclusive mechanism of rifampicin action has not been fully elucidated, but it has been suggested that it could act by binding directly to the developing plaque [65] and/or by acting as a free radical scavenger [66]. Panacea Pharmaceuticals had also developed a pair of α-synuclein inhibitors, PAN-408 and PAN-527, that had progressed to preclinical trials. However, there have been no recent reports of compounds with these names [67, 68].

Polyphenolic compounds, like flavonoids or Congo red, have been proposed to be α-synuclein aggregation inhibitors [69]. Many of these compounds are derived from natural sources and have low micromolar IC_{50} values for protein aggregation inhibition. Baicalein (**Fig. 1.3**), a flavonoid isolated from the Chinese Skullcap plant (*Scutellaraia baicalensis*), can directly bind to a single site on α-synuclein with submicromolar affinity and inhibit oligomerization [70]. It is likely that a quinone oxidation product of this compound is responsible for the observed inhibitory activity (**Fig. 1.3**) [70]. Interestingly, this compound can inhibit α-synuclein aggregate nucleation but does not affect fibril elongation

or dissolve aggregates, suggesting that the molecule may act by stabilizing the monomeric α-synuclein [70]. This mechanism could be beneficial, as plaque disruption could generate free protofibrils and lead to increased cellular damage. Circular dichroism studies confirmed that binding of baicalein stabilized the semi-folded state of α-synuclein [70]. Unfortunately, baicalein also stabilized an oligomeric species of α-synuclein as well as the monomer [70]. It is not known whether the oligomeric species stabilized by baicalein has neurodegenerative properties, however this finding does not bode well for this family of polyphenoic compounds as inhibitors of α-synuclein function. Unfortunately, it is possible that these molecules could stabilize the formation of the protofibrils that, as the current hypothesis states, are the pathogenic factor in protein aggregation diseases.

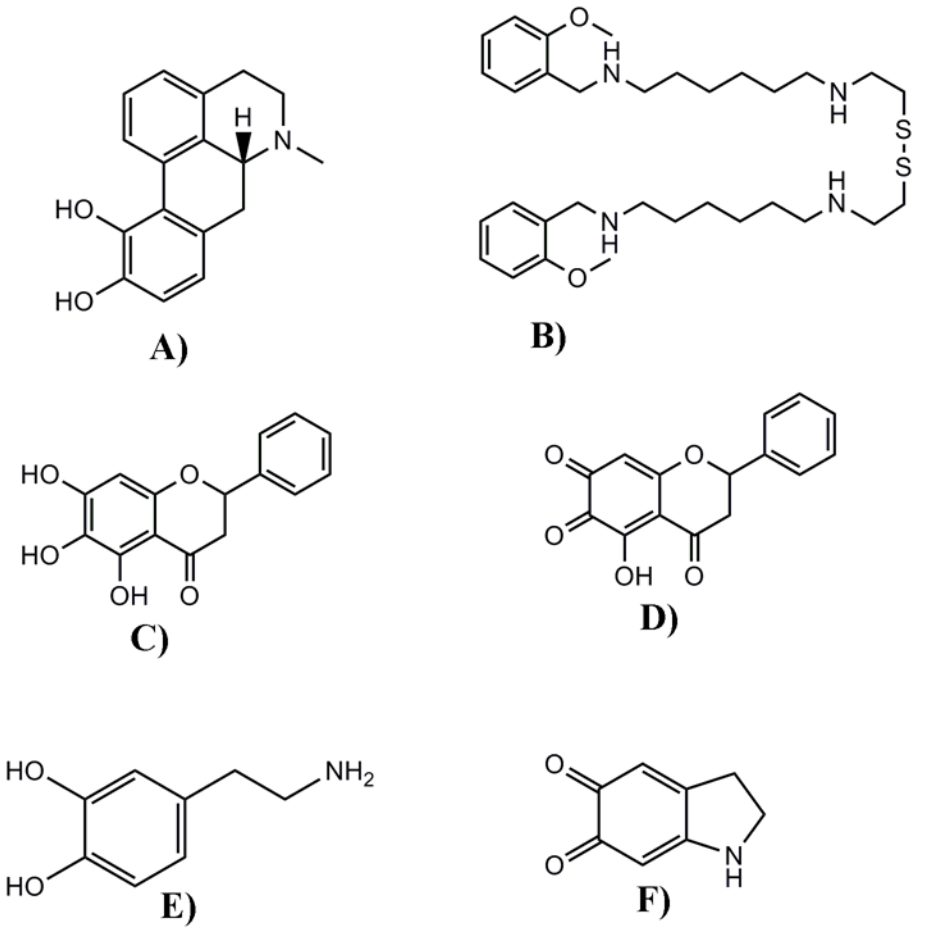

Figure 1.3 Examples of small molecule α-synuclein aggregation inhibitors. **A)** One member of a series of catechols known to inhibit α-synuclein aggregation [54]. **B)** A non-catechol inhibitor from the same series [54]. **C)** Baicalein, a natural product from the Chinese skullcap (*Scutellaraia baicalensis*) [68]. **D)** The presumed active oxidation product of baicalein that inhibits α-synuclein aggregation [68]. **E)** Dopamine, a known inhibitor of α-synuclein aggregation [54, 59] and **F)** dopaminochrome, one of the oxidation products of dopamine that has anti-aggregation properties [59].

Protein aggregation diseases are a major cause of morbidity in the elderly population of first world countries. While there are a number of therapeutics currently in use to treat the symptoms of these diseases [23, 67, 68, 70], there are very few if any actual treatments that stop or reverse disease progression. If the hypothesis that protein oligomers are the primary pathogenic factor in these diseases is correct, then small molecules that prevent or reverse protein

oligomerization may provide a mechanism to target the actual cause of the disease. There has been substantial work put forth to develop inhibitors of protein oligomerization and significant progress has been made. There is, however, much more work that needs to be done in this field before a clinically useful agent will be available for general use.

Modulating Signal Transduction through inhibiting protein-protein interactions:

Signal transduction cascades are required for nearly all biological functions. The importance of these systems is further illustrated by the fact that a large proportion of all clinically used therapeutics modulate signaling [23]. The most common method to modulate information processing through a signal transduction pathway is to alter activity of the most upstream molecule in the system: the receptor. These receptors come in many forms including G-protein coupled receptors, intracellular steroid/glucocorticoid receptors, and tyrosine kinase linked receptors. Currently ~30-50% of all clinically used drugs target GPCRs and a substantial portion of the remaining drugs target other receptor systems [23]. While many of these drugs are effective therapeutics, targeting regulation systems or molecules further downstream in the signaling pathway may provide advantages not readily available when solely modulating receptor activity.

Targeting downstream signaling molecules in a signal transduction pathway requires overcoming several significant hurdles in drug development,

including cell permeability of the compound, achieving pathway specificity, and avoiding unwanted or unexpected side effects. There are currently several examples of clinically used drugs or drug candidates that target downstream signaling molecules in a pathway. The majority of these are kinase inhibitors, exemplified by Gleevec, that inhibit an enzymatic step in a signal transduction cascade [71]. As compared to a standard PPIs, enzymes are much more amenable to small molecule targeting due to their well-defined active site binding pocket. Furthermore, kinases represent a critical step in the signal transduction pathway that can be selectively inhibited. With all of these qualities, it is easy to understand why a kinase inhibitor could be a useful therapeutic.

Many signal transduction steps do not rely upon an enzymatic process but rather use PPIs to relay information, often in the context of a signalosome that is tightly regulated by scaffolding components in the cell (e.g. lipid rafts, scaffold proteins). Targeting these steps requires the development of small molecules that inhibit the PPIs required for signal transduction. One of the more obvious drug targets in this case would be the scaffolding proteins that pull these signalosomes together.

Inhibition of PDZ interactions:

PDZ domains are important scaffolding components in many signaling systems, with an extensive role in the development and maintenance of both pre- and post-synaptic structures [72, 73]. Development of reversible small molecule inhibitors that target PDZ domains would provide useful tools to probe the many

functions of these important scaffolds [74]. Of all canonical PPIs, PDZ domains are possibly the most similar to a traditional ligand-receptor interaction, as the interaction interface is comprised of a groove on the PDZ domain binding to the last few (3-5) amino acid residues in its partner (**Fig. 1.4A**) [73]. The small interaction interface requires that the few amino acids compromising the PDZ ligand contribute a great deal to the energetics of binding. Having such a small PPI interface might suggest that these interactions would be amenable to small molecule disruption. To this end, there have been a few PDZ inhibitors described based either upon rational design or from random high-throughput screening (**Fig. 1.4B**) [75-82]. Rational design of PDZ inhibitors would appear to be relatively straightforward, as the PDZ ligand is comprised of so few residues and the binding pockets of many PDZ domains have been characterized structurally by NMR or crystallographically. Indeed, several peptidomimetic scaffolds have been developed that inhibit PDZ interactions (**Fig. 1.4B**) [76, 77, 80, 81]

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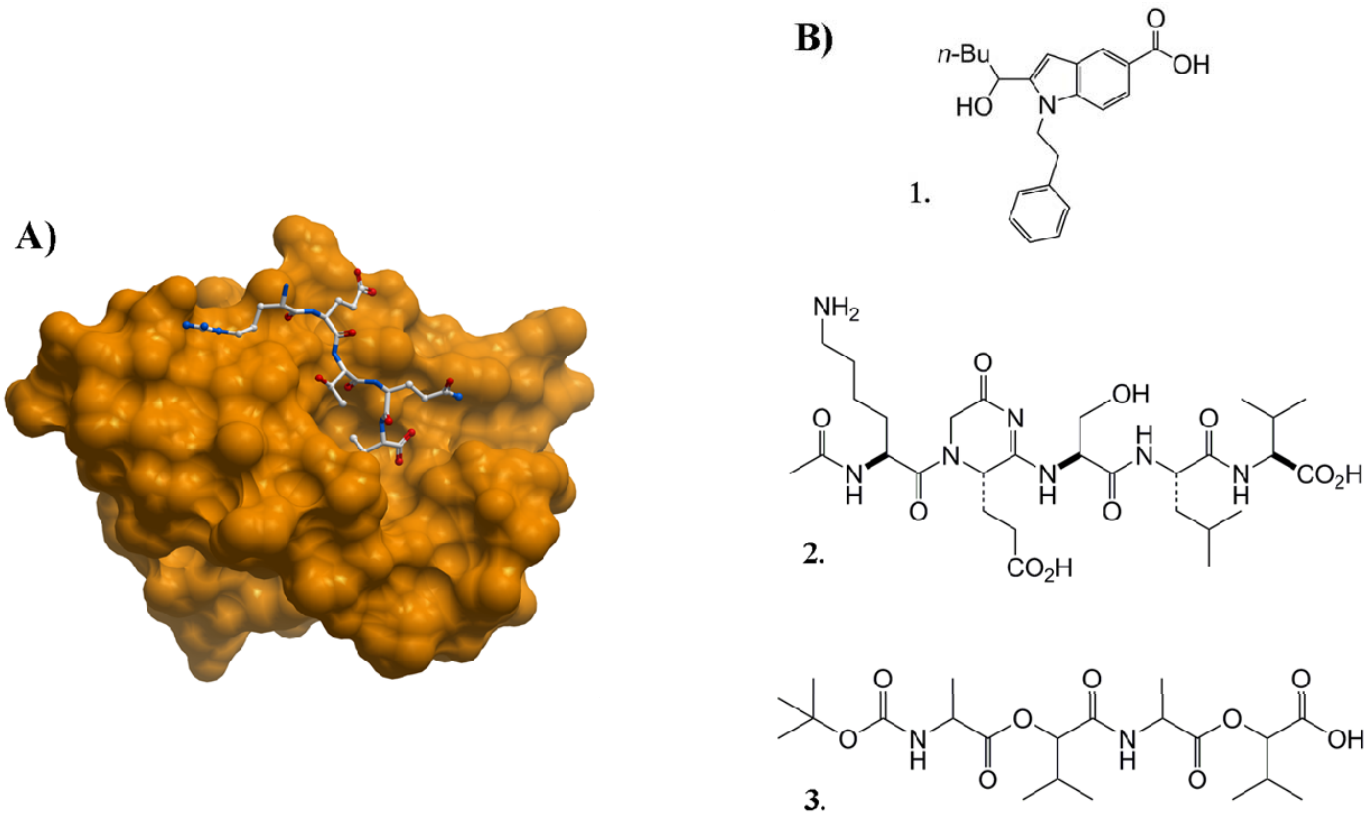

Figure 1.4 Crystal structure of the first PDZ domain from MAGI bound to the PDZ ligand of HPV18 E6. **A)** MAGI shown in surface with HPV18 E6 shown as sticks and balls. Note how in this protein-protein interaction, only a few (generally 3-5) residues play a predominant role in the binding energetics. Structure from PDB ID 2I04. [83] **B)** Examples of small molecule PDZ inhibitors. 1. General scaffold for a wide array of PDZ domains [77-79]. Analogs of this structure have been shown to inhibit the second PDZ domain of NHERF1 [78]. 2. Beta-hairpin peptidomimetic developed to inhibit the α1-syntrophin PDZ domain [80]. 3. Peptidomimetic developed to inhibit the NHERF1 PDZ domains [81].

Cell permeant small molecule inhibitors of PDZ domains will provide a mechanism with which to probe the complex functions of these scaffolding proteins. For example, the Na^+/H^+ exchanger regulatory factor 1 (NHERF1) contains two PDZ domains and has been shown to have altered expression in many cancers [84-88]. The role of NHERF1 in cancer is complicated and appears to be dependent upon cellular context. Outside the realm of oncology, NHERF1 has been shown to be a multifunctional scaffolding protein that is capable of regulating the trafficking and localization of many membrane associated proteins [89]. Clearly, a tool which would allow for the acute and reversible inhibition of NHERF1 PDZ function could provide a powerful mechanism with which to determine the physiological role of this protein in different cellular contexts.

Currently, the best defined PDZ inhibitors are directed against the dishevelled and NHERF1 PDZ domains. These compounds were originally designed as a treatment for beta-catenin dependent tumor growth or to study the controversial role of NHERF1 in cancer progression, respectively. While these compounds have limited utility as centrally acting agents, they provide a clear example of how a PDZ inhibitor could be developed for one of the many PDZ domains that are important in neural functioning (see for reviews [19, 72, 73, 90-94]).

The first cell permeable PDZ inhibitor was developed by Fujii, et al [77]. This irreversible inhibitor was rationally designed to bind to the second PDZ

domain of MAGI [77]. The compound dose-dependently (IC_{50} ~10-30 μM) inhibited the binding of a peptide corresponding to the PDZ ligand of the lipid phosphatase PTEN to the membrane-associated MAGI protein in a fluorescence polarization assay. It also increased the activity of PKB (or Akt) in cells, consistent with increased phosphatidyl inositol 3,4,5 trisphosphate levels due to reduced PTEN recruitment to the membrane [77]. Eventually, this indole scaffold was developed into a reversible, albeit weak (IC_{50} ~1 mM), inhibitor of the second PDZ domain of MAGI [76]. A similar indole scaffold was used to develop an inhibitor of the disheveled PDZ domain, an important scaffold in the Wnt/β-catenin pathway [79]. This compound, named FJ9, blocked the interaction between the PDZ ligand at the C-terminus of the 7TM receptor Frizzled 7 with the disheveled PDZ domain both *in vitro* and in cells (*In vitro* IC_{50} 30-60 μM). It also suppressed the growth of tumor cells in a β-catenin dependent manner [79]. Another inhibitor of the disheveled PDZ domain has been described. This relatively weak (~200 μM IC_{50}) inhibitor was identified in a virtual screen against the disheveled PDZ domain and it inhibited Wnt signaling in a zebrafish embryo model of Wnt signaling [82]. Inhibition of PDZ domains has the potential to provide very useful pharmacologic tools for the study of protein trafficking, synaptic function, and other scaffolding-dependent processes. While current compounds still have only modest affinities, a selective inhibitor of some particular PDZ domains may also provide useful therapeutic agents, although this hypothesis needs to be tested.

Targeting elements of the G-protein signaling pathway:

Another class of PPIs for which drug targeting is attractive is the multitude of PPIs formed by heterotrimeric G protein subunits. These trimeric proteins are formed of α and βγ subunits which, when activated (and thus dissociated into free α and βγ subunits), interact via PPIs with a large number of downstream effectors, including adenylate cyclase, phosphoinosotide-3 kinase, phospholipase Cβ, voltage gated Ca^{2+} channels, G protein coupled inwardly-rectifying potassium channels, and others. G-proteins, especially Gα subunits, also bind to regulatory proteins that can alter the temporal and spatial signaling pattern. Developing specific inhibitors of various G-protein/effector or G-protein/regulator interactions could provide a mechanism to selectively modulate GPCR signaling pathways. It is not difficult to imagine several scenarios whereby modulating GPCR signaling could provide significant therapeutic benefit, either by potentiating positive actions of a drug or by inhibiting undesirable side effects. The progress in this field will be discussed throughout the rest of this thesis, including the contributions that I have made during the course of my Ph.D work.

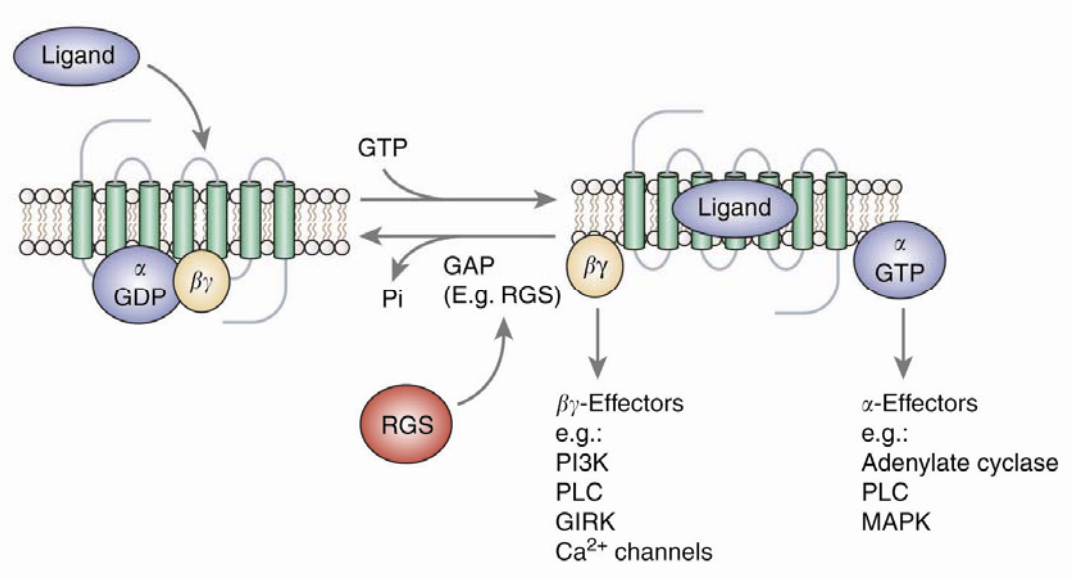

Figure 1.5 Canonical G-protein signaling mechanism. See text for explanation. Note that the hydrolysis of GTP by Gα is accelerated by members of the RGS family. Reproduced with permission from Nature Publishing Group from [1].

Selective Gβγ Inhibitors:

Due to the prevalence of clinically important drugs that target GPCRs, there has been great interest in the therapeutic modulation of signaling downstream of these receptors. Canonical signaling through GPCRs (**Fig. 1.5**) progresses through the activation of a receptor by ligand binding, which stimulates the exchange of GDP for GTP on the Gα subunit of a heterotrimeric G protein. The GTP-bound Gα subunit and Gβγ subunit of the G protein then dissociate or at least undergo a conformational change to expose interaction surfaces to act upon downstream effectors in the signaling pathway. Since the first signaling molecule downstream of a GPCR is the G protein heterotrimer, it has become an interesting target for small molecule inhibition. While there have been no published reports of a small molecule inhibitor of Gα/effector PPIs, there have been a family of compounds identified by Smrcka and colleagues that bind

to Gβγ and selectively inhibit its interaction with downstream effectors [95-97]. The strategy used to identify these inhibitors provides a clear example of a protocol being used to identify small molecule PPIIs. The first step that the investigators took was to screen a random-peptide phage display library to identify binding sites on Gβγ [98]. A series of peptides was identified and one inhibited the Gβγ regulation of PI3K and PLCβ. It did not however, inhibit regulation of type I adenylate cyclase or N-type Ca^{2+} channels, suggesting that effector selectivity may be possible with small molecule modulators of Gβγ activity. By analyzing the crystal structure of $G\beta_1\gamma_2$ bound to this selective peptide inhibitor, it was possible to define the binding pocket for the peptide. Using this site as a binding pocket in virtual screening, the investigators identified 85 small molecules (top 1% in the screen) that were predicted to bind to the Gβγ 'hotspot [95]. Analysis of these compounds using an ELISA assay based upon displacement of the peptide ligand identified 9 compounds with reasonable IC_{50} values (100 nM – 60 μM). One of these compounds, M119 (**Fig. 1.6A**), inhibited the Gβγ stimulation of PLCβ and PI3Kγ activity *in vitro* and it also inhibited the Gβγ-dependent calcium release from activation of the G_i-linked N-formyl peptide receptor in differentiated HL-60 cells. The compound had no inhibitory activity upon the calcium mobilization initiated by carbachol in HEK cells stably expressing the G_q-linked M3 muscarinic receptor, showing that M119 is selective for Gβγ-dependent calcium mobilization. M119 also showed *in vivo* activity when tested in a morphine antinociception assay in mice. PLCβ3$^{-/-}$ mice have been shown to be ten times more sensitive to the antinociceptive effects of morphine

and an intracerebroventricular injection of M119 recapitulated this augmentation of morphine activity in wild type animals [95, 99]. Since opioid receptors have many Gβγ-dependent functions, the fact that M119 potentiates morphine-induced antinociception instead of inhibiting it provides evidence that this compound is not globally inhibiting Gβγ activity. Another structurally distinct compound identified by this approach, M201, also showed an interesting selectivity profile in its ability to inhibit Gβγ-effector interactions. This compound showed no ability to inhibit $PLC\beta_2$ activity, potentiated $PLC\beta_3$ and PI3K activity, and inhibited GRK2 binding. The discovery of effector-selective modulators of Gβγ signaling M119 & M201, has thus provided a clear example of how targeting downstream signaling molecules can be a viable approach to modulating the pharmacological properties of a common drug (e.g. morphine).

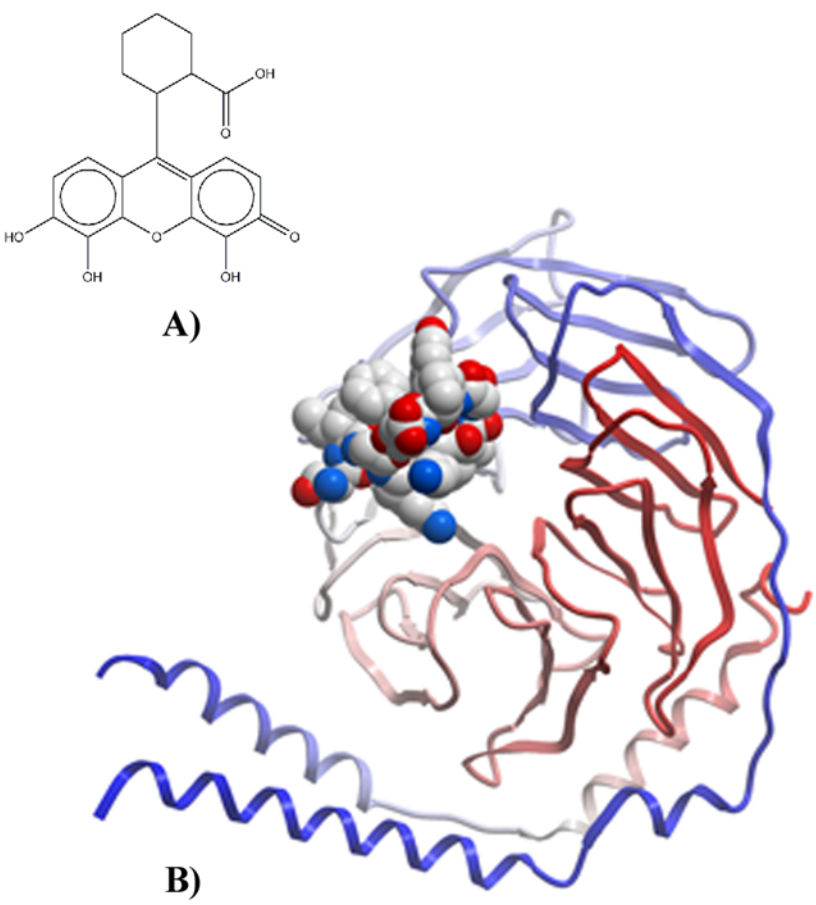

Figure 1.6 Structure of M119, a pathway selective inhibitor of Gβγ signaling **A)** Chemical structure of M119 [95]. **B)** Crystal structure of Gβγ bound to peptide SIGK. This peptide, identified by phage display, binds to the identified "hot spot" on Gβγ [100]. Structure from PDB ID 1XHM.

RGS Proteins:

Another approach to modulating GPCR signaling is to alter the activity of key proteins that regulate signal transduction. The G protein pathways are regulated by a number of proteins including scaffolding proteins such as the PDZ domains discussed previously, RGS proteins, G protein coupled receptor kinases (GRKs), and arrestins. These molecules are critical for the proper temporal and spatial regulation of GPCR signaling. By selectively modulating the actions of

these molecules it might be possible to more finely tune GPCR signaling for therapeutic purposes.

A particularly interesting approach to modulating GPCR signaling is to target RGS proteins. These molecules function as GTPase accelerating proteins (GAP), by binding directly to Gα subunits and accelerating the intrinsic hydrolysis rate of GTP (**Fig. 1.1, 1.5**) [18, 101, 102]. The discovery of these proteins provided a solution to the paradox of how rapid regulation of GPCR signaling could occur given the slow intrinsic rate of GTP hydrolysis by purified Gα subunits. They explain the subsecond regulation of G protein signals observed in excitable cells [18, 101, 103, 104] and RGS proteins can strongly inhibit cellular responses [105-107]. There are over twenty identified RGS proteins that interact with limited selectivity for most Gα subtypes (**Table 1.1**, [101, 103, 108-110]). The only exception to this is $G\alpha_s$, for which no RGS interaction has been confirmed. There are increasing reports of RGS selectivity for signaling by specific GPCRs, suggesting that targeting an RGS may provide a mechanism to selectively augment signaling through a particular GPCR [111, 112]. It is also increasingly appreciated that RGS proteins are heterogeneously expressed throughout the body, including in individual neuron types in specific brain regions [113-115]. The distinct expression patterns, presumed GPCR selectivity, and the dependence on an active signaling pathway for function all suggest that small molecules that modulate RGS activity could potentially be useful therapeutics. Indeed, mice expressing a mutated (G184S) form of $G\alpha_{i2}$ or $G\alpha_o$ that render these G proteins insensitive to RGS effects exhibit markedly enhanced potency

of agonists and substantial physiological phenotypes [116-119]. Specifically, mice with the $G\alpha_{i2}$ G184S mutation show reduced fat mass and resistance to high fat diet, possibly due to CNS actions [119]. They also show behaviors consistent with enhanced 5-HT_{1A} signaling and a spontaneously antidepressant-like state as well as 10-fold increased potency of 5HT-based antidepressant drugs [120]. Mice with the RGS-insensitive mutant $G\alpha_o$ show increased antiepileptiform activity in hippocampal slices by α_{2a} agonists [121]. Strikingly, the effects are quite specific where the 5-HT_{1A} potentiation is only seen for antidepressant-like and not for hypothermia effects [120]. This exquisite specificity suggests that RGS proteins may play a targeted role in the regulation of different physiological effects that are elicited through the same receptor. Since there are a number of thorough reviews of RGS structure and function (e.g. [1, 101-103, 108, 115, 122-129]), I will focus primarily upon RGS4 and its potential as a drug target.

RGS Family	Notable Accessory Domains	RGS name	Suranames	Gα Selectivity	Ref.
R4	N-terminal amphipathic helix	RGS1	BL34	Gi/o/q	[130]
		RGS2	GOS8	Gq	[131, 132]
		RGS3		Gi/q/11	[133]
		RGS4		Gi/q	[134]
		RGS5		Gi/o/q	[135]
		RGS8		Gi/o/q	[136]
		RGS13		Gi/q	[137]
		RGS16		Gi/o/q/13*	[135, 138]
		RGS18		Gi/q	[139]
		RGS21		Gi/q	[140]
R7	DEP, GGL	RGS6		Go	[141]
		RGS7		Go/i	[142]
		RGS9	RGS-r	Go/Gt	[142, 143]
		RGS11		Go/i	[142]

Table 1.1 Current list of mammalian RGS proteins that are known to bind to Gα subunits. DEP: Dishevelled/EGL10/Plextrin homology domain; GGl: Gγ-like domain; PDZ: PSD95/Dlg/Z0-1/2 domain; PTB: phosphotyrosine binding domain; RBD: Ras binding domain; GoLoco: guanine nucleotide-dissocation inhibitor domain; DH: Dbl homologous domain; PH: Plextrin homology domain; DAX: Domain present in Dishevelled and Axin; S/T kinase: serine/threonine kinase domain; RhoGEF: Rho guanine nucleotide exchange factor; GRK: G-protein coupled receptor kinase. *RGS16 inhibits signaling through G13, but not via GAP activity. **No GAP activity, data remain to be independently confirmed. *** Weak or no GAP activity, physiological importance in question.

RGS Family	Notable Accessory Domains	RGS name	Suranames	Gα Selectivity	Ref.
R12	PDZ, PTB, RBD, GoLoco	RGS10		Gi/o/z	[144, 145]
		RGS12		Gi/o (not q)	[146]
		RGS14		Gi/o	[147, 148]
RZ	Cysteine String, PDZ ligand (RGS19)	RGS17	RGSZ2	Gi/q	[115]
		RGS19	GAIP	Gi1/3 > Gi2	[149]
		RGS20	RGSZ1	Gz	[150]
Axin	β-Cateninin Binding, GSK-3β binding, DAX	Axin 1		G12**	[151]
		Axin 2	Conductin	G12**	[151]

RGS Family	Notable Accessory Domains	RGS name	Suranames	Gα Selectivity	Ref.
RhoGEFs	DH/PH	LARG		G12/13/q**	[152, 153]
		P115 RhoGEF		G12/13	[154]
		PDZ RhoGEF		G12/13	[155]
GRK	S/T Kinase, PH (PH on GRK2/3 only)	GRK1	Rhodopsin Kinase	None Known	[124]
		GRK2	βARK1	Gq***	[156, 157]
		GRK3	βARK2	Gq***	[156]
		GRK4	IT-11 kinase	G13, Gs***	[158]
		GRK5		None Known	[124, 156]
		GRK6		None known	[124, 156]
		GRK7		None Known	[124]

The RGS Homology Domain:

The RGS Homology (RH) domain is comprised of nine helices that form a bi-lobed structure (**Fig 1.7**). This fold, originally identified for RGS4 in complex with $G\alpha_{i1}$, has been observed in at least 22 other proteins and bears little to no homology with the folds of small G-protein GAPs. The α4-α7 helices predominantly provide the structural components and stability required for interaction with Gα subunits, but the loop regions between helices α3-α4, α5-α6, and α7-α8 form the primary interaction interface with the G protein. These loops bind to the three 'switch' regions of Gα. The GAP activity of RGS is believed to stem from the stabilization of these 'switch regions into the transition state conformation. As such, the RGS protein actually does not provide any residues that are crucial for catalytic activity, as is seen with some GAPs for small GTPases. [124]

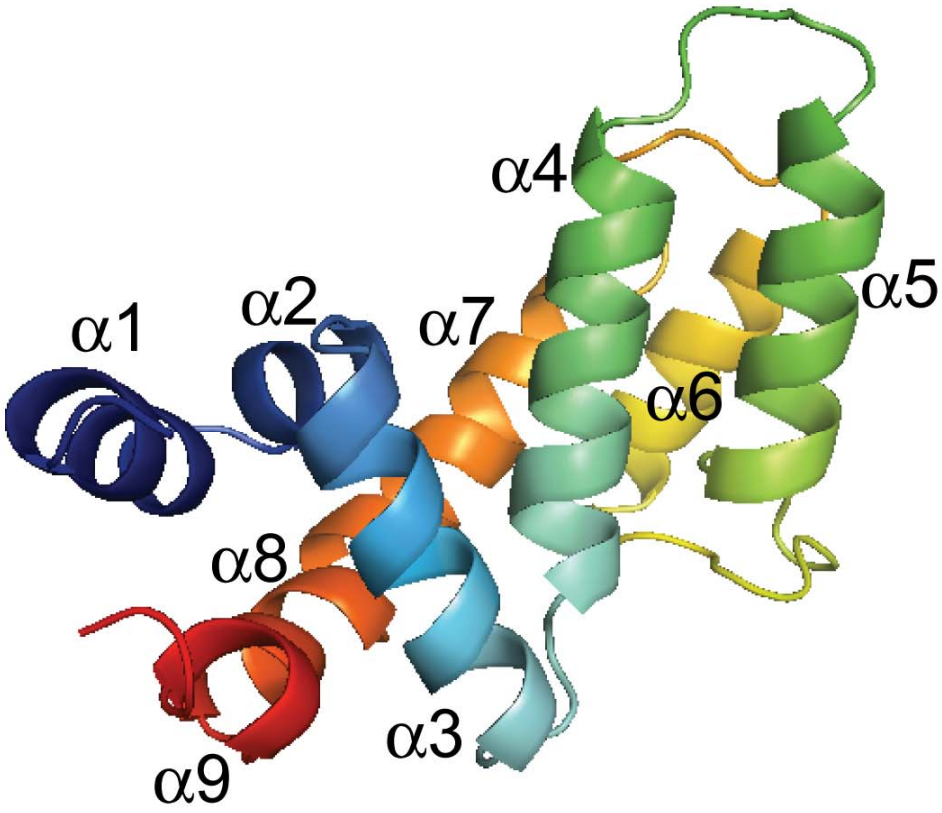

Figure 1.7 The RGS homology (RH) domain fold of RGS4. That the loop regions between α3-α4, α5-α6, and α7-α8 provide the surface required for Gα binding. Structure from PDB ID 1AGR [18].

A particularly promising feature of the RH domain of RGS4 as a target for small molecule inhibitors is that it contains an endogenous small molecule allosteric regulatory site. Kurachi and colleagues showed that (3,4,5)P_3-phosphotidyl inositol (PIP_3) and lysophosphatidic acid – but not other phosphoinositides - directly bind to an allosteric site on the RGS and inhibit its activity [159]. This binding inhibits RGS4 GAP activity *in vitro* and also inhibits the effects of RGS4 upon the muscarinic control of GIRK currents in a reconstituted *Xenopus* oocyte system [159].

While there is no direct structural information (e.g x-ray crystal structure) of the phospholipid interactions with the RGS, there is significant evidence that the binding of PIP_3 occurs at a site that is independent of the G-protein binding interaction interface. There are four conserved lysine residues in the α4/α5 helices (K99, K100, K112, K113) of RGS4 that have been shown to be important for PIP_3 binding (**Fig. 1.8**). Charge-swapping mutation of these lysines (e.g. K99E/K100E) renders RGS4 unable to bind activated $G\alpha_{i1}$ as well as inhibiting RGS modulation of the M2-activation of G-protein inwardly rectifying potassium (GIRK) channels [159].

Two of these lysine residues (K99/K100) lie in a site analogous to the site of binding of adenomatous polyposis coli protein to the RH domain of Axin, suggesting that this site might be a more generalized accessory site for protein/small molecule binding to RH domains. On RGS4, this site has also been shown to bind calmodulin in a Ca^{2+}-dependent manner [159, 160]. Mutation of K99/K100 to glutamate in this system inhibited the interaction with calmodulin, suggesting that the binding sites of acidic phospholipids and calmodulin overlap [161]. Further strengthening this notion, binding of Ca^{2+}-calmodulin reverses the PIP_3–induced RGS4 inhibition [160]. Since RGS4 has been shown to be efficient at inhibiting calcium release induced by Gq-coupled receptors ([162], Chapter IV) this calcium-dependent activation of RGS4 is likely to be a physiological feedback mechanism for dampening overactive Gq signaling [160, 163].

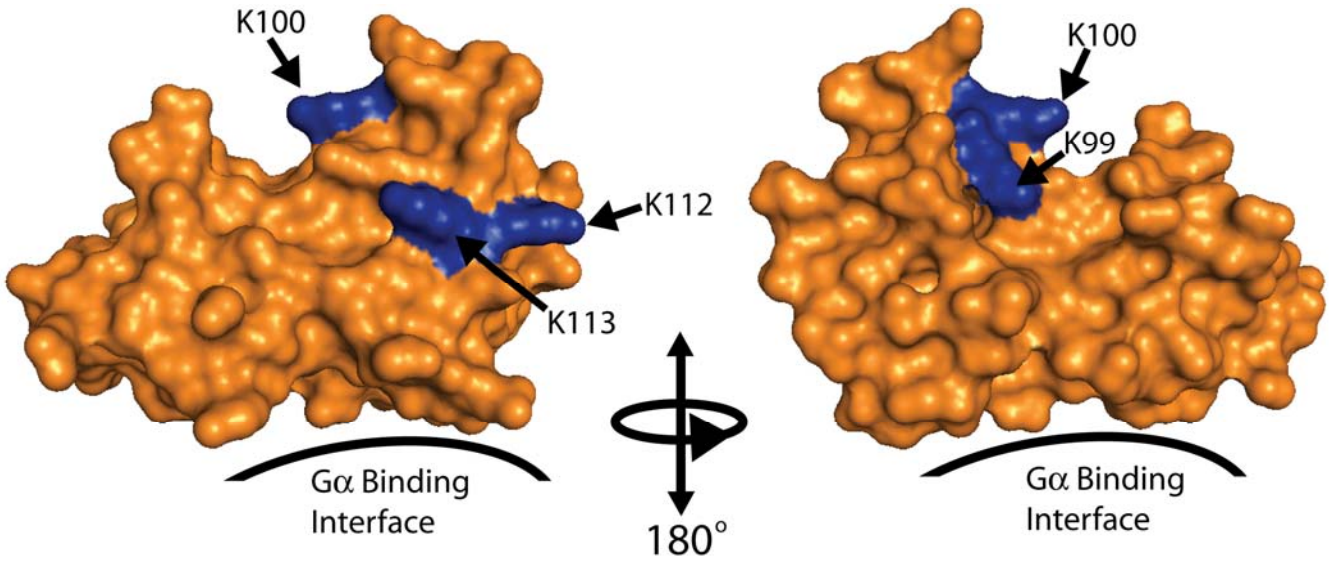

Figure 1.8 Conserved lysine residues in RGS4 that have been implicated in calmodulin and acidic phospholipid binding. The "B" site region of RGS4 contains K100 and K99. Mutating these residues to glutamate dramatically reduces calmodulin binding, $G\alpha_{i1}$ binding and GAP activity [159]. Residues K112 and K113 are also implicated in mediating the effects of $(3,4,5)P_3$-phosphotidyl inositol and calmodulin on RGS4.

RGS4 as a Drug Target:

There is substantial interest in developing small molecule or peptide modulators of RGS proteins [101, 103, 122, 123, 129, 164]. An RGS inhibitor given alone would be expected to accentuate signaling initiated by endogenous ligands, a treatment that could be useful in a variety of neurological conditions such as depression (via enhancement of serotonin signaling), early stage Alzheimer's or Parkinson's diseases (via enhancement of cholinergic or dopaminergic signaling, respectively). They could also be used as an adjunct with a GPCR agonist by increasing the potency or selectivity of the drug by accentuating signal transduction through the receptor. One could imagine that an

RGS9 inhibitor that accentuated dopaminergic signaling selectively in the striatum where RGS9 is expressed could be a useful adjunctive therapy with L-dopa or synthetic dopamine agonists in Parkinson's disease (**Fig. 1.9**). Furthermore, an RGS inhibitor that selectively accentuated opioid signaling in neurons in the pain pathway may provide a mechanism to selectively increase the analgesic properties of opioids but might leave alone the undesirable actions of these drugs (i.e. constipation, abuse liability) which might be regulated by different RGS proteins. Thus, RGS inhibitors could serve as GPCR agonist potentiators but would also enhance agonist specificity in a cell-type or pathway-specific manner.

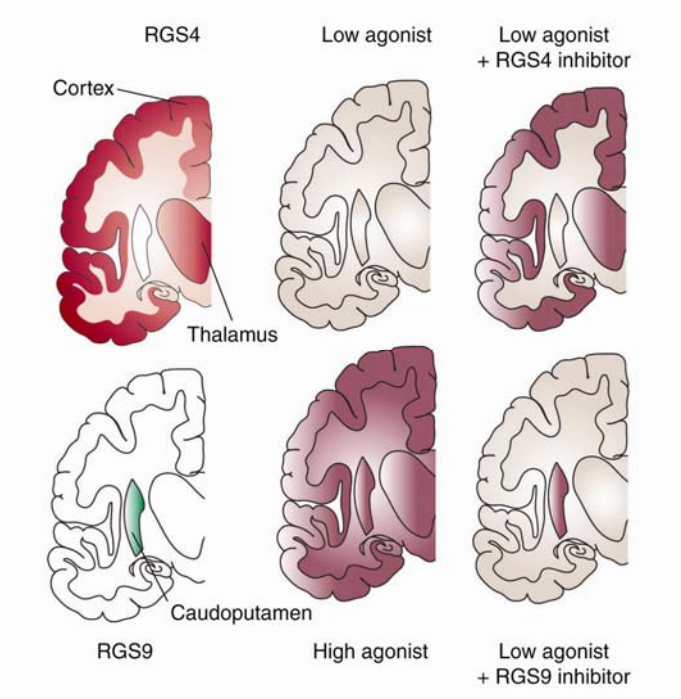

Figure 1.9 RGS-inhibitors increase the tissue specificity of an agonist. *Left Panel:* Graphical representation of the RGS4 & RGS9 protein expression patterns in the brain [151]. The Red/Green color denotes regions of high expression, specifically cortex and thalamic regions for RGS4, and basal ganglia for RGS9. *Center Panel:* Upon addition of a low or high concentration of agonist, a response would be seen across all brain regions that express the receptor. *Left Panel:* In the presence of an RGS inhibitor, there would be tissue-specific enhancement of agonist effect in the tissues where the RGS is expressed. Reproduced with permission from Nature Publishing Group from [1].

RGS4 is a prototypical RGS protein that is widely expressed throughout the CNS with limited expression in peripheral tissues. It controls a variety of signaling systems, and has been implicated as a risk factor for schizophrenia [165-188]. RGS4, like other RGS proteins, interacts strongly with several members of the $G\alpha_{i/o}$ and $G\alpha_q$ families and shows limited selectivity between these proteins in *in vitro* binding and functional studies [18, 189-191]. The interaction interface between RGS4 and $G\alpha_{i1}$ was revealed by the crystal structure by Tesmer et al [18]. This relatively flat interface includes the three switch regions in $G\alpha_{i1}$ (residues 179-185 in switch 1, 204-213 in switch 2, and 235-237 in switch 3) and several of the loops in the RH domain of RGS4 [18]. The interaction interface covers approximately 1100$Å^2$ and utilizes primarily van der Waals and hydrogen bond interactions (**Fig. 1.10**). The binding affinity of the Gα/RGS complex is dependent upon the conformational state of the Gα. There is very little to no interaction when Gα is in the GDP-bound state, some moderate affinity when bound with GTP, and a strong interaction (K_d ~1-5nM) when the Gα is bound to GDP-aluminum fluoride, a state believed to serve as a mimic for the transition state of GTP hydrolysis [189, 192].

The physiological effects of RGS4 have been studied in a number of systems. Two lines of RGS4 knockout mice have been developed [193, 194]. The original whole body knockout of RGS4 had a subtle phenotype [193], which was attributed in part to compensation by other RGS proteins. Recently, whole-body or brain-region specific inducible RGS4 knockout strains of mice have been

developed [194]. The whole-body knockout displayed reduced sensitivity to fentanyl and methadone but wild-type sensitivity to morphine, presumably through complex neurological interactions that are as of yet misunderstood. Interestingly, these animals displayed worsened withdrawal effects to morphine as compared to their littermate wild-type controls, suggesting that RGS4 plays a role at distinct sites in the reward and withdrawal pathways.

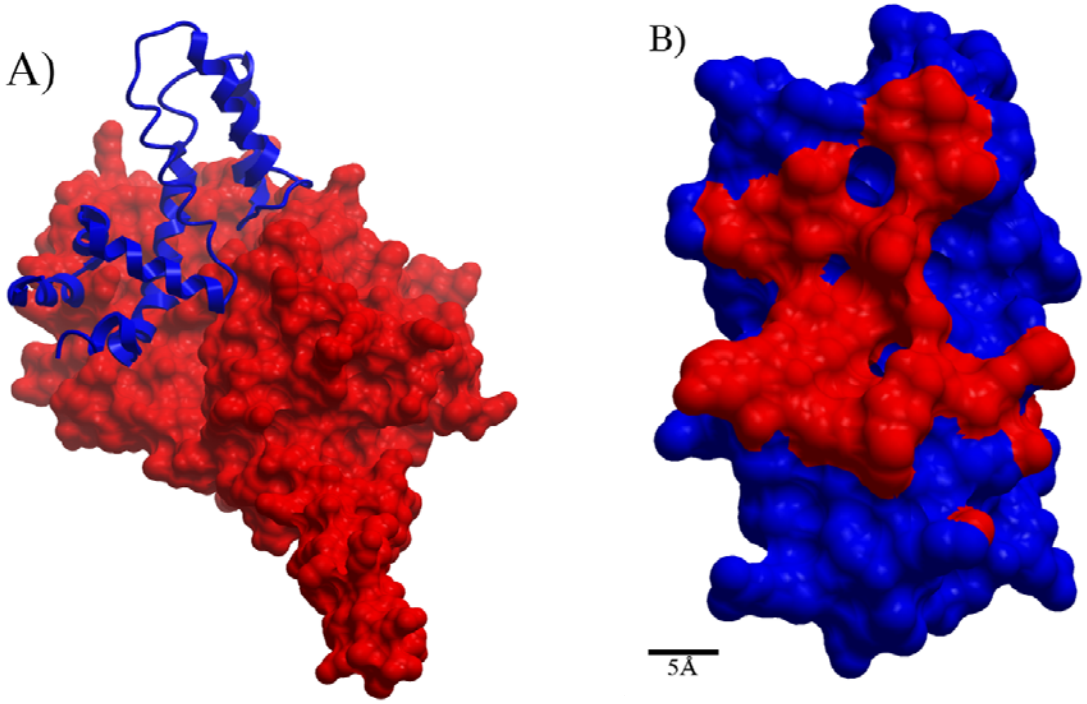

Figure 1.10 Crystal structure of RGS4 in complex with $G\alpha_{i1}$ **A)** RGS4 (Blue ribbon) binding to $G\alpha_{i1}$ (red surface). This stereotypical protein-protein interaction buries approximately 1100Å^2 and is relatively featureless in terms of readily identifiable small molecule binding sites. **B)** Structure of RGS4 that has been rotated to display the regions of the surface (red) that lie within 5Å of $G\alpha_{i1}$. Note the large diffuse contact interface. Structures from PDB ID 1AGR [18].

The brain-region specific RGS4 knockout animals are particularly useful for probing the physiological role of RGS4 in pain processing and opiate reward mechanisms [194]. When RGS4 was depleted from the nucleus accumbens, the animals showed decreased sensitivity to fentanyl antinociception. Deletion of

RGS4 from the accumbens also had no effect upon stereotypical withdrawal behavior. This is in clear contradiction with the whole body knockout animals, suggesting a complex role of RGS4 *in vivo.* Because of the redundancy of RGS proteins, future studies utilizing tissue- or cell-type specific deletions of RGS proteins are likely to provide much more compelling information regarding the true physiological roles of RGS proteins.

RGS proteins also play a role in glucose homeostasis. Mice expressing an RGS-insensitive (G148S) mutation in $G\alpha_{i2}$ display improved insulin sensitivity as compared to wild-type controls [119]. Another recent study showed that RGS4 plays a role in regulating the muscarinic control of glucose-stimulated insulin release (GSIS) from the pancreatic β cells [162]. Activation of the M3 muscarinic receptor potentiates insulin release in response to high glucose both *in vitro* and *in vivo* [195, 196]. Using siRNA knockdown of RGS4 in the MIN6 insulinoma cell line, Wess and colleagues were able to cause a left-shift the dose-response curve for oxotremorine-M potentiated GSIS [162]. Islets isolated from RGS4 knockout animals also displayed increased sensitivity to oxotremorine-M potentiation of GSIS. Furthermore, those authors showed that the RGS4 knockout animals had a potentiated insulin release response to the muscarinic agonist bethanecol as compared to wildtype littermate controls. Taken together, these data strongly suggest a role of RGS4 in the regulation of parasympathetic control of pancreatic β cell function. In this setting, an RGS4 inhibitor could function to potentiate insulin release.

Importance of selectivity in RGS inhibition:

From a research standpoint, an exquisitely selective RGS inhibitor would be of great value in elucidating the physiological role of RGS proteins *in vivo*. However, from a clinical standpoint an RGS inhibitor that targeted a small select group of RGS proteins might be more valuable. Most diseases (e.g. cardiac failure, depression) coincide with changes in a complex set of signaling pathways that are regulated by several different RGS proteins. While still hypothetical, it is possible to imagine that an RGS inhibitor with a specific activity profile against several different RGS proteins might have very different physiological effects than an inhibitor specific for one RGS protein. Furthermore, one could imagine that the activity profile that would provide the most prominent effect for one disease may not be the best for another disease. Therefore, it is possible that an RGS inhibitor with a specific set of activity against several different RGS proteins might be very valuable in the treatment of certain diseases. However, which RGS proteins to target and in what combination for a given disease are questions that remain to be fully answered.

Current RGS Inhibitors:

Two groups have published independent series of peptide inhibitors of RGS4 function. One series from our lab was rationally designed to mimic the switch I region of $G\alpha_i$ and expanded by screening of a constrained peptide library [190, 197]. The other peptide inhibitor series was developed by a random yeast-two hybrid screening campaign [198]. The lead peptide from this latter campaign

bears no resemblance to the sequence of any known RGS4 interacting protein and its mechanism of action is unclear. Both of these series produced lead peptides with modest (mid-low micromolar) activity in both binding and functional assays, suggesting that small molecule inhibition of RGS function may be more tractable than previously thought [190, 198]. The first small molecule inhibitor of RGS4 was published in 2007 [189]. This compound, CCG-4986 (4-chloro-N-[N-(4-nitrophenyl)methoxysulfanyl]benzene-1-sulfonamide), was identified through a flow-cytometry protein interaction assay (FCPIA)-based high throughput screen on a diverse compound library. This compound has a 4 micromolar IC_{50} value for the inhibition of RGS4 binding to $G\alpha_o$ and shows significant selectivity for RGS4 over RGS8, its closest relative based upon sequence homology. The activity of the compound was confirmed by single turnover GTP hydrolysis assay, in which CCG-4986 blocked the GTPase accelerating protein (GAP) activity of the RGS. The compound bound directly to RGS4 as determined by changes in intrinsic fluorescence of the RGS upon compound addition. Further study of the mechanism of CCG-4986 action showed that it did not function in a cellular environment. Subsequent mechanistic studies determined that the compound irreversibly forms a covalent adduct with the RGS in both orthosteric (i.e. at the site of Gα binding) [199],[200] and allosteric interaction sites [200]. While this lack of cellular activity limits the utility of the compound as a pharmacological tool, the development of CCG-4986 was nonetheless exciting, as it clearly shows that RGS proteins are susceptible to small molecule inhibition and also to allosteric modulation which may provide greater specificity among RGS proteins. Current

efforts toward developing small molecule RGS inhibitors using high-throughput screening and rational design approaches are ongoing and are the main focus of this thesis.

While RGS inhibitors have the potential to accentuate signaling through GPCRs, it is also possible to imagine a scenario where augmenting RGS activity with “RGS agonists” could be therapeutically beneficial. As mentioned above, RGS4 has been shown to be reciprocally regulated by acidic phospholipids and calmodulin [159-161]. A small molecule that inhibits the interaction of RGS4 with acidic phospholipids or calmodulin could provide a mechanism to ‘activate’ RGS4. By activating RGS4, it may be possible to attenuate signaling from aberrant or overactive GPCR neurotransmitter receptors. Phosphodiesterase γ (PDEγ) can also positively modulate RGS activity. PDEγ has been shown to selectively potentiate the GAP activity of RGS9 but not RGS4, 16, or 19. It does this in part by forming a ternary complex with Gα and the RGS9/Gβγ complex [201-204]. It may be possible to develop compounds that mimic PDEγ or alter its ability to bind to the Gα-RGS complex providing a novel mechanism to enhance RGS activity.

Whether the goal is to produce RGS inhibitors or RGS activators, there are clear challenges as most actions do require modulation of PPIs. However, small molecules that regulate RGS function would provide a novel approach to the treatment of diseases stemming from or benefited by changes in GPCR signaling.

Overview of the Thesis:

My work focused upon identifying small molecule inhibitors of the RGS-Gα interaction. The compounds generated from this work should provide pharmacological probes to better understand the physiological role of RGS proteins both *in vitro* and *in vivo*. Furthermore, these compounds may serve as leads for the development of therapeutics for a number of different disease states (e.g. depression [120], diabetes [119, 162]).

Chapter II describes results from a high-throughput screen that I developed using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay to monitor the interaction between RGS4 and $G\alpha_o$. From this screen of approximately 40,000 small molecules, the first series of reversible inhibitors of an RGS protein were identified. The prototype compound from this family, CCG-63802, selectively inhibits RGS4 in both the TR-FRET (IC_{50} 1 μM) and FCPIA (IC_{50} 10 μM) assays. Furthermore, CCG-63802 inhibits the GAP activity RGS4 upon $G\alpha_o$. Using biophysical studies, I have shown that the compound binds to the RGS domain and mutagenesis studies have narrowed down the potential binding sites on this protein to two interfaces that are known to play a role in the regulation of the RGS-Gα interaction. Structure-Activity studies in this family of compounds have determined a number of important factors regarding the mechanism of action of these compounds, including: 1) a reversible Michael addition of the compound via the vinyl cyanide moiety is crucial for activity, 2) all three heterocycles are important for full activity, and 3) optimization of the phenyl ring substituents can provide ½ Log improvement in

potency. The future development of this class of compounds is focused upon increasing potency, improving the cellular activity of these compounds, and increasing solubility.

In Chapter III, I characterize the most potent *in vitro* family of RGS4 inhibitors identified to date. The prototype compound from this family, CCG-50014 was originally discovered in a high throughput screen performed by a postdoctoral fellow in our laboratory [205]. I undertook the detailed characterization of the biochemical mechanism of action, structure-activity relationships, and cellular activity of this compound. CCG-50014 potently inhibits the RGS4-$G\alpha_o$ interaction *in vitro* (IC_{50} 30 nM). Like the compounds in Chapter II, this family of inhibitors blocks the RGS-Gα PPI in a variety of biochemical assays including single turnover GAP and FCPIA. The binding mechanism of CCG-50014 to RGS8 is studied in detail with thermal stability measurements, mutagenesis, and mass spectrometry. This mechanism of action is further examined through the synthesis and analysis of a number of analogs of CCG-50014. These analogs also allowed for the generation of structure-activity knowledge leading to the identification of more potent, more soluble compounds that possess less non-specific effects on other non-RGS functions.

In Chapter IV, I detail the ability of CCG-50014 and related analogs to function in a cellular environment. While it was known from the functional studies that CCG-50014 loses activity under reducing conditions that mimic the intracellular environment, the remarkable potency of this family of compounds prompted us to study the cellular action of these compounds. RGS4 is a cytosolic

protein that translocates to the plasma membrane in the presence of activated Gα subunits [206]. Using this information, I expressed a GFP-tagged form of RGS4 in HEK293T cells and induced membrane translocation by coexpression of $G\alpha_o$. The massive overexpression of the Gα pulled the RGS to the membrane in the absence of receptor stimulation. This interaction was blocked by several of the compounds tested, with observable activity down to 1 micromolar. While these data proved that we could inhibit the RGS4-Gα PPI in cells, it did not discern whether or not this effect altered a GPCR signaling pathway. To determine this, we tested CCG-50014 for its ability to block the RGS4 effect on the δ-opioid receptor (δ) using endogenous protein in SH-SY5Y neuroblastoma cells. It was previously shown that in these cells, endogenous RGS4 inhibits the δ signaling, but not μ. In this assay, CCG-50014 enhanced the response through δ but not μ, consistent with inhibition of RGS4. To further study the cellular activity of these compounds, I tested several analogs for their ability to accentuate signaling through the M3 muscarinic receptor after suppression of the receptor response by co-expression of RGS4. Surprisingly, many of these compounds produced a significant calcium transient on their own, suggesting an off-target effect. Of the analogs that did not have this effect, several partially reversed the RGS4-mediated suppression of M3 signaling. The ability of these compounds to function in a cellular system has prompted us to pursue future work with this class of compounds in isolated organ systems and in whole animals.

The work from this thesis significantly advanced the field by producing the first reversible small molecule RGS inhibitor, by characterizing the most potent RGS inhibitor identified to date, and by providing a fundamental framework for the development of future inhibitors with improved cellular and animal activity. It is possible that a promising lead compound for preclinical and clinical trials may be developed with further development of these two classes of RGS inhibitors.

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CHAPTER II:
Reversible, allosteric, small-molecule inhibitors of RGS proteins

Introduction:

. There has been significant interest in developing small molecule inhibitors of specific protein-protein interactions (SMPPIIs) for use as research probes and potential therapeutic agents [1-5]. The development of SMPPIIs has been difficult. One challenge has been the lack of clearly identifiable small molecule binding sites on the relatively featureless protein-protein interaction interface. A promising approach is the use of allosteric pockets on the protein target to bypass this problem and – as outlined in Chapter I - there has been solid progress in SMPPII development [1-4, 6-8]. The compounds described in this chapter appear to be acting in an allosteric manner to inhibit the RGS/$G\alpha_o$ PPI..

There is substantial interest in the therapeutic potential of small molecule modulators of RGS proteins [4, 9-12]. In brief, RGS inhibitors may potentiate signaling though GPCRs in a tissue-specific manner due to the localized expression patterns of many RGS proteins. This effect could be used to reduce side effects of clinically used GPCR agonists that stem from non-target tissue receptor activation (e.g. μ-opioid receptor dependent constipation during post-operative analgesia [13]). Due to the wealth of information on the structure and

function of the prototypical RGS, RGS4, we chose this protein as our primary target for validating the druggability of RGS proteins.

There have been several reported peptide inhibitors of RGS4 and related family members [14-16] and one disclosed small molecule inhibitor [17]. Due to the physical properties of the peptides, none of them function in a cellular environment unless they are introduced intracellularly (e.g. by dialysis via a patch pipette [15]). The small molecule compound CCG-4986 irreversibly inhibits RGS4 by reacting with one or more cysteine residues [18, 19]) and is inactive in a cellular setting Consequently, we undertook this study to identify novel RGS inhibitors that retain activity under reducing conditions and ones that have a reversible mechanism of action.

This chapter describes the identification and characterization of the first class of reversible small molecule inhibitors of an RGS protein, typified by CCG-63802 and CCG-63808. They were identified in a biochemical high-throughput screen that I developed based upon time-resolved fluorescence resonance energy transfer between fluorescently labeled RGS4 and $G\alpha_o$. The RGS-inhibitory activity of the compounds identified in this screen was confirmed using three different biochemical assays (TR-FRET, FCPIA, Single Turnover GTPase). FCPIA reversibility studies revealed that these compounds inhibit the binding RGS4 to $G\alpha_o$ in a reversible manner.

RGS and Gα thermal stability measurements showed that the compounds bind solely to the RGS. To identify the compound binding site on RGS4, I performed a series of mutagenesis studies. An initial finding was that compound

activity required the presence of at least one cysteine residue on the RGS. More detailed analysis showed that these compounds require the presence of at least two cysteines on the RGS for full activity. These two cysteine residues (Cys 148 and Cys 95) are located in an allosteric site on the RGS, suggesting that these compounds may be binding to the RGS at a site far from the Gα interaction interface.

A series of analogs of CCG-63802 were tested using FCPIA to determine the structure-activity relationships of this novel compound class. CCG-63802 contains three heterocycles with a vinyl cyanide linker. It was found that for full activity, all three heterocycles were necessary. The effects of modifying each of the heterocycles are described in detail. The vinyl cyanide group, a known Michael acceptor, is also required for activity of the compounds. This fact, in conjunction with the compounds dependence on cysteine residues for function, suggests that CCG-63802 acts by reversibly reacting with one or more cysteine residues on the RGS. These compounds represent an important step towards the development of tools for the study of RGS functions in physiological and pathophysiological situations.

The work in this chapter was performed by me with the following exceptions: 1) Dr. Stephen Husbands and Dr. Benjamin Greedy synthesized all of the compounds; 2) The thermal stability experiments were performed by Alfred Chung under my direct guidance. I also independently replicated these experiments using a different thermal stability system and obtained similar results; 3) High throughput screening was performed in conjunction with Martha

Larsen; 4) Analysis of the screening data was performed in conjunction with Rez Halze, Paul Kirchhoff, and Dave Roman; 5) Thermal stability experiments on RGS4c were performed under my guidance by Andrew Storaska. Parts of this chapter have been compiled into a publication in Molecular Pharmacology [20].

Materials & Methods:

Reagents:

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) and were reagent grade or better. AlexaFluor™-488 succinimidyl ester and LanthaScreen™ Thiol reactive Tb chelate were obtained from Invitrogen (Carlsbad, CA). γ[^{32}P]GTP (10 mCi/mL) and [^{35}S]GTPγS (12.5 mCi/mL) were obtained from Perkin Elmer Life and Analytical Sciences, (Boston, MA) and were isotopically diluted with unlabeled nucleotide before use. Amylose resin was purchased from New England Biolabs (Ipswich, MA). Ni-NTA resin was purchased from Qiagen (Valencia, CA). Avidin-coated microspheres were purchased from Luminex (Austin, TX). The screening library was comprised of a commercially available subset of compounds from Chem Div (San Diego, CA) provided through a collaboration between the University of Michigan Center for Chemical Genomics and the Novartis Institute for Biomedical Research. CCG-63802 (((2E)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(3-methylphenoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-3-yl]prop-2-enenitrile)) and CCG-63808 (((2E)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(4-fluorolphenoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-3-yl]prop-2-enenitrile)) (see structures in **Fig. 2.1**) were purchased

from ChemDiv (San Diego, CA) and compound identity was verified by NMR via ChemDiv and by independent complete synthesis in the laboratory of Dr. Steven Husbands (University of Bath). Some analogs of CCG-63802 were purchased from Chem-Div and the rest (as identified by the BUXXXXX ID number) were synthesized in the laboratory of Dr. Stephen Husbands.

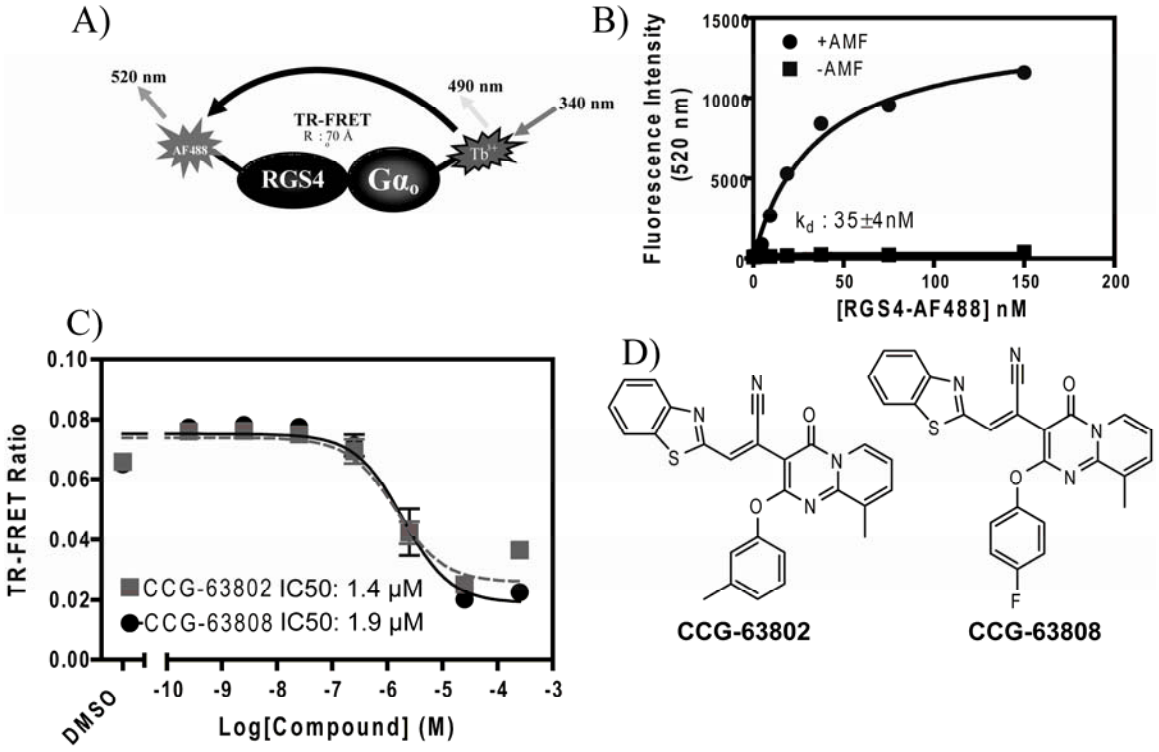


Figure 2.1 Characterization of the RGS4 TR-FRET high-throughput Assay. **A)** Schematic of RGS4-Gα_o TR-FRET assay. Gα_o is labeled with the LanthaScreen Tb-chelate donor fluorophore and RGS4 is labeled with an AlexaFluor-488 acceptor fluorophore. Excitation and emission maxima are listed for each fluorophore. **B)** Representative data showing the AlF_4^-/GDP dependence of the interaction between AF-488RGS4 and 10 nM of Tb-Gα_o. This saturable interaction has a K_d of 35±4 nM. Data are presented as mean±SEM **C)** Two compounds identified in the high throughput screen, CCG-63802 and CCG-63808, dose-dependently inhibit the TR-FRET signal between RGS4-AF488 and Tb-Gα_o with IC_{50} values of 1.4 (0.76; 2.6 μM) and 1.9 μM (1.02; 3.5 μM), respectively. Data are presented as mean (95% CI) **D)** The chemical structures of CCG-63802 and CCG-63808. n=3 for all data.

Compound synthesis:

Briefly, 2-Hydroxy-9-methyl-4H-pyrido[1,2-α]pyrimidin-4-one was prepared by the reaction of 2-amino-3-methylpyridine with diethyl malonate according to literature methods [21]. This material was firstly converted to 2-chloro-9-methyl-4-oxo-4H-pyrido[1,2-α]pyrimidine-3-carbaldehyde via Vilsmeier formylation, and this product was then heated with 4-fluorophenol to afford 2-(4-fluorophenoxy)-9-methyl-4-oxo-4H-pyrido[1,2-α]pyrimidine-3-carbaldehyde. Condensation of this compound with 2-benzothiazole acetonitrile using catalytic triethylamine in dichloromethane provided CCG-63808 as an orange crystalline solid (**Fig. 2.2**). CCG-63802 was prepared in a similar manner, except 4-fluorophenol was replaced with 3-methylphenol. Synthesized compounds were verified by 1H and 13C NMR using Jeol Delta-270-MHz instrument: 1H at 270 MHz, and Varian Mercury-400-MHz instrument: 1H at 400 MHz, 13C at 100 MHz; d in ppm, J in Hz with TMS as an internal standard, by electrospray mass spectrometry using micrOTOF (Bruker, Billerica, MA), and by microanalysis using a Perkin-Elmer 240C analyzer (Perkin-Elmer, Boston, MA).

Analogs lacking the vinyl nitrile moiety were synthesised depending on their functional group. Typically, analogs of the alkene were synthesised using a Horner-Wadsworth-Emmons reaction between the relevant aldehyde and a pre-prepared phosphonate. In several examples, reduction of the olefin was carried out, by hydrogenation using Pd/C under an atmospheric pressure of hydrogen. Further analogs, containing the amide functionality, were prepared using common amide coupling methodology.

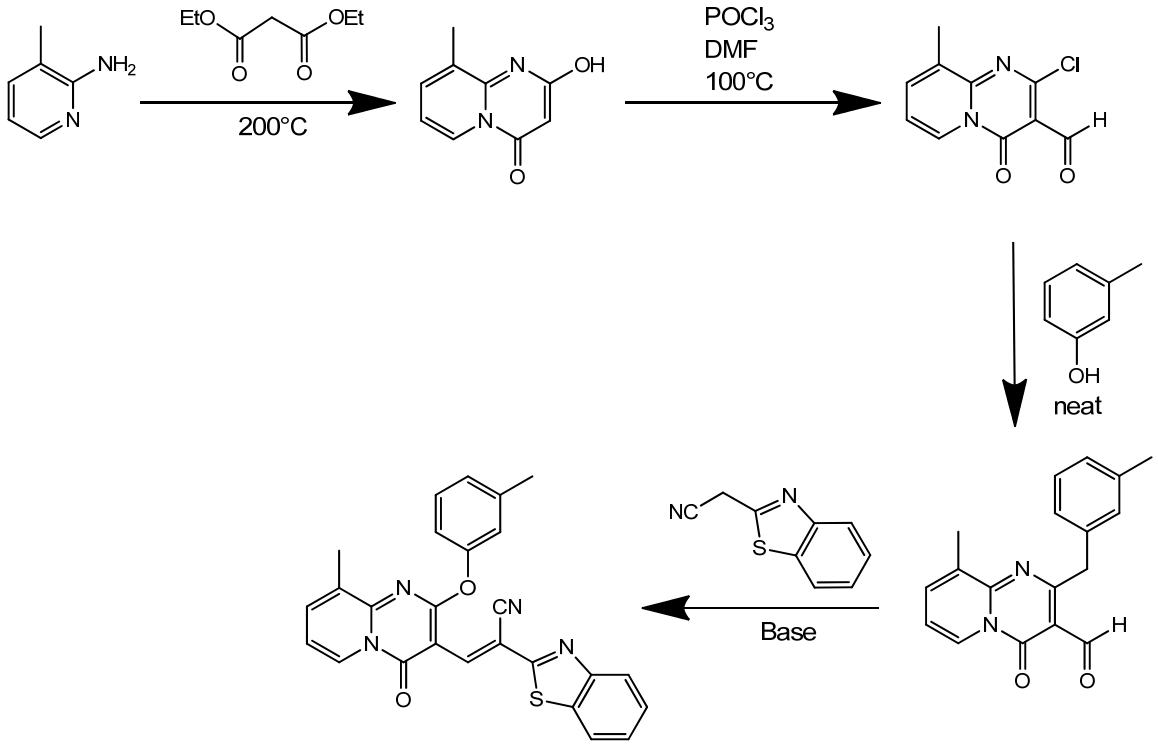


Figure 2.2 Reaction scheme for the synthesis of CCG-63802. Other analogs were synthesized using variations of this general scheme and will be described elsewhere.

Protein expression & purification:

Human RGS4 was expressed either from the pQE80RGS4 vector, which encodes 6x histidine tagged and N-terminally truncated form of RGS4 that lacks the first 18 residues (ΔN19RGS4) or from the pKMRGS4 vector, which encodes a maltose-binding protein-ΔN19RGS4 fusion protein. The ΔN form of RGS4 was selected because it provides better protein yield in prokaryotic expression systems. MBP-His6-RGS19ΔC11 (human), MBP-His6-RGS7 (human), MBP-His6-RGS8 (human) and MBP-His6-RGS16 (human) were expressed from constructs made with the pMALC2H10 vector as previously described [22]. For the mutagenesis studies, ΔN51RGS4 (rat) wild type and cysteine → alanine

mutants were expressed from the pMALC2H10 vector. Mutagenesis was performed as described [19] using the QuickChange Multi Site Directed Mutagenesis kit (Agilent, La Jolla CA) where one or more of the cysteine residues in the RGS domain of RGS4 were mutated to alanine.

All proteins were expressed in and harvested from BL21-DE3 *E. coli* via standard transformation, growth, and lysis protocols [16, 17, 23-25]. Histidine-tagged RGS4 was purified over a Nickel NTA affinity column (Qiagen, Valenica, CA) followed by cation exchange chromatography and size exclusion chromatography. MBP-tagged RGS proteins were purified with an amylose affinity column followed by size exclusion chromatography. Hexahistidine-tagged rat $G\alpha_o$ was expressed and purified as previously described [25]. G protein activity was determined by [^{35}S]GTPγS binding [26]. In all cases, proteins were purified to >90% homogeneity before use.

Chemical labeling of purified $G\alpha_o$ & RGS:

AlexaFluor-488 labeling of RGS4: ΔN19RGS4 was labeled with AlexaFluor-488 succinimidyl ester (Invitrogen, Carlsbad CA) at a 5:1 (label:protein) stoichiometry in a total volume of 2.0 mL of 50 mM HEPES pH 8.2 at 4˚C, 100 mM NaCl, 1 mM DTT. The reaction was performed while rotating samples in the dark for 1.5 hr at 4˚C. The reaction was quenched by the addition of 1 mM glycine for 10 minutes at 4˚C. Labeled RGS4 was resolved from the reaction mixture by size exclusion chromatography using a 20 mL Sephadex G-

25 desalting column (GE Biosciences, Piscataway NJ). Degree of labeling was determined spectroscopically to be approximately 1:1.

Tb chelate labeling of $G\alpha_o$: $G\alpha_o$ was labeled with the LanthaScreen™ Tb thiol reactive reagent (Invitrogen, Carlsbad CA) at a 5:1 (label:protein) stoichiometry in a total volume of 1.0 mL of 50 mM HEPES pH 7.25 at 4˚C, 100 mM NaCl, supplemented with 10 µM GDP and 0.8 mM TCEP. The reaction was allowed to proceed at 4˚C for 1.5 hr while rotating in the dark. The reaction was quenched by the addition 1 mM DTT for 20 minutes at 4˚C. Labeled protein was purified from the reaction mixture by size exclusion chromatography using a Sephadex G-25 desalting column (GE Biosciences, Piscataway NJ). Degree of labeling was determined spectroscopically to be approximately 1:1. The activity and effective concentration of the labeled G protein was determined by [^{35}S]GTPγS binding as described [26].

Biotinylation of RGS proteins: RGS protein was mixed at a 3:1 (label:protein) molar ratio with biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO) in a buffer of 50 mM HEPES pH 8.5 at 4°C, 100 mM NaCl, & 1 mM DTT. The reaction was allowed to proceed at 4°C while rotating for 2 hours and then was quenched by the addition of a large molar excess of glycine for 10 minutes. Labeled protein was purified from the reaction mixture by size exclusion chromatography using a Sephadex G-25 desalting column (GE Biosciences, Piscataway NJ).

AlexaFluor-532 labeling of $G\alpha_o$: $G\alpha_o$ labeling was performed as previously described [17]. Labeled protein was purified from the reaction mixture by size

exclusion chromatography using a Sephadex G-25 desalting column (GE Biosciences, Piscataway NJ).

Time Resolved FRET:

TR-FRET experiments were performed on a PHERAstar multipurpose microplate reader (BMG Labtech, Offenberg, Germany) using the LanthaScreen filter set. These experiments were based on the method of Leifert et al [27]. For the saturation experiments, Tb-$G\alpha_o$ was diluted to 20 nM in 50 mM HEPES pH 8.0, 100 mM NaCl, 0.1% lubrol, 30 µM GDP, 5 mM NaF, 5 mM $MgCl_2$, 5 µM $AlCl_3$ and allowed to activate for 10 minutes on ice before use. RGS4-AF488 was serially diluted in 50 mM HEPES pH 8.0 at room temperature, 100 mM NaCl, 0.1% lubrol (TR-FRET buffer). Ten microliters of the RGS4 dilution was added to a black non-stick low-volume 384 well plate (Corning 3676) with a minimum of duplicate measurements. Ten microliters of the Tb-$G\alpha_o$ was added (10 nM final) and the mixture was allowed to incubate at room temperature for 15 minutes in the dark. The non-specific TR-FRET signal was determined by excluding the $AlCl_3$, $MgCl_2$ and NaF from a set of samples. The fluorescence emission at both 490 and 520 nm was measured from 50 flashes of 340 nm excitation light per well. The data were collected in 10 µs bins and the delayed emission signal was integrated from 100 to 500 µs after each flash. TR-FRET data was analyzed as the ratio of emission at 520 nm/490 nm.

High Throughput Screening:

High throughput screening was performed with the University of Michigan Center for Chemical Genomics. The ca. 40,000 compound screening collection was provided by the Novartis Institute for Biomedical Research (East Hanover NJ) and was comprised of compounds selected from the ChemDiv screening library. Five microliters of 50 mM HEPES pH 8.0 at room temperature, 100 mM NaCl, 0.1% lubrol, 1 mM DTT (TR-FRET buffer) was dispensed with a Multidrop™ (Thermo Fisher Scientific, Waltham MA) into every well of a black, non-stick, low-volume 384-well plate. Two hundred nanoliters of each compound (2 mM stock, 20 μM final assay concentration) or DMSO control was added to the plate with a pin tool using a Beckman BioMek FX liquid handler (Beckman Coulter, Fullerton, CA). To this compound dilution, 5 microliters of 200 nM AlexFluor 488 labeled RGS4 was added and incubated for 15 minutes at room temperature in the dark. Then, 10 microliters of 20 nM Tb labeled $G\alpha_o$ was added to the mixture. For this assay, the positive inhibition control (i.e. no RGS4/$G\alpha_o$ binding) was Tb-labeled $G\alpha_o$ in the inactive GDP-bound state and the negative control (i.e. full RGS4/$G\alpha_o$ binding) utilized $G\alpha_o$ in the GDP/AlF_4 bound state. This mixture was incubated at room temperature in the dark for 15 minutes before analysis with the PHERAstar plate reader. Data were compiled and analyzed using the M-Screen database, an in-house chemoinformatics suite developed by the Center for Chemical Genomics at the University of Michigan. Compounds that inhibited the TR-FRET signal >2SD from the negative control

were considered “actives” and were chosen for dose response follow-up experiments.

TR-FRET Dose Response Experiments:

Actives from the primary screen were evaluated for concentration-dependent activity in the TR-FRET assay. Compound dilutions were performed in DMSO and 200 nL of diluted compound was spotted into the wells of a non-stick, low volume black 384-well plate that contained 5 µL of TR-FRET buffer. To the well, 5 µL of 200 nM AlexFluor 488 labeled RGS4 was added and incubated at room temperature in the dark for 15 minutes. Then, 10 µL of 20 nM Tb labeled $G\alpha_o$ GDP/AlF_4 was added to the mixture and incubated at room temperature in the dark for 30 minutes before analysis on the PHERAstar plate reader. Compound dilutions covered a final concentration range from 200 µM to 1.6 µM. Positive and negative controls were performed as in the primary screening assay. Compounds whose dose response curves were not fully defined by these concentrations were repeated using a more appropriate dilution scheme. Nonlinear least-squares regression fitting of the data was performed using the data analysis component of the MScreen database.

Flow Cytometry Protein Interaction Assay Concentration Dependence Experiments:

Compounds that confirmed in the follow up TR-FRET dose response assay were tested as described [17] in the Flow Cytometry Protein Interaction Assay (FCPIA). This was done in part to provide a complementary set of biochemical data to filter out any compounds that might produce spectroscopic artifacts in the TR-FRET assay. Briefly, biotinylated RGS proteins (5nM, final assay concentration) were immobilized on Luminex LumAvidin beads and incubated with diluted compound in 50 mM HEPES pH 8.0 at room temperature, 100 mM NaCl, 0.1% lubrol, 1 mM DTT, supplemented with 1% BSA. To each well of a 96-well PCR plate (Axygen, Union City, CA) was added AlexaFluor 532 labeled $G\alpha_o$ to a final concentration of 30nM. This mixture was incubated for 30 minutes at room temperature in the dark and then it was analyzed on a Luminex 200 flow cytometer for the bead associated fluorescence (median value). Nonlinear regression analysis of inhibition curves was performed with Prism 5.0 (Graphpad Software, San Diego CA).

FCPIA Reversibility Experiments:

RGS-coated beads were prepared as above and were treated with 50 μM compound or vehicle (DMSO) for 15 minutes at room temperature. The RGS-containing beads were then washed by resuspension in 1mL of phosphate buffered saline, pH 7.4 supplemented with 1% BSA, vortexing briefly, then pelleting the beads by centrifugation. This procedure was repeated a total of

three times before 1,000 beads were added to each quadruplicate well of a 96-well PCR plate that contained AlexaFluor-532-labeled $G\alpha_o$ at a final concentration of 20 nM in the presence or absence of 50μM test compound. The mixture was incubated for 30 minutes at room temperature and then analyzed on a Luminex 200 flow cytometer for bead-associated fluorescence. Data analysis was performed with Prism 5.0 (Graphpad Software, San Diego CA).

Single Turnover GTPase Measurements:

Compounds were tested for the ability to inhibit the RGS4-stimulated increase in GTP hydrolysis by $G\alpha_o$ as described previously [15, 17].

Thermal Stability Measurements:

Untagged ΔN19RGS4 or His_6-$G\alpha_o$ was added to the well of a 96-well ABI Prism optical reaction plate (Applied Biosystems, Foster City CA) to a final concentration of 5 or 2.5 μM, respectively in 50-60 μL of 50 mM HEPES pH 8.0 with 150 mM NaCl. Test compounds were added to the protein at the desired concentration and allowed to interact for 15 minutes at room temperature. To each well, Sypro Orange dye (Invitrogen, Carlsbad, CA) was added to a 5X final concentration (as described by the supplier) and the plate was sealed with an optically clear adhesive film. Sypro Orange fluorescence was measured continuously in an ABI HT7900 real-time PCR system during a stepwise gradient from ambient temperature to 90°C in 1°C steps lasting 30 seconds each. Data are analyzed by fitting the obtained curves to a Boltzmann model:

$$I = L\frac{(U-L)}{1+e^{\frac{(Tm-T)}{a}}}$$

Where I = Fluorescence Intensity (AU), L = the lower limit of the curve (°C), U = the upper limit of the curve (°C), T = temperature (°C), and a = is a slope factor. Values obtained after the fluorescence maximum occurred were excluded from the analysis.

Results:

Development of a High-Throughput TR-FRET RGS4-Gαo interaction screen:

I developed a biochemical TR-FRET assay using purified human RGS4 labeled with the AlexaFluor-488 acceptor fluorophore and purified $G\alpha_o$ labeled with the LanthaScreen Tb probe donor fluorophore (**Fig. 2.1A**). Using this system, I observed a saturable, aluminum fluoride-dependent interaction between RGS4 and $G\alpha_o$ that has an affinity consistent with other reports of this PPI in the literature (**Fig. 2.1B**) [17]. In collaboration with the Center for Chemical Genomics at the University of Michigan, this assay was scaled to 384-well format and used to screen ~44,000 small molecules for inhibition of RGS4/$G\alpha_o$ binding in the presence of a thiol reducing agent (**Table 2.1**). Compounds from this screen were re-tested in the primary screening assay to confirm the initial result and to assess the concentration dependence of the inhibition using the original TR-FRET assay. Of the 162 compounds that met the 2 standard deviation selection criteria for inhibition, 48 were either unavailable or predicted to be

chemically reactive and were not followed-up. The 114 selected compounds were retested in TR-FRET DRC and 11 were confirmed as inhibitors with IC_{50} values <400 μM and Hill Slopes <2.

Assay	Compounds tested	Active*	Hit Rate (%)
ChemDiv Library Subset	43878	162	0.37
TR-FRET DRC	114	11	0.025
FCPIA DRC	11	2	0.0046

Table 2.2 RGS4/Gαo TR-FRET high-throughput screening results. Actives were determined as follows: primary screen, >2SD from the negative control; TR-FRET Dose-response curve (DRC), IC_{50} value <400 μM; FCPIA DRC: IC_{50} value <500 μM.

The confirmed, active compounds were obtained from the supplier as fresh powders and tested using the Flow Cytometry Protein Interaction Assay (FCPIA), a method that measures the binding of fluorescently tagged $G\alpha_o$ to an RGS protein on beads [17]. Of the 11 compounds tested, 2 showed similar activity on RGS4 in both the TR-FRET dose response and FCPIA experiments (**Fig. 2.1C**). The 9 compounds that did not show activity in this secondary assay are presumed to have been spectral artifacts or small molecule aggregators that are likely to lose function in the relatively stringent conditions of the FCPIA assay buffer (50 mM HEPES, 100 mM NaCl, 1% BSA and 0.1% lubrol, pH 8.0).

The two active compounds that were identified from this primary screen were the closely related compounds, CCG-63808 and CCG-63802 (**Fig. 2.1D**). These compounds differ solely by the substituents on the phenyl moiety and have similar IC_{50} values in TR-FRET (RGS4 IC_{50} 1.4 and 1.9 μM for CCG-63802 and CCG-63808) and FCPIA (RGS4 IC_{50} 9 and 10 μM for CCG-63802 and CCG-

63808). The compounds also contain a vinyl cyanide moiety that may function as a Michael acceptor.

CCG-63802 & CCG-63808 selectively inhibit Gαo-RGS interactions:

Using TR-FRET to assess the RGS4-$G\alpha_o$ interaction, CCG-63802 and CCG-63808 had IC_{50} values of 1.4 and 1.9 μM, respectively (**Fig. 2.1C**). To determine the selectivity of these compounds for different RGS proteins, they were tested in an FCPIA competition experiment against a panel of 5 different RGS proteins (**Fig. 2.3**, **Table 2.2**). The compounds are 6- to 7-fold less potent in blocking $G\alpha_o$/RGS4 interactions when tested using FCPIA (IC_{50} 9 or 10 μM for CCG-62802 or CCG-63808) than with the TR-FRET method. This is probably due to the high level of BSA (1%) in the FCPIA buffer sequestering compound and decreasing its apparent concentration in the assay. These compounds did not inhibit Gα binding to RGS7, which is distantly related to RGS4, and they are two- to ten-fold more potent at RGS4 than on the other closely related R4 family members, RGS8 and RGS16 (**Table 2.2**). They are also active (IC_{50} 20-50 μM) on the one RZ family member tested, RGS19.

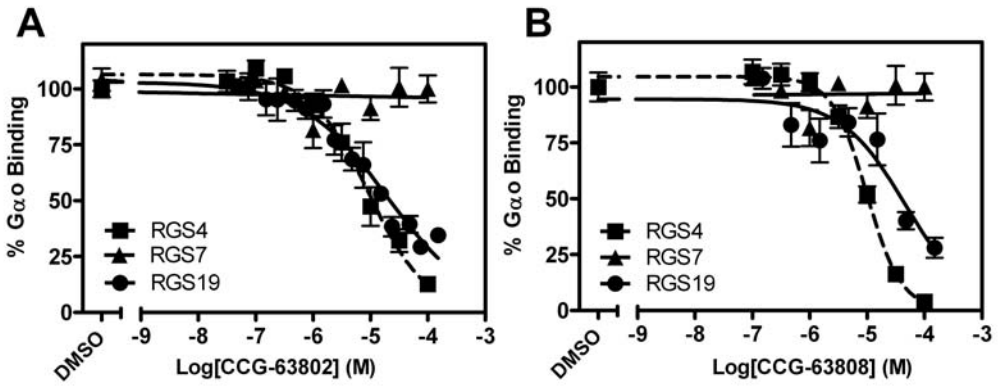


Figure 2.3 RGS specificity of CCG-63802 and CCG-63808 determined by multiplex FCPIA analysis (n≥3). RGS coated beads were treated with the indicated concentration of **A)** CCG-63802 or **B)** CCG-63808 for 15 minutes at room temperature, after which GDP/AlF_4 bound $G\alpha_o$-AF532 was added and allowed to incubate with the RGS/compound mixture for 30 minutes prior to analysis. See table 2.2 for IC_{50} values. All data was calculated using nonlinear least squares regression with the bottom of the curves constrained to 0% binding. Data are presented as mean ± SEM from 3 separate experiments.

	CCG-63802		CCG-63808	
RGS Protein	**IC_{50} (μM)**	**Hill Slope**	**IC_{50} (μM)**	**Hill Slope**
RGS4	9	-0.9	10	-1.4
RGS4c	>400	-0.4	>400	-0.8
RGS8	112	-0.6	74	-1.1
RGS16	42	-1.4	21	-2.1
RGS19	20	-0.6	46	-0.8
RGS7	NI	NI	NI	NI

Table 2.3 RGS specificity of CCG-63802 and CCG-63808 determined by multiplex FCPIA analysis (n≥3). All data was calculated from at least three independent experiments using nonlinear least squares regression with the bottom of the curves constrained to 0% binding. NI: No inhibition observed at highest concentration tested (100 μM).

CCG-63802 & CCG-63808 inhibit RGS4 GAP activity:

For RGS inhibitors to be functionally relevant, they need to inhibit the catalytic activity of the RGS in addition to blocking Gα/RGS binding. The two compounds inhibit the GAP activity of RGS4 as shown by measurements using the [^{32}P]GTP single turnover GAP assay (**Fig. 2.4**). Under these conditions, GTP hydrolysis by $G\alpha_o$ is accelerated ~10-fold by the addition of wtRGS4 and this effect can be inhibited by the previously described [17] RGS4 inhibitor CCG-

4986. At a concentration of 100 μM, CCG-63802 and CCG-63808 fully inhibit the RGS activity without affecting basal $G\alpha_o$ GTPase activity.

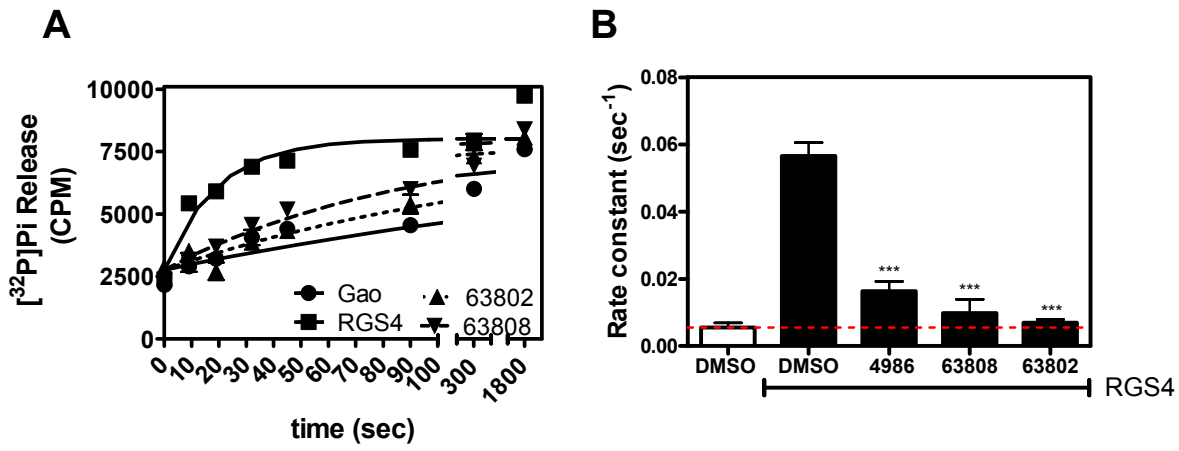


Figure 2.4 Single Turnover GAP analysis of small molecule RGS inhibitors with RGS4. **A)** RGS4 treated with 100 μM of CCG-4986, CCG-63808, or CCG-63802 lacks the ability to increase the intrinsic hydrolysis rate of $G\alpha_o$. Representative GAP data shown, however all experiments were performed a minimum of 3 times. **B)** Rate constants of GTP hydrolysis. Rate constants are presented as mean ± SEM from at least 3 independent experiments. ***p<0.001 vs. the DMSO treated RGS control.

CCG-63802 & CCG-63808 bind to RGS4 but not to $G\alpha_o$:

Because the studies presented so far assessed the binary interaction between two purified proteins, it was necessary to determine to which protein the compounds bound. The specificity for RGS4 over RGS7, 8, and 19 suggested, but did not prove that the compounds bound to the RGS rather than to the $G\alpha_o$. To directly identify the site of action of these compounds, we developed a thermal denaturation assay to assess compound binding. This methodology is based on the principle that the stability of a protein is often altered upon ligand binding [28, 29]. For proteins that have endogenous small molecule or peptide

ligands (e.g. enzymes or receptors), binding of the ligand often increases the thermal stability. Upon binding GDP, $G\alpha_o$ experiences a >5°C increase in melting temperature (T_m) when compared to nucleotide-free protein (**Fig 2.5**). This increases to a >20°C increase in T_m for $G\alpha_o$ binding the exceptionally high affinity nucleotide GTPγS. Using this assay, we observed a concentration-dependent 10°C reduction (See Discussion) in the melting temperature of RGS4 in the presence of CCG-63802 (**Fig. 2.6A**). The concentration dependence of this effect corresponds with the IC_{50} values obtained in the FCPIA assay. Even at a maximal concentration of CCG-63802 (100 μM), there was no change in the melting temperature of $G\alpha_o$ (**Fig. 2.6B**).

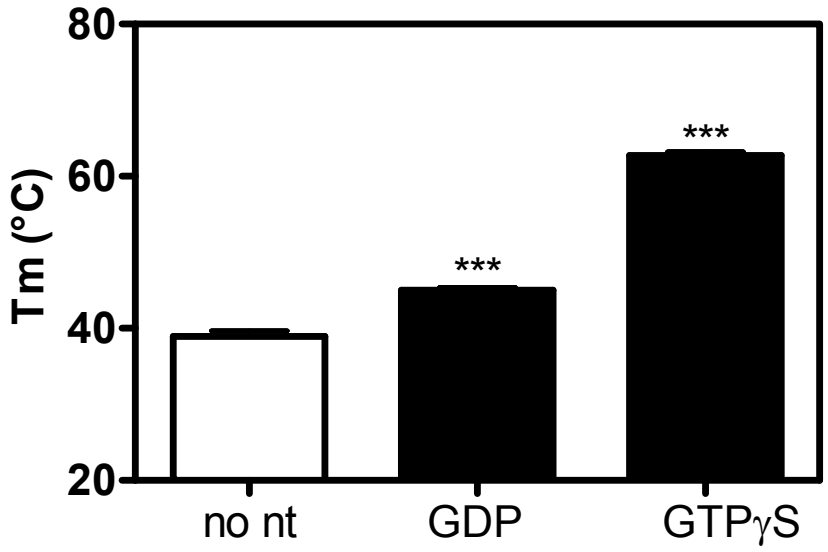


Figure 2.5 $G\alpha_o$ is thermally stabilized in presence of nucleotide. Purified $G\alpha_o$ was stripped of nucleotide by gel filtration in a buffer containing EDTA. Lack of nucleotide was confirmed by spectroscopic analysis. The melting temperature (T_m) of $G\alpha_o$ (2.5 μM) was determined by the thermal stability assay as described (see Methods) in the presence or absence of 50 μM GDP or GTPγS. Nucleotide free (no nt) $G\alpha_o$ has a T_m of 38.9±0.7°C. The protein is stabilized in the presence of 50 μM GDP by 6°C (T_m: 45.0±0.3C°) and is stabilized by 23°C in the presence of 50 μM GTPγS (Tm 62.7±0.5°C). Data are presented as mean ± SEM from 3 separate experiments.

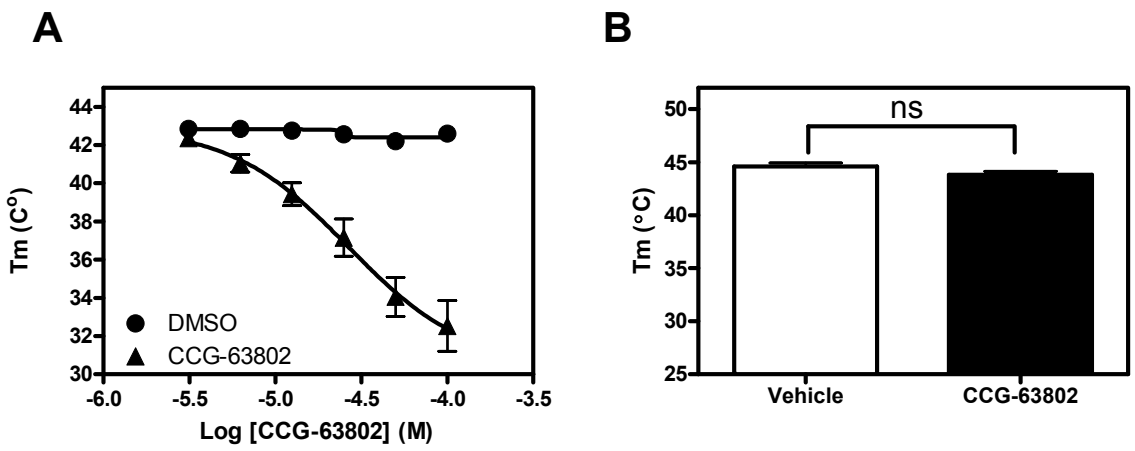


Figure 2.6 CCG-63802 specifically binds to RGS4 and not to $G\alpha_o$. **A)** Purified RGS4 shows a dose dependent change in melting temperature in the presence of CCG-63802 (EC_{50} 26 μM). **B)** A saturating concentration of CCG-63802 (100 μM) does not affect the melting temperature of $G\alpha_o$. Data are presented as mean±SEM of 3 separate experiments.

CCG-63802 and CCG-63808 are reversible inhibitors of the Gαo-RGS interaction:

The effects of CCG-4986, our previously described RGS4 inhibitor [17], could not be reversed by dilution and washing away the compound, showing that it acts irreversibly to inhibit the function of RGS4 [18, 19], and **Fig. 2.7**). Also, its activity was blocked in the presence of reducing agents. These effects are likely due to the formation of a covalent adduct of the compound with a cysteine residue in the RGS [18, 19]. Since our new compounds were identified through screens in the presence of DTT, we tested the reversibility of their inhibition. RGS-coated microspheres were treated with 50 μM compound or vehicle (DMSO), extensively washed (see Methods for details), and then assayed for $G\alpha_o$ binding (**Fig 2.7**). In contrast to the effects of CCG-4986, full binding was restored to compound-treated RGS-beads after washing (**Fig 2.7**), showing that

CCG-63802 and CCG-63808 are reversible on the 10-minute time scale required for the washing procedure. Consequently, these new compounds represent the first examples of reversible small molecule inhibitors of an RGS protein.

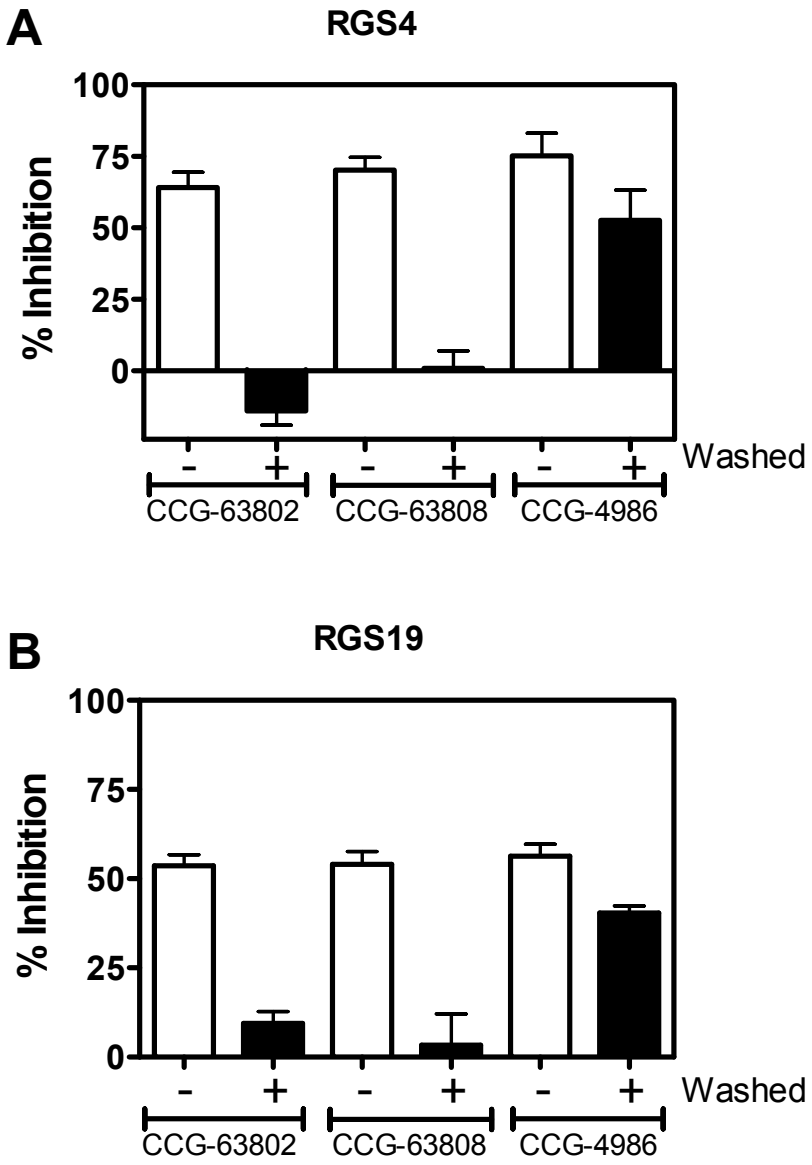


Figure 2.7 CCG-63802 and CCG-63808 are reversible inhibitors of **A)** RGS4 and **B)** RGS19. CCG-4986 is an irreversible inhibitor of RGS4 and RGS19. In all cases, RGS coated FCPIA beads were treated with 50 μM of compound (or vehicle, DMSO) and then extensively washed. The beads were then split into two groups and tested for the ability to interact with $G\alpha_o$-AF532 in the presence or absence of 50 μM Compound. Data shown are the mean±SEM of three separate experiments.

Cysteine Dependence of CCG-63802 and CCG-63808:

To further explore the mechanism of these compounds and the role of cysteines in their action, they were tested on a mutant of RGS4 where all cysteines in the RGS domain were mutated to alanine (RGS4c). In FCPIA measures of Gα binding to RGS4c, CCG-63802 and CCG-63808 show only modest activity, indicating a role for RGS cysteines in the actions of these compounds (**Fig 2.8**, **Table 2.3**). Consequently, we tested CCG-63808 and CCG-63802 with a panel of RGS4 RGS domain cysteine mutants using FCPIA (**Table 2.3**). The G protein binding affinity of these RGS mutants has been previously described [30] and the Kd values ranged from 3-12 nM, not drastically different from that of wild type RGS4. No single cysteine could fully account for the effects of these compounds, but it appears that three cysteines, Cys 148, Cys 132, and Cys 95 are important for full sensitivity to CCG-63808 and CCG-63802. Cysteine 95 and Cys 148 are located close to each other on RGS4, however they are at a site distinct from the Gα interaction interface. It appears that Cys 95 plays a more significant role than Cys 148, possibly suggesting that the compound docks onto the RGS at a site that is either closer to this cysteine or that requires this residue for proper formation of the compound binding pocket.

RGS4 Mutant	IC_{50} (μM)	pIC_{50} Log(M)	Hill Slope	Inhibition at 100μM (%)	n
WT	9	5.02 ± 0.07	-0.86 ± 0.11	87	9
C148A	43	4.37 ± 0.07	-0.95 ± 0.16	63	3
C132A	41	4.39 ± 0.07	-0.97 ± 0.18	66	3
C95A/C132A	32	4.50 ± 0.13	-0.78 ± 0.20	70	3
C148A/C132A	92	4.04 ± 0.07	-0.75 ± 0.11	57	3
C148A/C132A/C95A	~3000	2.55 ± 0.64	-0.33 ± 0.12	16	3
RGS4c	~8000	2.10 ± 1.50	-0.36 ± 0.30	13	6
A148C	~390	3.41 ± 0.17	-0.62 ± 0.14	30	3
A132C	174	3.76 ± 0.19	-0.80 ± 0.29	31	3
A95C	170	3.77 ± 0.23	-1.20 ± 0.82	30	3
A148C/A132C	33	4.47 ± 0.05	-1.48 ± 0.23	92	3
A148C/A95C	17	4.77±0.12	-1.06±0.28	100	3
A95C/A148C/A132C	16	4.79 ± 0.12	-0.63 ± 0.12	64	3

Table 2.4 RGS4 cysteine mutant sensitivity to CCG-63802. IC_{50} values calculated from FCPIA concentration-response experiments. Data are presented as mean ±SEM.

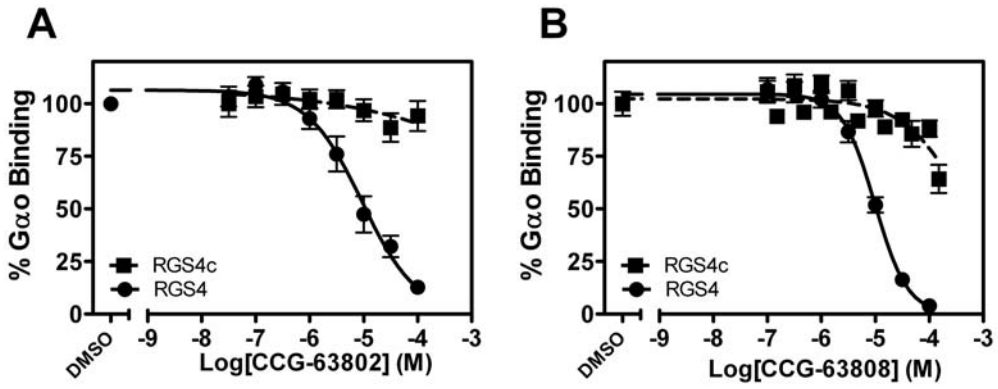


Figure 2.8 CCG-63802 and CCG-63808 are much less potent on a mutant form of RGS4 that lacks cysteine residues in the RH domain. FCPIA dose-response curves for **A)** CCCG-63802 and **B)** CCG-63808. Data are presented as mean ± SEM from 3 separate FCPIA experiments.

Since thiol-reactive compounds may have difficulty functioning in the reducing environment of a cell, it is important to assess the activity of any such leads under conditions mimicking the intracellular environment. Therefore, CCG-63802, CCG-63808, and CCG-4986 were tested for activity using FCPIA in the presence of 2 mM reduced glutathione (**Fig. 2.9**). This concentration of

glutathione was selected because it is similar to intracellular concentrations. CCG-63802 and CCG-63808 lose approximately 0.5-1 Log of potency (IC_{50} 6 μM → 40 μM for CCG-63802; 4 μM → 21 μM for CCG-63808) in the presence of 2 mM glutathione, but still retain the ability to fully inhibit the interaction between RGS4 and $G\alpha_o$. In contrast, CCG-4986 loses over 2-Logs in potency (IC_{50} from1.4 μM → 215 μM) in the presence of 2 mM glutathione and it is not capable of fully inhibiting the RGS-$G\alpha_o$ interaction up to concentrations nearing its aqueous solubility (**Fig. 2.9**).

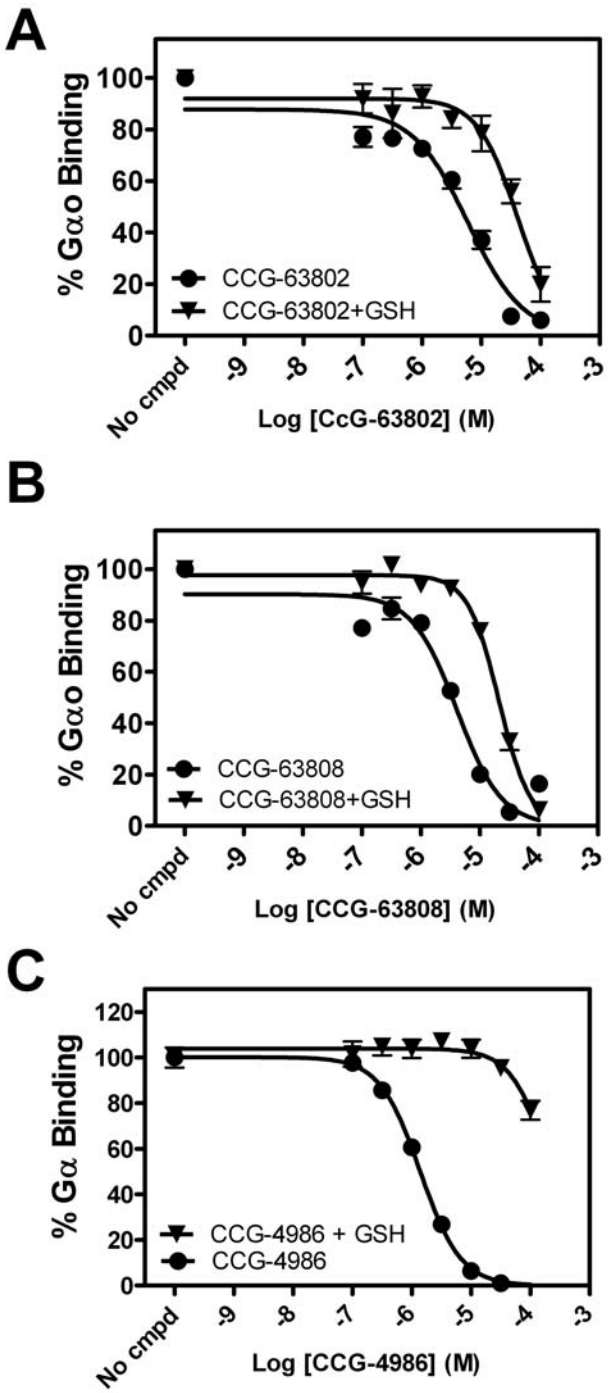


Figure 2.9 CCG-63802 is less sensitive to glutathione than other RGS4 inhibitors. **A)** CCG-63802 and **B)** CCG-63808 retain full inhibitory activity in the presence of 2 mM glutathione, the potency is right shifted by approximately 0.5-1 Log (CCG-63802: pIC_{50} 5.25±0.07 to 4.39±0.07; CCG-63808: pIC_{50} 5.39±0.06 to 4.68±0.03). In contrast, **C)** CCG-4986 loses over two logs of potency (IC_{50} 5.87±0.03 to 3.66±0.15) in the presence of glutathione. n=2; n=3 for CCG-4986. Data presented as mean±SEM.

I originally found that CCG-63802 and CCG-63808 inhibit the single turnover GAP activity of RGS4c (**Fig. 2.10**) mutant despite their much lower potency to inhibit $G\alpha_o$/RGS4c binding in FCPIA (**Fig 2.8**). These data suggested that this class of compounds can inhibit the functional activity of the cysteine-null RGS4 mutant, while having much less effect on the high-affinity binding to GDP-AMF bound $G\alpha_o$ (see Discussion). This inhibitory effect does not appear to be due to compound aggregation, as is not reversed in the presence of 0.01% Triton X-100 (data not shown), which generally blocks the activity of promiscuous small molecule aggregators [31]. Unfortunately, the compounds did not have an effect on RGS4c in steady state GTPase assays and thermal stability experiments (data not shown, experiments performed by Andrew Storaska). While it is possible that the single turnover data are correct, the lack of consistency with other biochemical assays raises questions regarding the validity of the single turnover data. As such, it is believed that while these data are solid, their physiological significance is likely to be limited. Although disappointing, this result is in line with the observed cysteine dependence in FCPIA.

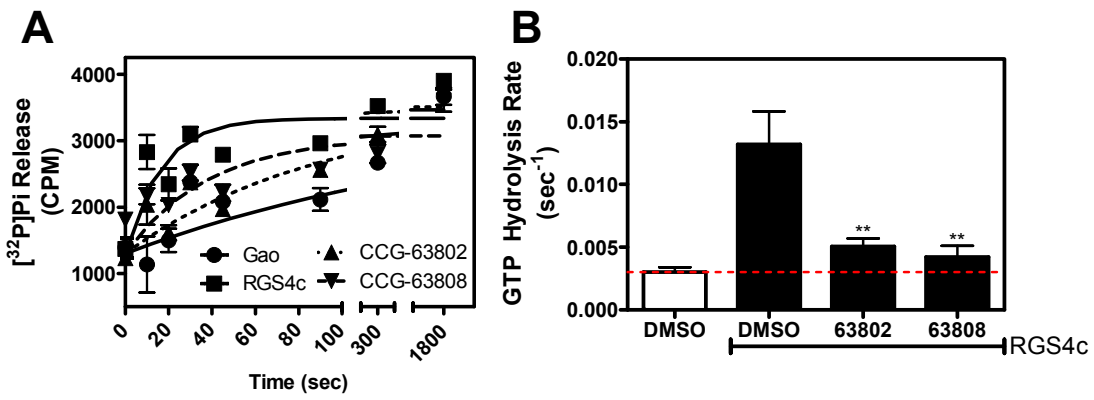


Figure 2.10 CCG-63802 and CCG-63808 inhibit the GAP activity of a cysteine-null RGS4 mutant. **A)** CCG-63802 or CCG-63808 (100 μM) inhibits the ability of RGS4c to accelerate the rate of GTP hydrolysis by $G\alpha_o$. Representative data shown, however all experiments were performed a minimum of 3 times. **B)** Rate constants of GTP hydrolysis. Rate constants are presented as mean ± SEM from at least 3 independent experiments. **p<0.01 vs. the DMSO treated RGS control.

Structure-Activity Studies of the CCG-63802 class of compounds:

In order to understand the structure-activity landscape surrounding the CCG-63802 class of compounds, we developed a series of analogs to test specific hypotheses about the mechanism of action of this compound family. In general 4 main questions were asked: 1) Are any of the heterocycles unnecessary? 2) Is the vinyl cyanide moiety (a known Michael Acceptor) critical for activity? 3) Is the benzothiazole (a suspected thiol reactive moiety) necessary for activity? 4) Can significantly improved potency be achieved by optimizing the substituents on the phenyl moiety?

To answer these questions, I tested a total of 74 analogs of CCG-63802/CCG-63808 for their ability to inhibit the RGS4-$G\alpha_o$ PPI using FCPIA. To first determine the smallest functional unit of the compound, a series of analogs of the parent compounds were studied (**Table 2.4**). In general, these compounds lacked one or more of the three heterocycles present in the parent compound.

Compounds CCG-203673-5 contain primarily the pyridopyrimidone ring structure and did not inhibit RGS4- or RGS8-$G\alpha_o$ interaction at any concentration below 100μM. Compounds CCG-203676-80 contain the pyridopyrimidone ring with the fluorophenoxy moiety present in CCG-63808. These likewise did not show inhibitory activity against the RGS4/RGS8-$G\alpha_o$ PPI in FCPIA or GAP (**Fig. 2.11**). The compounds CCG-203686 and CCG-203687 contain the pyridopyrimidone/fluorphenoxy rings and the vinyl cyanide moiety (CCG-203686) or the cyano group lacking the vinyl (CCG-203687). Neither of these compounds have activity, suggesting that the benzothiazole is important in the mechanism of action of this compound.

The presence of the vinyl cyanide is important for the Michael-style reactivity of this group. To determine the importance of this reactivity in the mechanism of action of CCG-63802, I tested three analogs (CCG-203683-5) that do not contain a vinyl cyanide group (**Table 2.4**). In place of this group, these compounds contain an alkyl chain (CCG-203685), a alkene (CCG-203684) or an alkyl chain with a cyano group (CCG-203683) It was found that none of these compounds have activity towards RGS4, suggesting that the vinyl cyanide moiety is a major driving force between the compound-RGS interaction.

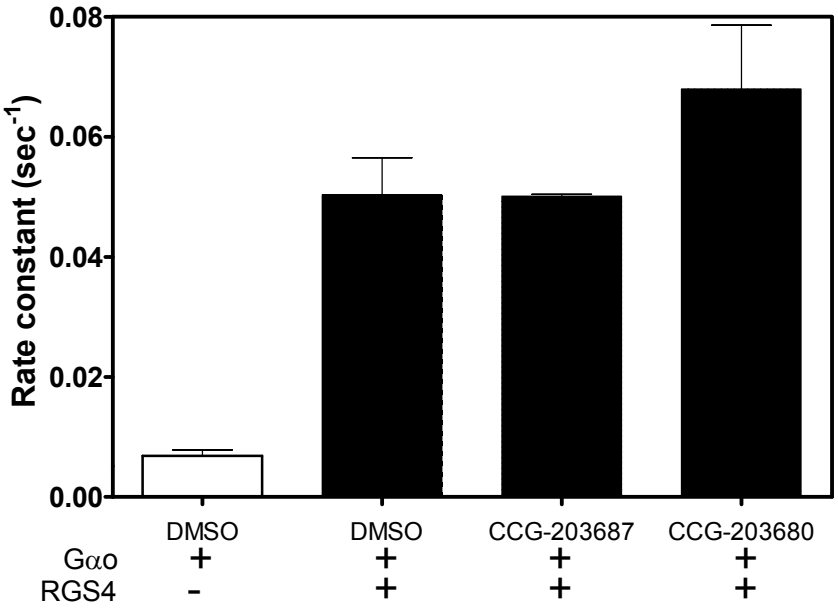


Figure 2.11 CCG-203687 and CCG-203680 (100 μM) are incapable of inhibiting the ability of RGS4 to accelerate the rate of GTP hydrolysis by $G\alpha_o$. Rate constants are presented as mean ± SEM from 2 independent experiments.

Because it is known that there is some cysteine dependence of the compound for full activity and since the vinyl cyanide is a Michael acceptor whose reactivity may be potentiated by the benzothiazole, it could be hypothesized that this portion of the molecule would be disproportionately important in driving the energetics of the compound-RGS interaction. To test this hypothesis, a series of compounds (CCG-203697,CCG-203703-18) were synthesized (**Table 2.4**). These compounds contain the benzothiazole and vinyl cyanide portions of CCG-63802 and have an increasingly bulky series of ring structures opposite the benzothiazole (i.e. in place of the pyridopyrimidone/methylphenoxy component of CCG-63802). Of these

compounds, only CCG-203714 showed any activity, although this activity was weak (>200 μM IC_{50}) and inconsistent. Thus, these data suggest that the vinyl cyanide/benzothiazole groups on this class of compound are necessary, but not sufficient for RGS inhibitory activity. It will be shown below, however, that with the appropriate ring structure, the benzothiazole can be replaced. When taken in conjunction with the pyridopyrimidone analog data, these data suggest that all three rings and the vinyl cyanide are required for full activity of this class of compounds. In certain instances (e.g. CCG-203687 and CCG-203680, **Fig. 2.11**) compounds from these groups of compounds were tested at a high concentration (100 μM) in a single turnover RGS4 GAP assay to confirm the observations that were made using FCPIA. In all cases, there was direct correlation between lack of activity in FCPIA and lack of activity in the single turnover GAP assay.

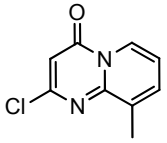
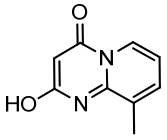
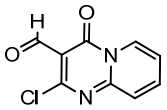
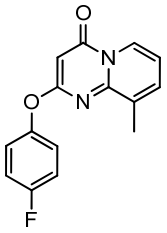
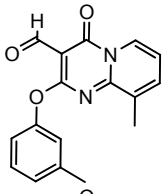
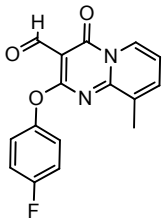
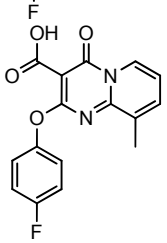
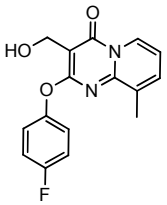
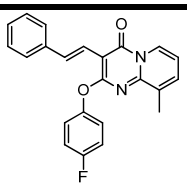
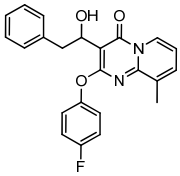
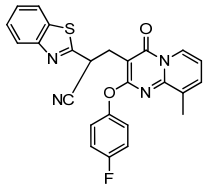
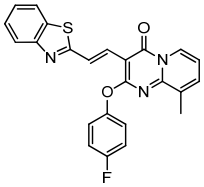
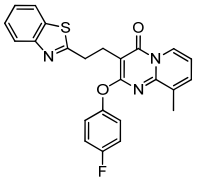
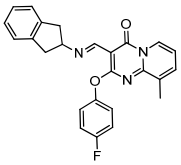
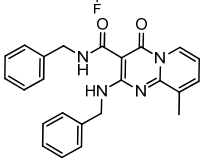
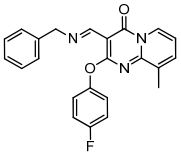
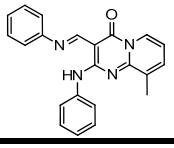
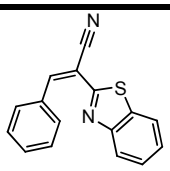
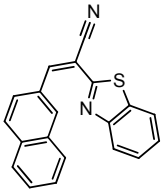
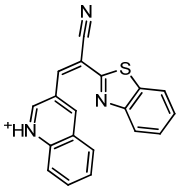
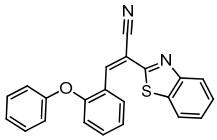
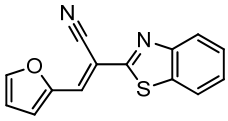
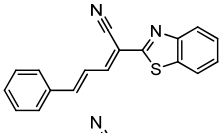
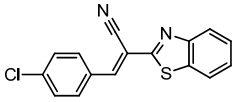
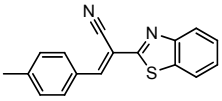
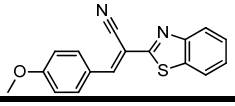
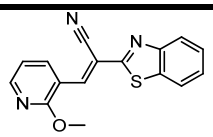
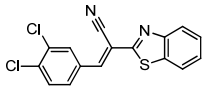
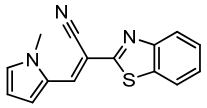
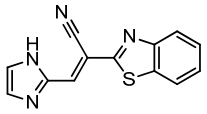
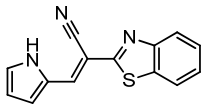
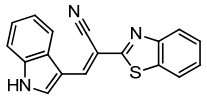
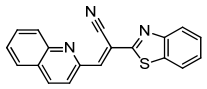
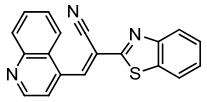
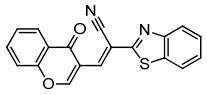
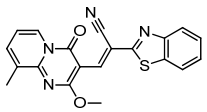
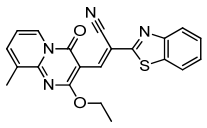
CCG Number	Structure	ID Number	Molecular Weight	RGS4 IC_{50} (μM)	RGS4c IC_{50} (μM)	RGS8 IC_{50} (μM)
203673		BU08057	194.62	NA	NT	NA
203674		BU08058	176.17	NA	NT	NA
203675		BU08059	222.63	NA	NT	NA
203676		BU08060	270.26	NA	NT	NA
203677		BU08061	294.3	NA	NT	NA
203678		BU08062	298.27	NA	NT	NA
203679		BU08063	314.27	NA	NT	NA
203680		BU08064	300.28	NA	NT	NA

Table2.5 Inactive analogs of CCG-63802. Compounds were tested in FCPIA against RGS4 and RGS8. All compounds were synthesized in the laboratory of Dr. Stephen Husbands. n=2

CCG Number	Structure	ID Number	Molecular Weight	RGS4 IC_{50} (μM)	RGS4c IC_{50} (μM)	RGS8 IC_{50} (μM)
203681		BU09003	372.39	NA	NT	NA
203682		BU09004	390.41	NA	NT	NA
203683		BU09005	456.49	NA	NT	NA
203684		BU09006	429.47	>100	NT	NA
203685		BU09007	431.48	NA	NT	NA
203691		BU09015	413.44	>100	NT	NA
203692		BU09016	398.46	NA	NT	NA
203693		BU09017	387.41	NA	NT	NA
203694		BU09018	354.4	>100	NT	NA

CCG Number	Structure	ID Number	Molecular Weight	RGS4 IC_{50} (μM)	RGS4c IC_{50} (μM)	RGS8 IC_{50} (μM)
203697		BU09025	262.33	N/A	NT	NT
203703		BU09033	312.39	NA	NT	NT
203704		BU09034	313.38	NA	NT	NT
203705		BU09035	354.42	NA	NT	NT
203706		BU09038	252.29	NA	NT	NT
203707		BU09039	288.07	NA	NT	NT
43223		BU09040	296.77	NA	NT	NT
203708		BU09041	276.36	NA	NT	NT
203709		BU09042	292.35	NA	NT	NT

CCG Number	Structure	ID Number	Molecular Weight	RGS4 IC_{50} (μM)	RGS4c IC_{50} (μM)	RGS8 IC_{50} (μM)
203710		BU09043	293.34	NA	NT	NT
203711		BU09044	331.22	NA	NT	NT
203712		BU09045	265.33	NA	NT	NT
203713		BU09046	252.29	NA	NT	NT
203714		BU09047	252.29	>100	NT	NT
203715		BU09048	301.37	NA	NT	NT
203716		BU09049	313.38	NA	NT	NT
203717		BU09050	313.38	NA	NT	NT
203718		BU09051	330.36	NA	NT	NT
203719		BU09052	372.42	NA	NT	NT
203720		BU09053	388.44	>100	NT	NT
N/A	Mixture of 63802 degradation products	BU09019	N/A	3.4	NT	NA

To further probe the necessity of the benzothiazole/vinyl cyanide groups in the action of this class of compounds, two more sets of analogs were created: one set where the benzothiazole was replaced with a series of different ring structures; and a set of compounds lacking various components of the vinyl cyanide group. These sets of analogs were tested using FCPIA and, when appropriate, verified with single turnover GAP.

In the first series of compounds, the benzothiazole moiety was replaced with a hydrogen (CCG-203686), a napthyl (CCG-203695), or a benzodioxole (CCG-203696) (**Table 2.4; Fig. 2.12**). Of these compounds, the napthyl and benzodioxole analogs showed significant, yet reduced, potency on inhibiting RGS4 in the FCPIA and GAP assays (**Fig. 2.12**). The fact that these two compounds show activity does suggest that a large hydrophobic ring structure is important at this position, with preference towards the benzothiazole.

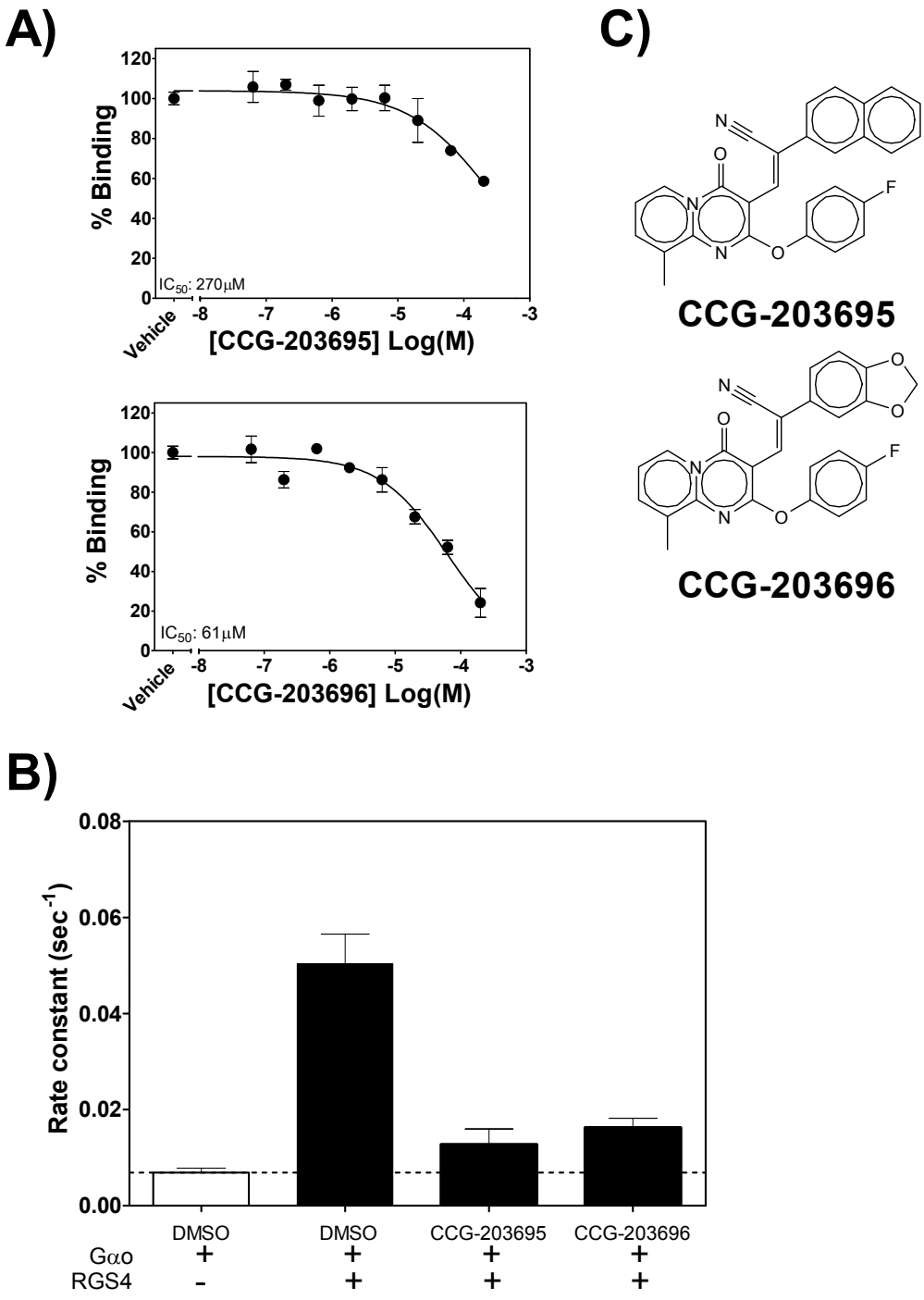
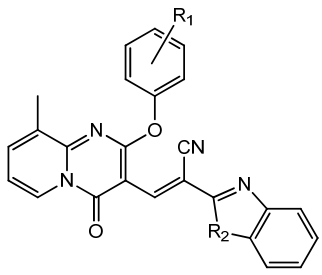


Figure 2.12 CCG-203695 and CCG-203696 dose-dependently inhibit the RGS4-Gαo interaction in FCPIA with IC_{50} values of 270 and 61μM, respectively (n=2). **A)** FCPIA Dose response curves. **B)** CCG-203695 and CCG-203696 (100μM) inhibit the ability of RGS4 to accelerate the rate of GTP hydrolysis by $G\alpha_o$. Rate constants are presented as mean ± SEM from 2 independent experiments. **C)** Structures of CCG-203695 and CCG-203696.

The second series of these compounds were designed to test the importance of the sulfur atom in the benzothiazole group. These compounds were of particular interest because of the potential importance of cysteine reactivity in the mechanism of action of the compound. In this series, several benzothiazole analogs with different substituents on the phenyl moiety (the importance of which is discussed below) were compared to the benzimidazole analogs thereof. Five such parings of these analogs were tested using FCPIA (**Table 2.5**). In the majority of cases, the benzimidazole compounds were not able to inhibit the RGS4-$G\alpha_o$ interaction, due at least in part to solubility limitations. In the one instance where there was appreciable activity (CCG-63798), compound solubility was less of an issue and therefore it is believed that it is possible that a benzimidazole is an acceptable group at this position, as long as solubility can be maintained.

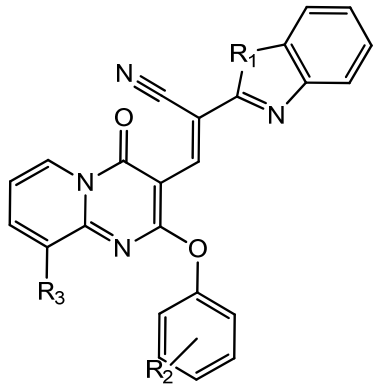


CCG ID	R_1	R_2: S (Benzothiazole) IC_{50} (μM)	R_2: N (Benzimidazole) IC_{50} (μM)
63804, 63806	*p*Et	4	>100
203669, 203670	H	7	>100
63826, 63828	*m*Bis-Me	8	>100
63742, 63776	*p*tBu	10	>100
203673, 63798	*p*OMe	28	31

Table 2.6 Benzothiazole vs. Benzimidazole analogs of the CCG-63802 class of compounds. IC50 values are presented as the mean from at least 2 FCPIA experiments measuring the inhibition of $G\alpha_o$ binding to RGS4.

This class of compounds requires at least two of the three heterocycles for full activity – the benzothiazole (or some other bulky substituent) and the pyridopyrimidone. To better understand the chemical space around the phenyl

moiety we developed a series of compounds with varying substituents on or in place of the phenyl group (**Table 2.6**). It was found that replacing this group with an alkyl chain (e.g. CCG203719-20) results in compounds with weak, if any, activity. If however, the phenyl ring is maintained but the substituents thereon are varied, limited variation (3 to 30 μM IC_{50}) in the structure-activity landscape can be observed. While the increase in potency achieved is small, it can be concluded that the optimal moiety on this phenyl ring is a small, hydrophobic substituent in the *para* position. Furthermore, this site can accommodate larger hydrophobic substituents (up to t-butyl); however, it is at the cost of reduced potency and compound solubility.



CCG ID	R1	R2	R3	RGS4 IC50 (μM)	RGS8 IC50 (μM)
63804	S	*p*Et	CH3	4	53
63794	S	*p*Br	CH3	4	50
63770	S	*m,o*Me	H	5	57
203671	S	*p*Me	CH3	5	67
63766	S	*m*Me, *p*Cl	H	7	>100
203701	S	*p*Bu	CH3	7	>100
203669	S	H	CH3	7	57
63826	S	*m*Bis-Me	CH3	8	41
63764	S	*p*F	H	8	65
63772	S	*o,p*Me	H	8	80
63762	S	*p*Br	H	9	>100
63768	S	*m*Bis-Me	H	9	>100
63742	S	*p*tBu	H	10	>100
203666	S	*p*Et	H	11	>100
203665	S	H	H	14	>100
203700	S	*p*Isopropyl	CH3	23	NT
203675	S	*p*Isopropyl	H	23	>100
203672	S	*p*Cl	H	24	81
203677	S	*p*propyl	H	25	>100
203702	S	*p*propyl	CH3	25	NT
203699	S	*p,*mCl	CH3	27	NT
203698	S	*p*OMe	CH3	27	NT
203674	S	*p,m*Cl	H	27	>100
203673	S	*p*OMe	H	28	>100
63798	N	*p*OMe	H	31	>100
203668	N	*m*Me, *p*Cl	H	>100	>100
203667	N	*o*Cl	H	>100	>100
63806	N	*p*Et	CH3	>100	>100
63776	N	*p*tBu	CH3	>100	>100
96665	N	*m*Me	CH3	>100	>100
63828	N	*m*Bis-Me	CH3	>100	>100
63830	N	*o*OMe	CH3	>100	>100
203670	N	H	CH3	>100	>100

Table 2.7 Substituents on the phenyl moiety contribute to the potency of CCG-63802 analogs on RGS4. All compounds were tested using FCPIA. Data are presented as the mean of at least two independent experiments.

Discussion:

RGS proteins play a strong modulatory role in GPCR signaling leading to substantial interest in small molecule inhibitors targeting this class of proteins [9-12, 32]. The localized expression of RGS proteins [33] suggested that RGS inhibitors could provide enhanced tissue specificity for GPCR agonist actions [9, 10, 32]. Furthermore, up-regulation of RGS proteins in various disease states, for example RGS4 in neuropathic pain models [34], also provides an important rationale for targeting RGS proteins. In this study, we report the second family of RGS SMPPIIs. Unlike the our previously reported RGS inhibitor, CCG-4986 [17] which is irreversible and loses function in the presence of reducing agents ([18, 19]), the new compounds identified here, act reversibly and retain substantial function in the presence of glutathione, a predominant intracellular reductant. These compounds, with their reversibility and activity in glutathione, therefore represent a significant step forward in the development RGS SMPPIIs. Unfortunately, these compounds do not appear to possess significant cellular activity, likely due permeability issues stemming from the large polar surface area of the most potent members of this family.

Similar to CCG-4986, CCG-63802 and CCG-63808 are relatively selective for RGS4 over other R4 family members, including the closely related RGS8 and RGS16. They have no detectable activity for the more distantly related RGS7. They also have dependence on cysteine residues because they very weakly inhibit the cysteine-null (C→A) mutant of RGS4 (RGS4c) in the FCPIA assay. However, both compounds at 100 μM fully inhibit the GAP activity of RGS4c.

There are a few potential explanations for this discrepancy. First, the compounds, which are of modest affinity (10 μM) in the FCPIA studies, may have a very short RGS-bound lifetime and therefore have difficulty competing with the constitutive binding of AlF_4^-/GDP-bound $G\alpha_o$ to the RGS. In the GTPase assay they may be more efficient at inhibiting the transient interaction between GTP-bound $G\alpha_o$ and RGS4 during the catalytic cycle. Also, since the compounds appear to act via an allosteric site (see below), the induced conformational change in RGS4 may have a more dramatic impact on binding to or GAP activity at the Gα-GTP than for the GDP-AlF_4^- conformation of the Gα subunits. While this explanation is tempting, subsequent studies using a steady state GTPase assay and a thermal stability assay with RGS4c failed to reproduce the effects observed in the single turnover assay, lessening the strength of the single turnover data (Data not shown, experiments performed by Andrew Storaska). The limited and inconsistent activity of CCG-63802 and CCG-63808 on RGS4c corresponds with the cysteine reactivity that is believed to play a role in the mechanism of action of this family of compounds.

The partial cysteine-dependence of the actions of these compounds suggests a tethering model in which a reactive group binds to an RGS cysteine residue. This is supported by the cysteine mutagenesis studies and also by the presence of the potential Michael acceptor functionality (vinyl cyanide) in both of the compounds. Tethered ligands can provide enhanced potency for small molecules acting on difficult targets [35, 36]. Our ability to detect these compounds in the original screen may have derived from potency enhancement

from a slow off-rate due to tethering. The reaction, however, is clearly reversible on the 10-minute time scale and attempts to demonstrate covalent binding by mass spectroscopy have been unsuccessful. While uncommon, there are other well-described examples of reversible Michael acceptor reactions with thiols [37, 38]. While most drug molecules are designed to avoid such reactive groups, there are a number of examples of clinically used drugs (e.g. omeprazole) or drug candidates (CI-1033) that are thiol reactive [39, 40]. The clear margin of safety and commercial success of these drugs suggests that irreversible inhibitors may not be as intractable as therapeutic agents as previously thought. Furthermore, tethered ligands have been used to develop SAR in the context of the higher affinity starting structure that is then transferred to analogs without the reactive group [41].

I have shown that wild type RGS4 is inhibited at 10 μM by CCG-63802 (**Table 2.2, Table 2.3**). Mutation of all four cysteines in the RH domain to alanine completely desensitizes the RGS to this family of compounds. By using this discrepancy, I was able to determine the cysteines that are important for the modulation of RGS function by CCG-63802 and related analogs. The compounds described here require two cysteines for full potency of RGS4 inhibition: Cys 95 and Cys 148. These residues are positioned in the “B site” of RGS proteins [10] which is proposed to participate in the allosteric modulation of RGS4 by acidic phospholipids and calmodulin [42, 43]. The presence of either Cys 95 or Cys 148 alone provided only modest sensitivity to CCG-63802, however, a mutant that

contains both Cys 95 and Cys 148 displays nearly wild-type levels of sensitivity to this family of compounds (**Table 2.3**).

Cysteine 132 is located on the outer edge of the Gα interaction interface. It is tempting to hypothesize that labeling this cysteine with CCG-63802 would provide significant steric occlusion of the RGS-$G\alpha_o$ PPI. However, that contained only Cys 132 displayed modest (IC_{50}: 174 μM, **Table 2.3**) sensitivity to CCG-63802. Furthermore, addition of the Cys 132 to the double-add back mutant that contains Cys 95 and Cys 148 was unable to further potentiate the effects of CCG-63802 (**Table 2.3**). These data strongly suggest that, while Cys 132 may play a minor role in the action of these compounds, the primary driving force behind CCG-63802 inhibition is through the interactions with Cys 95 and Cys 148.

The binding of CCG-63802 induces a destabilizing effect on RGS4 in the thermal stability studies. This reduced stability of the RGS4 may be related to conformational perturbation induced upon compound binding to the cysteines in the allosteric site. In most instances, proteins with endogenous small molecule ligands (e.g. Gα proteins) are stabilized by the presence of their ligand [44, 45]. This notion was recently borne out by the crystallization of several GPCRs [46-50]. In all cases (the notable exception being opsin), crystals were only obtained in the presence of a small molecule antagonist. This strongly suggests that these ligands are important for the structural stability of this class receptor in solution. Furthermore, our data (**Fig. 2.5**) and others [51, 52] also confirm that binding of natural or artificial ligands to sites that have evolved the capacity for small

molecule binding causes a stabilization of the protein. This stabilizing effect may be due to the decrease in free energy derived from the binding event and also the conformational restriction required for high-affinity ligand-protein interaction.

On the surface, it would appear that this paradigm is contradicted by the compounds CCG-63802 and CCG-63808 which potently destabilize RGS4 even though they appear to bind close to the site on RGS4 that binds native acidic phospholipids (See **Introduction** for more details). It is possible that these compounds bind to a site near, yet independent of, the acidic lipid site on the RGS and binding to this non-natural site might not be expected to produce the same stabilization effect as binding of small molecules to sites that have evolved the capacity for such small molecule-protein interactions. Also, insertion of the compounds into the 4-helix bundle, stabilized by the reversible Michael addition to a cysteine thiol, could unfold the RGS4 structure leading to destabilization.

When a chemical entity is discovered to have a particularly interesting biological activity, it is wise to explore the chemical space surrounding the molecule in search of higher potency compounds or for compounds with better physicochemical properties (e.g. improved solubility). To characterize the structure activity landscape surrounding the CCG-63802 class of compounds, we synthesized (or purchased, if available) a series of analogs to test specific hypotheses about the mechanism of action of CCG-63802. These compounds were tested using FCPIA and those compounds of particular interest were further studied in follow up experiments using a single turnover GAP assay. In total, 74 analogs were tested for their RGS inhibitory activity. Analysis of the IC_{50} vales of

the compounds using FCPIA (**Tables 2.4-2.6**) reveals the following: 1) the compound can accommodate a series of substituents on the phenyl ring while maintaining an IC_{50} value within ½ Log of the parent compound (4-30 μM); 2) the benzothiazole is generally required for full activity, yet measurable activity is retained when the benzothiazole is replaced with a benzodioxole (IC_{50} 61 μM), napthyl (IC_{50} 270 μM), or in one instance, a benzimidazole (IC_{50} 31 μM); 3) the minimal structural unit of the compound class includes all three heterocycles and the vinyl cyanide. The GAP data available correlate well with the FCPIA data, suggesting that the PPI-inhibitory effects we observe are correlated to functional inhibition of RGS activity – at least *in vitro*.

Overall, the SAR landscape surrounding the CCG-63802 class of molecules is particularly steep when altering any of the moieties that are believed to relate to its reactivity. Outside of that, the SAR is rather shallow. The primary observation from the analogs that retain full reactivity (e.g. that contain the vinyl cyanide and benzothiazole moieties) is that a small hydrophobic substituent on the phenyl moiety, preferably in the *para* position provides the greatest increase in potency. The dependence upon reactivity suggests that a large part of this compound's mechanism of action is through a reversible adduct formation with a cysteine residue in RGS4, most likely at the 95/148 positions in the molecule. While this is discouraging from a compound development standpoint, this class of compounds does show that RGS proteins are capable of being reversibly inhibited – an important proof of concept. It is likely that future work focusing up on this family of compounds, especially the elucidation of the structural contacts

of CCG-63802 with RGS4, should provide valuable insight into the development of future generations of small molecule RGS inhibitors.

Conclusions:

In this study we have identified the first examples of reversible SMPPIIs that disrupt RGS protein function. CCG-63808 and CCG-63802 are selective inhibitors of the RGS-Gα interaction and R4 family GAP activity. Their mechanism appears to, at least in part, involve an allosteric action at the “B” site on the RGS [10], which has been implicated in the physiological allosteric modulation of RGS proteins by acidic phospholipids and calmodulin [43, 53]. While these compounds have yet to show any cellular activity, it is possible that future generations of the CCG-63802 family of compounds that have better physicochemical properties (e.g. improved solubility and lower polar surface area), and improved potency will provide more tractable lead compounds for cellular and whole-animal studies.

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Chapter III:
Biochemical Evaluation of Class of Small Molecule RGS inhibitors with Cellular Activity

Introduction:

In the prior chapter I characterized the mechanism of action of the first family of reversible RGS inhibitors. While the CCG-63802 class of compounds serves as a useful proof-of-concept, the structure-activity relationship studies thus far have not yielded compounds with significant cellular activity. In this chapter I characterize the biochemical mechanism of action of the first example of an RGS inhibitor that can potentiate G protein signaling in a number of living-cell systems. Like the previously identified RGS inhibitor, CCG-4986, this compound is an irreversible modifier of cysteine residues.

The development of cysteine-reactive small molecule inhibitors into useful research probes and therapeutic agents is particularly challenging. A major difficulty is obtaining sufficient target specificity. Overly reactive compounds are often capable of non-specifically reacting with most solvent accessible thiols, leading to deleterious off-target effects in a physiological setting. Strikingly, covalent interactions of certain compounds (e.g. sulfonamide metabolites) with plasma proteins can lead to potentially life-threatening immune responses [1]. The reducing intracellular environment of the cell is also biased against cysteine-reactive compounds. The major intracellular reductant is glutathione - a cysteine-containing tripeptide present in the cell at a concentration of ~2 mM. Thiol-

reactive compounds also are likely to have poor pharmacokinetic profiles due to the number of metabolic enzymes that act upon cysteine residues. These issues constitute the major challenges to the development of the compound class described in this chapter.

There are, however, a few successful therapeutics that function by covalently binding to cysteine. For example, the acid-reflux drug omeprazole operates in the stomach by covalently modifying a proton exchanger [2]. In this case, the compound does not reach the systemic circulation to any great extent, so side effects are minimized. There is also a class of cysteine-reactive irreversible tyrosine kinase inhibitors, typified by CI-1033, that are currently in clinical trials [3]. Cysteine reactive compounds thus have a place in modern pharmacology. To be truly useful however, these compounds require significantly more development than their non-reactive counterparts.

The compound discussed in this chapter, CCG-50014, was discovered in a high throughput biochemical screen designed to identify inhibitors of 5 different RGS proteins. This screen was performed by a postdoctoral fellow in our laboratory and has been previously described in the literature [4]. CCG-50014 was identified as the most potent inhibitor from this screen with an IC_{50} value <300 nM. In this chapter I characterize the biochemical mechanism of action of this compound. The information obtained from these studies allowed for the development of novel analogs of CCG-50014 (see **Chapter IV**) that have cellular activity.

All work in this chapter was performed by myself, with the following exceptions: 1) The computational modeling of CCG-50014 docking to RGS8 was performed by Haoming Zhang in the laboratory of Dr. Paul Hollenberg; 2) LC-MS analysis of CCG-50014 or vehicle-treated RGS proteins was performed by Haoming Zhang in the laboratory of Dr. Paul Hollenberg and myself; 3) CCG-50014 and analogs were synthesized by Dr. Benjamin Greedy and/or Dr. Emma Casey in the laboratory of Dr. Stephen Husbands.

Methods:

Reagents:

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) and were reagent grade or better. γ[^{32}P]GTP (10 mCi/mL) and [^{35}S]GTPγS (12.5 mCi/mL) was obtained from Perkin Elmer Life and Analytical Sciences, (Boston, MA) and was isotopically diluted before use. Amylose resin was purchased from New England Biolabs (Ipswich, MA). Ni-NTA resin was purchased from Qiagen (Valencia, CA). Avidin-coated microspheres were purchased from Luminex (Austin, TX). CCG-50014 (4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione) and analogs were purchased from Fisher Scientific (Waltham, MA) from the Maybridge compound collection or were synthesized in the laboratory of Dr. Stephen Husbands.

Protein expression and purification:

With the exception of RGS8 mutants, all RGS and G proteins were prepared as previously described [5]. For the RGS8 cysteine → serine mutants, site directed mutagenesis was performed using the following primers for 107C (C160S) (Sense: 5'-GCAGGAGCCATCCCTGACTAGCTTTGACCAAG-3'; Antisense: 5'-CGTCCTCGGTAGGGACTGATCGAAACTGGTTC-3'), and 160C (C107S) (Sense: 5'-TGGAATTCTGGTTGGCCAGTGAGGAGTTCAAGAAG-3'; Antisense: 5'-ACCTTAAGACCAACCGGTCACTCCTCAAGTTCTTC-3'). Mutagenesis was performed using the QuickChange Multi-site Directed Mutagenesis kit (Agilent, La Jolla CA). G protein activity was determined by [^{35}S]GTPγS binding [6]. In all cases, proteins were purified to >90% homogeneity before use.

Chemical labeling of purified $G\alpha_o$ and RGS proteins:

RGS proteins were biotinylated and $G\alpha_o$ was labeled with AlexaFluor-532 as previously described [7].

FCPIA Dose Response and Reversibility experiments:

FCPIA was performed as previously described using chemically biotinylated RGS proteins and AlexaFluor-532 labeled $G\alpha_o$. ([4, 8])

Single Turnover GTPase Measurements:

Compounds were tested for the ability to inhibit the RGS4 and RGS8-stimulated increase in GTP hydrolysis by $G\alpha_o$ as described previously [9, 10].

Thermal Stability Measurements:

The thermal denaturation of RGS8 and $G\alpha_o$ was measured using a ThermoFluor Instrument (Johnson & Johnson, Langhorne, PA). Protein (5 μM RGS8 or $G\alpha_o$) was incubated with CCG-50014 or vehicle control for 15 minutes at room temperature in 50 mM HEPES pH 8.2, 500 mM NaCl, 5% glycerol in a volume of 15 μL in a black 384-well PCR microtiter plate (ThermoFisher Cat # TF-0384/K). To this mixture was added 1-anilinonapthalene-8-sulfonic acid to a final concentration of 200 μM. The samples were overlaid with 5 μL of silicone oil and subjected to a temperature ramp using the following parameters: ramp temperature range: 30-90°C; temperature increment: 1°C; image collection temperature: 25°C; temperature holds: 30 seconds for ramp temperature, 15 seconds for image collection temperature. The samples were cooled to 25˚C between temperature increments for image capture to maximize signal:noise. Melting temperatures (T_m) were calculated from the data using the sigmoidal fitting procedure in the ThermoFluor^{++} software package (version 1.3.7).

Analyses of the protein adduct of RGS by ESI-LC/MS:

The molecular mass of the RGS protein was analyzed by ESI-LC/MS using a LCQ ion-trap mass spectrometer (ThermoScientific, Waltham, MA). RGS8 wild-type or mutant proteins were diluted to 2 μM in 50 mM potassium phosphate buffer, pH 7.4 and CCG-50014 or an equivalent volume of DMSO was added to the sample. Following treatment with CCG-50014, an aliquot (~50 μL)

of the protein solution was applied to a reverse-phase Zorbax 300-SB C3 column (2×150 mm, 5 μm) (Agilent Technologies, CA). The RGS protein was subjected to high performance liquid chromatography with a binary solvent system consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using the following gradient: 30% B for 5 min., linearly increased to 90% B in 20 min., and held at 90% B for 30 min. The flow rate was 0.25 mL/min. The mass spectrometer was tuned with horse heart cytochrome *c* and the instrumental settings for the mass spectrometer were: spray voltage, 3.5 kV; capillary temperature, 220^{o}C; sheath gas flow, 80 (arbitrary units); auxiliary gas flow, 20 (arbitrary units). The molecular masses of the unmodified and inhibitor-modified RGS proteins were determined by deconvolution of the apoprotein charge envelopes using the Bio-works software (Thermo Scientific, Waltham, MA).

Papain Activity Assay:

Papain (Sigma-Aldrich, St. Louis, MO) activity was monitored by the increase in fluorescence caused by the liberation of fluorescein from auto-quenched fluorescein-conjugated casein (AnaSpec, San Jose, CA). Papain (0.625 U) was diluted into 20 mM sodium acetate pH 6.5, 2 mM EDTA. The enzyme was treated with iodoacetamide, N-ethyl maleimide, CCG-50014, or vehicle control for 30 minutes at room temperature. To this, FITC-casein was added to a final concentration of 250 nM. The reaction was allowed to proceed at room temperature in the dark. At time various points along the reaction, the

fluorescence intensity (ex. 485nm, em. 520 nm) was measured using a Victor II plate reader (Perkin Elmer, Boston, MA). As a control, CCG-50014 was tested at pH 6.5 and it retains full inhibitory activity against the RGS4-$G\alpha_o$ PPI in FCPIA.

Docking of CCG-50014 to RGS8:

The energy-based docking software Autodock (ver. 4.0) was used to explore the potential binding sites of CCG-50014 on RGS8. The coordinates of RGS8 were obtained from the Protein Data Bank (PDB ID 2IHD). Water and other hetero atoms were removed from the structure prior to docking. The coordinates of the CCG-50014 ligand were built using the ChemBioOffice 2008 software suite (CambridgeSoft, Cambridge, MA) and the geometry of CCG-50014 was optimized using the semi-empirical quantum PM3 method included in the ChemBioOffice 2008 software suite. For unbiased docking, the grid box of the RGS was set at 60×60×60 $Å^3$ to encompass the entire RGS protein. The flexible CCG-50014 ligand was docked to the rigid RGS using a Lamarckian Genetic Algorithm (LGA) with the following parameters: mutation rate, 0.02; cross-over rate, 0.8; maximal number of generations, 2.7×10^4.

Results:

FCPIA characterization of RGS inhibitory activity:

CCG-50014 (**Fig. 3.1**) was originally identified as a potential inhibitor of RGS8 and RGS16 in a polyplex high throughput screen to identify inhibitors of the RGS-Gα interaction [4]. This activity was confirmed by analyzing the effect of

CCG-50014 on several different RGS proteins with freshly reordered compound using multiplexed FCPIA. CCG-50014 fully inhibited several different RGS proteins including RGS4, 8, 16, and 19, but did not have activity on RGS7 or a mutated form of RGS4 that lacks cysteine residues (**Fig. 3.2A, Table 3.1**). The 30 nM IC_{50} value observed for the inhibition of RGS4 makes CCG-50014 the most potent small molecule RGS inhibitor discovered to date.

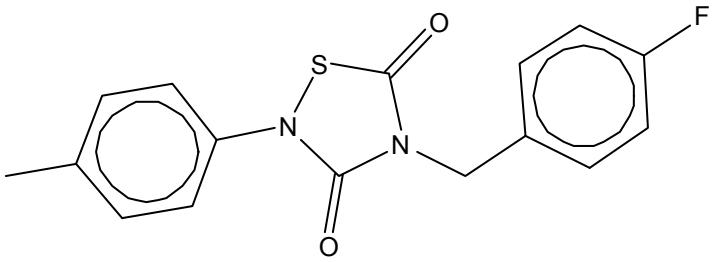


Figure 3.1 The chemical structure of CCG-50014 (4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione).

RGS	IC_{50} (μM) ± SEM	Hill Slope
RGS4 wild Type	0.030 ± 0.006	-1.53
RGS4 Cys-null Mutant	N/A	N/A
RGS8	11 ± 2	-0.57
RGS16	3.5 ± 2.4	-1.33
RGS19	0.12 ± 0.02	-0.61
RGS7	N/A	N/A

Table 3.1: CCG-50014 shows >100 fold specificity for RGS4 over other RGS proteins in the FCPIA assay. Data are presented as: mean IC_{50} values ± SEM from at least three independent experiments (for RGS4 and RGS8, n >28). N/A: No inhibition below the aqueous solubility limit of the compound.

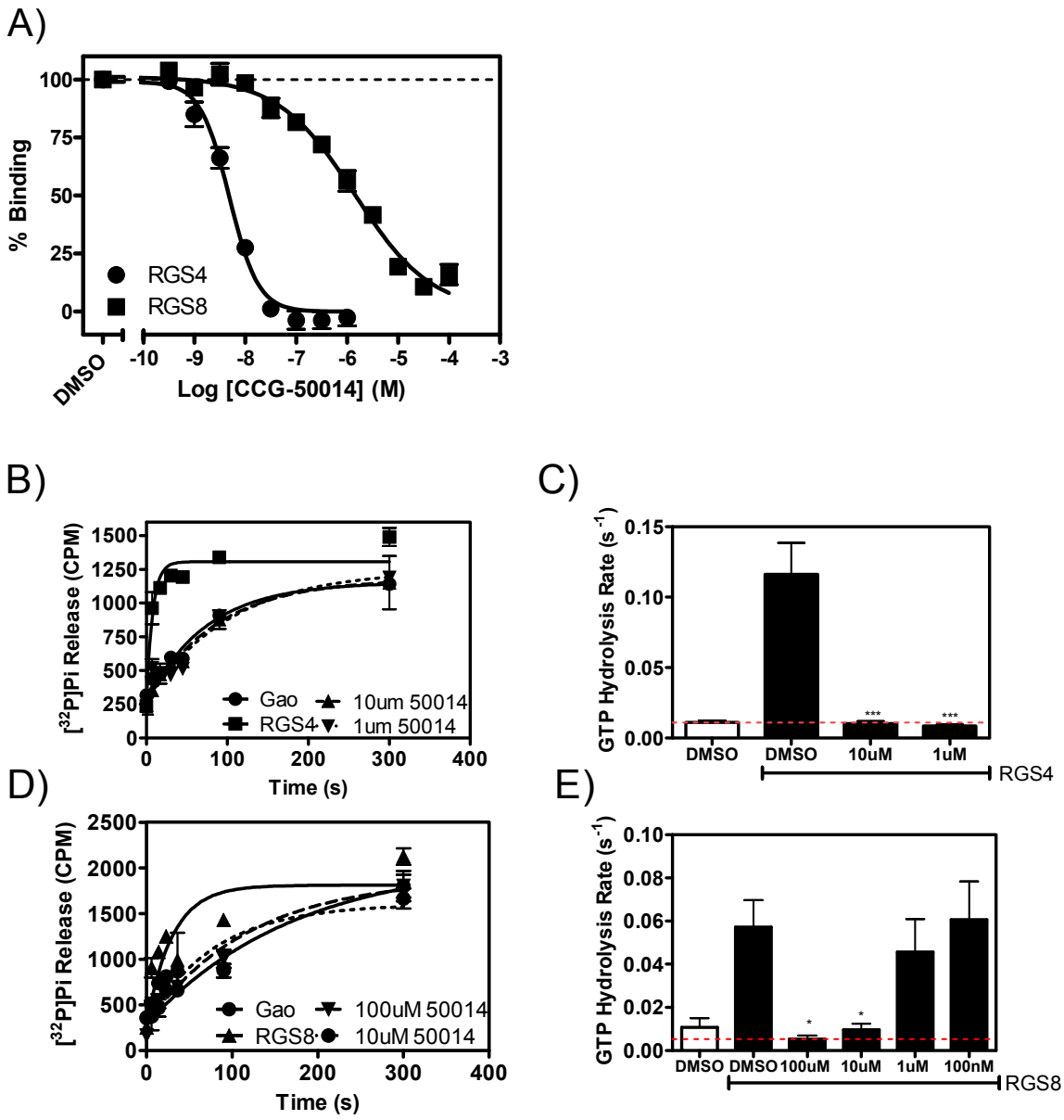


Figure 3.2 CCG-50014 inhibits RGS4 and RGS8 binding and function. **A)** CCG-50014 dose-dependently inhibits the binding between aluminum fluoride-activated $G\alpha_o$ and RGS4 or RGS8. Data shown are an average of three independent experiments. This experiment has been independently repeated 28 times, producing average IC_{50} values of 30 nM against RGS4 and 1.1 μM against RGS8. **B,C)** CCG-50014 also inhibits the GAP activity of RGS4 and **D,E)** RGS8. Using a single-turnover GAP assay, CCG-50014 dose-dependently inhibits the GAP activity of both RGS4 and RGS8. * P <0.05, *** P <0.0001. All experiments were independently repeated a minimum of three times.

CCG-50014 inhibits the catalytic GTPase accelerating activity of RGS8 and RGS4:

In a single turnover GAP assay, CCG-50014 inhibited the GAP activity of RGS8 and RGS4 on $G\alpha_o$ (**Fig. 3.2B**). Under these assay conditions, RGS8 and RGS4 accelerate the rate of GTP hydrolysis by approximately 5 and 10 fold, respectively. CCG-50014 inhibited that activity of both RGS proteins. At a saturating concentration (100 μM), CCG-50014 did not alter the intrinsic rate of GTP hydrolysis by $G\alpha_o$, proving that the compound does not act by altering the enzymatic activity of the G protein, at least under single-turnover conditions (**Fig. 3.3**).

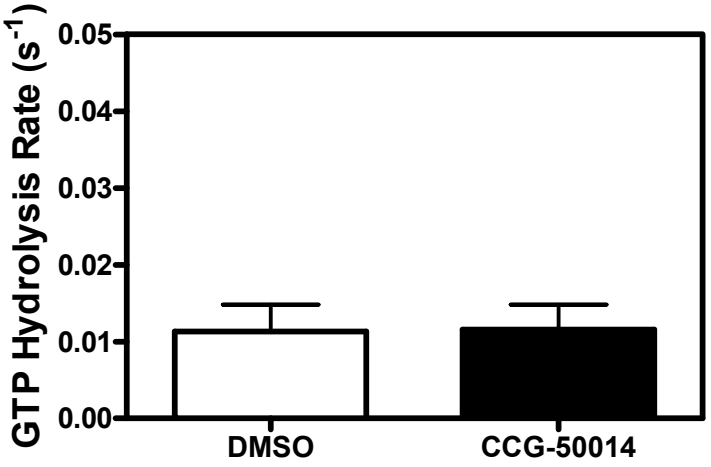


Figure 3.3 CCG-50014 does not affect the intrinsic rate of GTP hydrolysis by $G\alpha_o$. The rate of GTP hydrolysis as measured using the single turnover GTPase assay was not significantly different in the absence or presence of 100 μM CCG-50014. Data are presented as the average of four independently replicated experiments.

CCG-50014 irreversibly inhibits RGS proteins:

FCPIA-based reversibility experiments were performed to probe the mechanism of action of the compound (**Fig. 3.4**). RGS-coated polystyrene beads

were incubated with a saturating concentration (100 μM) of CCG-50014 for 15 minutes before being thoroughly washed by repeated centrifugation and resuspension (theoretical dilution of ~78,000 fold). These beads were then analyzed for $G\alpha_o$ binding by FCPIA. Washing of the beads did not restore $G\alpha_o$ binding activity by the RGS proteins, suggesting that the compound was irreversibly bound to the protein. This inhibition was partially reversed by washing the beads with buffer containing 1 mM dithiothreitol (DTT), suggesting the mechanism of reactivity could be through sulfhydryl modification, a mechanism in common with the previously described RGS inhibitor, CCG-4986 [9].

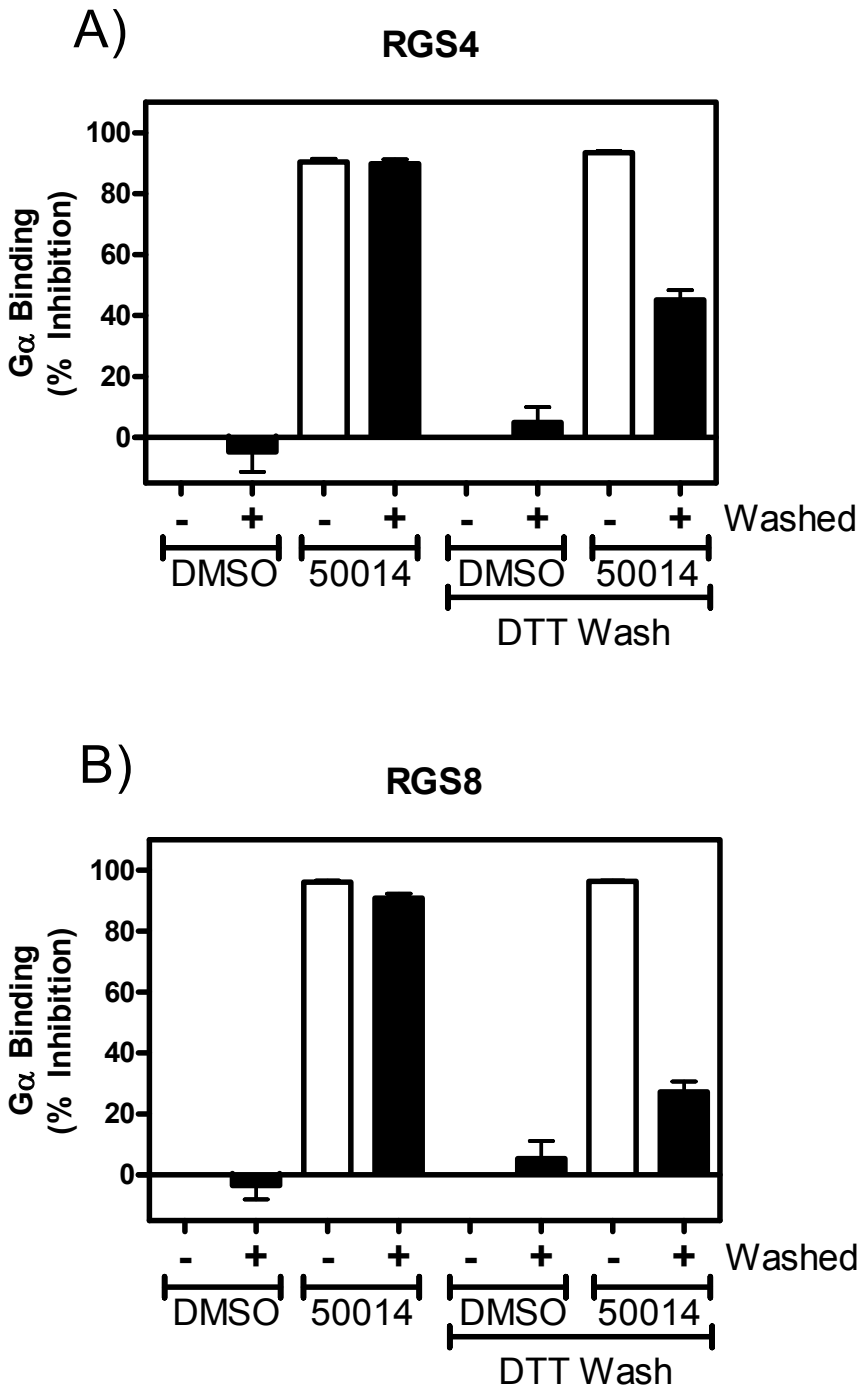


Figure 3.4 CCG-50014 is an irreversible inhibitor of RGS4 and RGS8 and its effects are partially reversed the thiol reductant DTT. **A)** RGS4 and **B)** RGS8 were treated for 15 minutes with 100 μM CCG-50014 prior to vigorous washing to remove any unbound compound. To determine if the compound was reacting in a thiol-sensitive manner, washing was performed in the absence or presence of 1 mM DTT. Data are presented as the mean±SEM from at least three independent experiments.

CCG-50014 Binds to RGS Proteins but not to $G\alpha_o$:

The melting temperature of a protein is often influenced by the binding of small molecules [11-13]. Using a ThermoFluor® instrument (Johnson & Johnson, Langhorne, PA), I characterized the thermal denaturation of RGS8 and $G\alpha_o$ in the presence and absence of CCG-50014 (**Fig. 3.5**). Using this technique, I observed a large, dose-dependent destabilization of RGS8 but no effect on $G\alpha_o$. This suggests that the compound is interacting exclusively with the RGS protein. This result was further confirmed by Liquid Chromatography-Mass Spectral (LC-MS) analysis as described below.

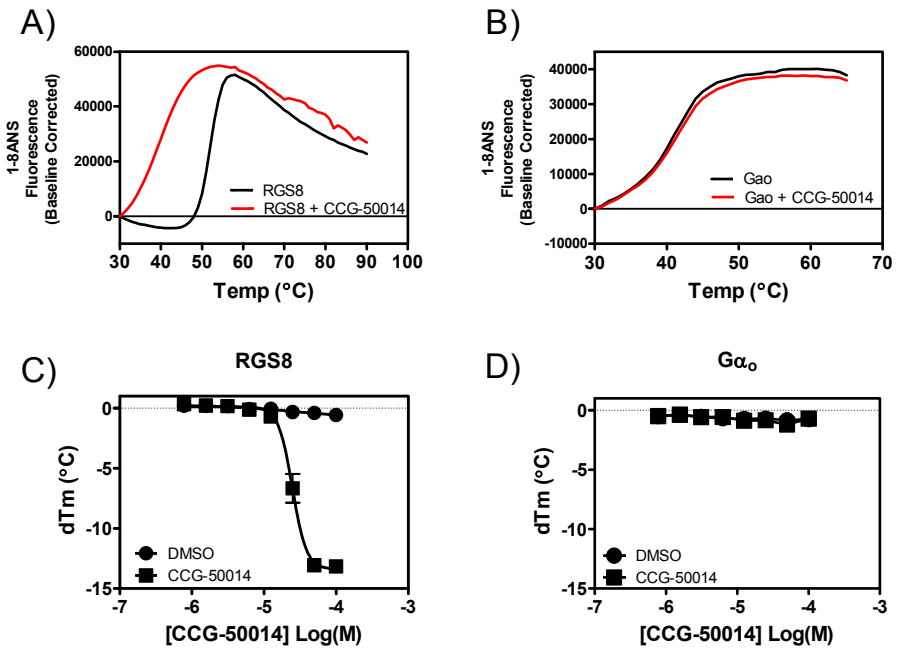


Figure 3.5 CCG-50014 thermally destabilizes RGS8 in a dose-dependent manner, but has no effect on the thermal stability of $G\alpha_o$. Representative melting traces of **A)** RGS8 and **B)** $G\alpha_o$ in the absence (black trace) and presence (red trace) of a saturating concentration of CCG-50014. Dose-response curves showing the thermal destabilization effects of CCG-50014 on **C)** RGS8 and **D)** $G\alpha_o$. Data are presented as the mean±SEM of three independent experiments.

CCG-50014 depends on cysteine residues to inhibit the AlF_4-$G\alpha_o$/RGS interaction:

To identify the potential cysteine targets of CCG-50014, we studied the compound's effects on RGS8. This protein only contains two cysteines in the RGS homology domain, making it a simpler model system to study than other RGS proteins. Each cysteine from the RGS8 RGS homology (RH) domain was individually mutated to serine and the activity of the compound was analyzed via FCPIA (**Fig. 3.6**). These mutants have been named according to the cysteine residue that they maintain (e.g. 107C contains Cys 107 and a serine at position 160). Neither cysteine was fully necessary for function of the compound, but mutating both cysteines reduced the potency of CCG-50014 by >100 fold. An interesting trend was noticed whereby the Hill coefficients for the inhibition of each individual mutant was significantly shallower than that of the wild-type protein, possibly suggesting some form of cooperativity between the two cysteine residues. However, this interpretation is dependent upon the assumptions of the Hill equation, including that the binding has reached equilibrium. In the case of an irreversible inhibitor, clearly this is not the case. Therefore, these data could also be explained by differences in the rate of compound reaction with these two mutants. The insensitivity of the RGS8 cysteine null mutant corresponds well with the insensitivity of the RGS4 cysteine null mutant (**Table 3.1**), suggesting a similar mechanism of action across the two proteins.

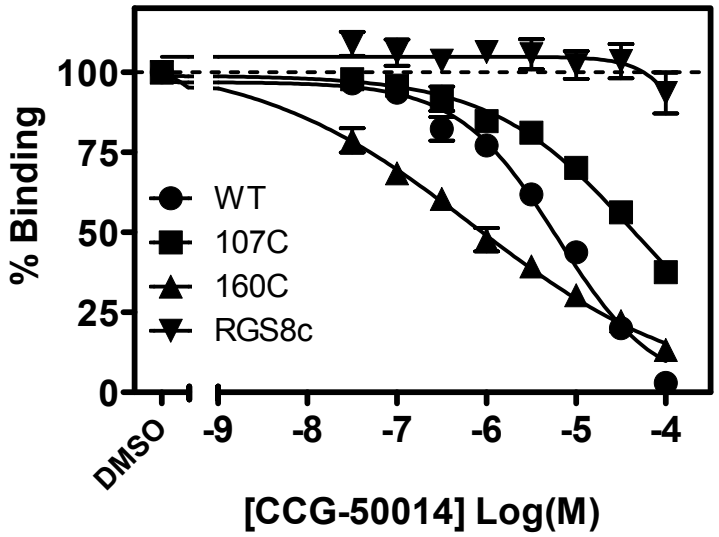


Figure 3.6 CCG-50014 requires at least one cysteine residue on RGS8 for full activity. Mutating both cysteines to serine (RGS8c) produced a protein that was completely insensitive to the effect of CCG-50014. The presence of either Cys 107 (107C) or Cys 160 (160C) provided sensitivity to CCG-50014. The inhibition parameters (IC_{50} (μM), Hill Coefficient) for CCG-50014 on these proteins were as follows: wildtype RGS8 (wt): 6.1 μM, -0.79; 107C: 46.5 μM, -0.54; 160C: 0.71 μM, -0.36; Cysteine-null RGS8 (RGS8c): >100 μM. Data are presented as the mean±SEM of three independent experiments.

CCG-50014 is a covalent sulfhydryl modifier of RGS8:

The data thus far suggest that CCG-50014 covalently modifies RGS proteins. To test this hypothesis, we performed high performance liquid chromatography–mass spectral analysis on RGS8 samples treated with CCG-50014 (**Fig. 3.7**). After compound treatment, there was a peak shift in RGS8 corresponding to a full mass adduct of CCG-50014. At high concentrations of CCG-50014 (100 μM) with wild-type RGS8, a second minor peak corresponding to two full adducts was also observed, suggesting that CCG-50014 at this concentration can react with both cysteine residues in the protein. To confirm that this action was via cysteine reactivity, the mutant RGS8 where the two cysteines in the RH domain were mutated to serine was also analyzed and no adduct was

observed (**Fig 3.7B**). RGS8 individual cysteine mutants (107C, 160C) were also tested for covalent adduct formation in MS. A single adduct was observed on 107C, while no adduct was observed on 160C under the conditions tested. The lack of an observable adduct on 160C was of particular interest since this mutant was also irreversibly modified by CCG-50014. Indeed, it was inhibited more potently than the 107C mutant. To further probe this observation, WT RGS8 and the two cysteine mutant proteins were treated with a saturating concentration (100 μM) of CCG-50014 before removal of the compound via gel filtration chromatography (**Fig. 3.8**). CCG-50014-treated wild-type RGS8 showed a minor mobility shift compared to vehicle-treated WT RGS8 and showed a 14-fold decrease in its ability to compete for $G\alpha_o$ binding to RGS8 beads. The CCG-50014- and vehicle-treated 107C mutant protein migrated through the column in an identical manner and no discernable difference in $G\alpha_o$ binding was observed. The 160C mutant, however, formed aggregates upon treatment with a saturating concentration of CCG-50014 and no monomeric, soluble protein was recovered from the experiment. This suggests that labeling at Cys 160 causes a dramatic decrease in protein stability. It is likely that this aggregation accounts for the lack of an observable adduct in the mass spectral experiments, as these aggregates would not migrate on the HPLC as a standard protein peak for MS analysis.

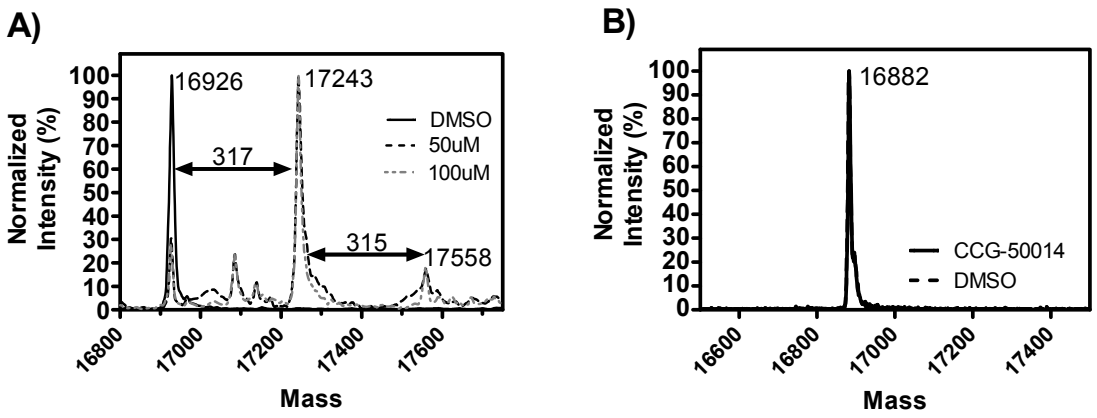


Figure 3.7 CCG-50014 forms a covalent adduct on RGS8. **A)** Protein was treated with CCG-50014 before analysis via LC-MS. After treatment with compound a predominant peak appeared with a mass shift of 317 as compared to the vehicle-treated protein, correlating to the addition of a full compound adduct (CCG-50014 MW: 316.4). A second minor peak with an additional mass shift of 315 was observed, which correlates to the addition of two full MW adducts of CCG-50014. **B)** No adducts are observed on the cysteine-null (C→S) form of RGS8 (RGS8c).

To further probe the mechanism of action of this compound, we studied the development of irreversible inhibition of the RGS8 mutants. Using a standard reversibility experiment (Fig. 3.9), the effect of CCG-50014 on 160C is completely irreversible, while the effect on 107C can be partially reversed by washing away the compound. These data, along with the gel filtration data add credence to the hypothesis that labeling at Cys 160 causes dramatic destabilization of the protein while labeling at Cys 107 produces inhibition that can be reversed.

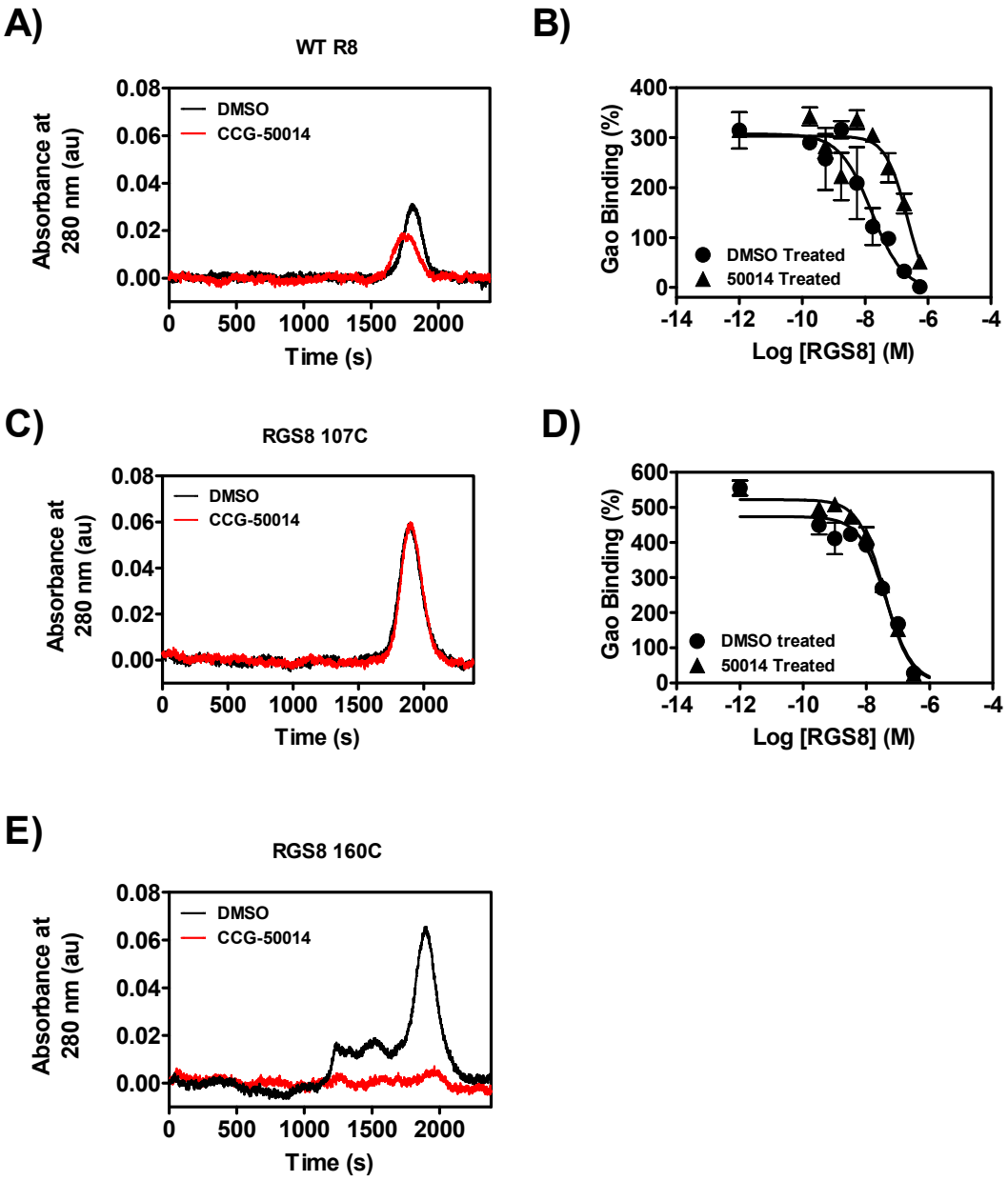


Figure 3.8 CCG-50014-induced protein aggregation is dependent on the presence of 160C. **A,B)** Wild type, **C,D)** 107C, or **E)** 160C RGS8 was treated with a 5-fold excess of CCG-50014 before removal of the compound via gel filtration. Shown are representative UV chromatogram traces and data from the corresponding competition experiments to test the activity of the recovered protein. The wild-type RGS8 chromatogram shows a slightly left shifted and suppressed peak after CCG-50014 treatment, which coincides with a 14-fold decrease in protein activity. The 107C mutant protein is completely insensitive to the effects of CCG-50014, while the 160C mutant protein completely (and visually) aggregates upon compound treatment and is removed by the prefiltration of the samples.

Because Cys 160 is buried in the core of RGS8 and Cys 107 is closer to the surface of the protein, I hypothesized that the compound might interact more rapidly with Cys 107 than Cys 160. Due to the fact that there are differential effects by CCG-50014 (reversible inhibition vs protein aggregation) depending on which cysteine is labeled, this hypothesis was tested using FCPIA reversibility experiments. The experiment was designed to monitor the development of irreversible inhibition on wild-type RGS8 and the two RGS8 mutants by CCG-50014 as a function of time (**Fig 3.10**). Wild-type, 107C, or 160C RGS8 were immobilized on beads and treated for varying periods of time with 20 μM CCG-50014 before extensive washing. The beads were then probed for $G\alpha_o$ binding using FCPIA and compared to RGS-coated beads that had been treated with DMSO alone. At this concentration of CCG-50014, the 107C RGS8 was ~20% irreversibly inhibited and 160C was ~50% irreversibly inhibited at all time points tested, suggesting that the compound rapidly exerted it's effect on the RGS protein. The wild-type protein showed a delayed development of irreversible inhibition, whereby at early time points, the inhibition was ~20% and increased to, but did not exceed, ~50% over 30 minutes. This suggests that there is a differential mechanism of action of the compound on the two individual mutants that is combined in the wild type protein. Furthermore, it suggests that reaction with Cys 107 is kinetically preferred in the wild type protein.

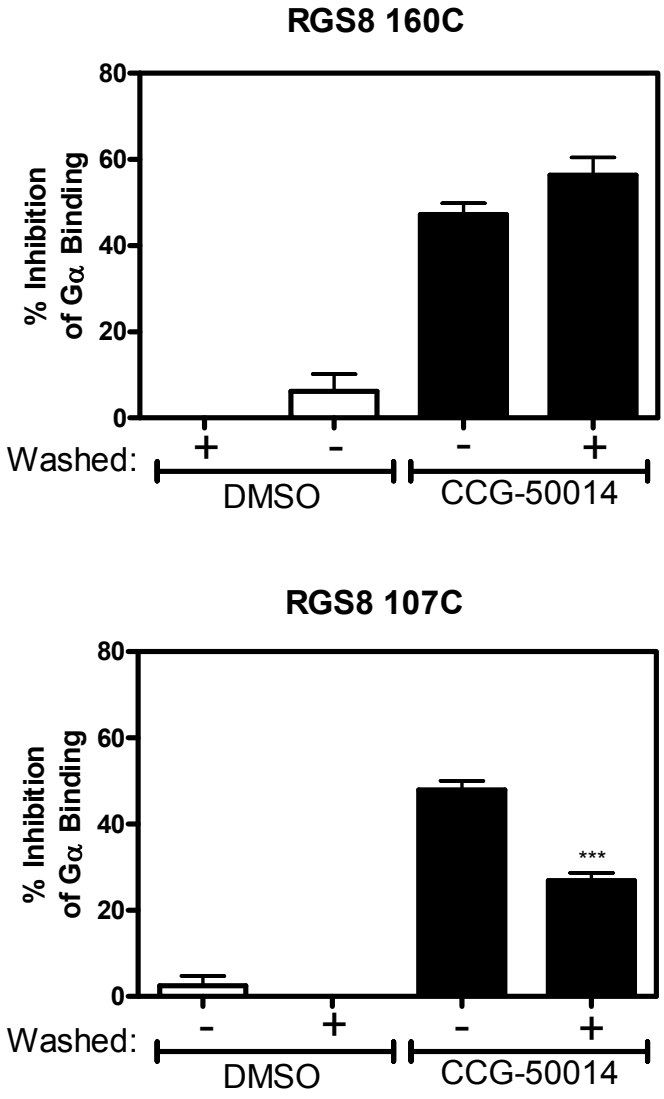


Figure 3.9 Irreversible inhibition of RGS8 is predominantly mediated by Cys 160. Mutant proteins were exposed to 20 μM CCG-50014 and reversibility experiments were performed as in Fig. 3.4. Data are presented as the mean±SEM from three independent experiments. ***P<0.0001 using an unpaired t test.

A potential confounding factor in this analysis is that there is a significant difference in basal melting temperatures between 107C RGS8 (Tm 53.2±0.2) and 160C RGS8 (Tm 42.5±0.1), whereby 160C is dramatically less stable overall. It is possible that labeling of the 160C mutant may have a more exacerbated sensitivity to CCG-50014 than that of the 107C mutant, solely due to

the intrinsic instability of this protein. One could therefore imagine that labeling of the 160C mutant with smaller, less disruptive, analogs CCG-50014 may not always force protein unfolding in the wild-type protein.

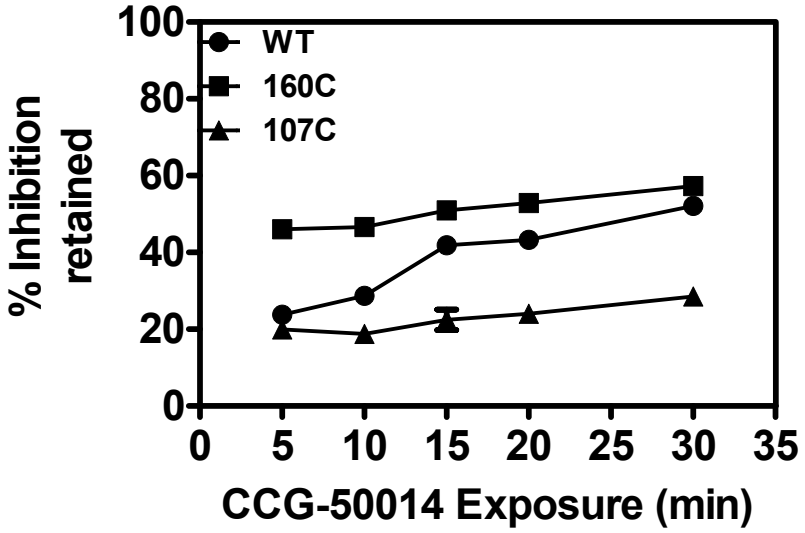


Figure 3.10 Development of irreversible inhibition after exposure to CCG-50014 differs between the individual cysteine mutants and provides a means to understand the compound's mechanism of action. Wild-type, 160C or 107C RGS8 was treated with 20 μM CCG-50014 for the desired amount of time before compound removal by extensive washing. The developed amount of irreversible inhibition was quantified by comparing the G-protein binding of CCG-50014 treated beads to DMSO treated beads. Data are presented as the mean±SEM from three independent experiments.

CCG-50014 is not a general cysteine alkylator:

Cysteine reactive compounds might be expected to have more off-target effects than non-reactive compounds. To determine if this compound could bind to and inhibit any reactive cysteine, we tested the ability of CCG-50014 and a known general cysteine alkylator (iodoacetamide) to inhibit a standard cysteine protease (**Fig. 3.11**). Iodoacetamide inhibited the proteolytic activity of papain in a dose-dependent manner. However, even at high concentrations (100 μM), CCG-50014 had no effect on papain. This suggests that there is at least a basal level of selectivity of this class of compounds for cysteines in the RGS over other

reactive cysteines. It is also possible that the compound cannot enter the active site of papain and therefore it would be prudent to extend these studies to a panel of physiologically relevant thiol-dependent processes.

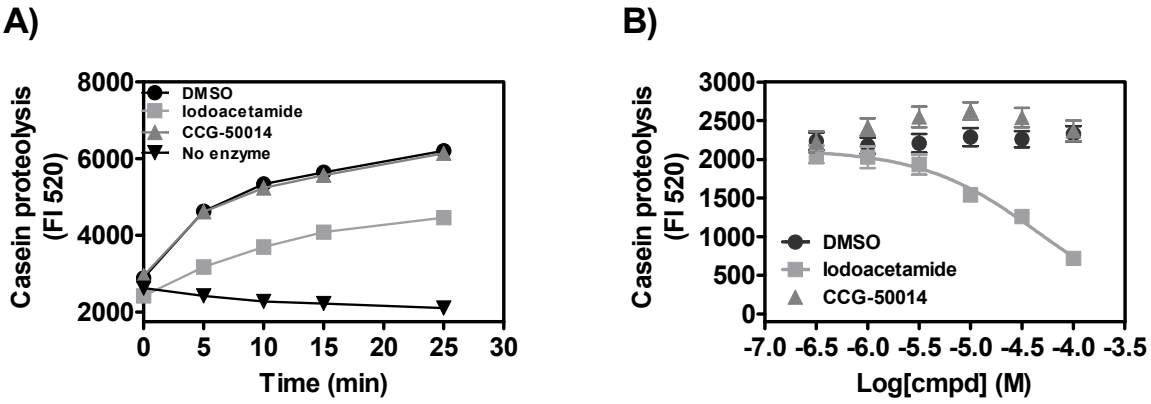


Figure 3.11 CCG-50014 does not inhibit the general cysteine protease, papain. **A)** Papain (0.625 U) was mixed with self-quenching FITC-conjugated casein and the liberated fluorescence that results from casein-dependent proteolysis was observed as a function of time in the presence of different cysteine alkylators. Even 100 μM CCG-50014 is incapable of inhibiting casein proteolysis by papain. **B)** The effect of the cysteine alkylator iodoacetamide on inhibiting papain activity is dose-dependent. Once again, CCG-50014 is incapable of inhibiting papain activity. Data are presented as the mean±SEM from three independent experiments.

General cysteine alkylators do not inhibit RGS proteins:

The RGS selectivity of CCG-50014 could be explained by RGS proteins being particularly sensitive to thiol modification. To test for this, I analyzed the RGS inhibitory activity of two general cysteine alkylators, N-ethyl maleimide and iodoacetamide (**Fig. 3.12**). Iodoacetamide had no effect on $G\alpha_o$ binding to any of the RGS proteins tested. At high concentrations (IC_{50}: 30 μM), N-ethyl maleimide inhibited RGS4, however it had no effect on RGS8 or papain (data not shown). These data show that CCG-50014 is more than 3.5 orders of magnitude more potent on RGS4 than either of the general cysteine alkylators tested. This strongly suggests that RGS proteins are not particularly sensitive to cysteine

modification and the effect observed by CCG-50014 is more than just random thiol alkylation.

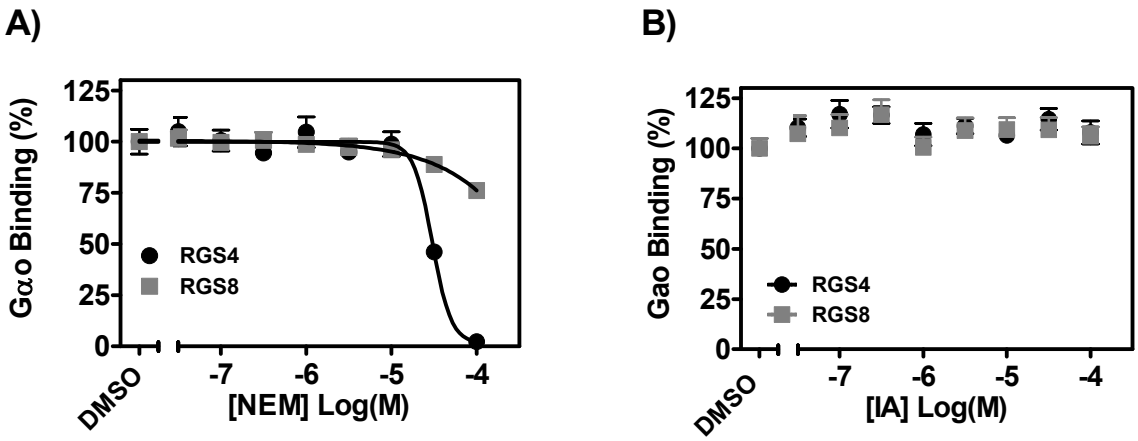


Figure 3.12 CCG-50014 is a much more potent RGS inhibitor than two general cysteine alkylators N-ethyl maleimide (NEM) and iodoacetamide (IA). Dose response curves for **A)** NEM and **B)** IA. The only protein that displayed any sensitivity to the alkylators tested was RGS4, which was inhibited by NEM with an IC_{50} value >3.5 Log higher than that of CCG-50014. Data are presented as the mean±SEM from three independent experiments.

Computational modeling of the CCG-50014-RGS8 interaction:

To identify potential binding sites for CCG-50014 on RGS8, I in collaboration with Haoming Zhang performed an unbiased molecular docking simulation. CCG-50014 docked preferentially to a site on the RGS that is located near the region of the surface of RGS8 that corresponds to the "B"-site of RGS4 (**Fig. 3.13**). The compound docked at this site with a calculated free energy of -6.4 kCal/mol, which translates to an estimated K_i of 18 μM. This affinity is approximately in line with experimentally derived K_i value of 0.3μM (**Table 1**). This binding site places the compound a considerable distance from the two cysteine residues known to play a role in the compound's inhibitory activity (**Fig.**

3.13B). It would require a substantial change in the conformation of the protein for the compound to dock at this site and react with a cysteine residue.

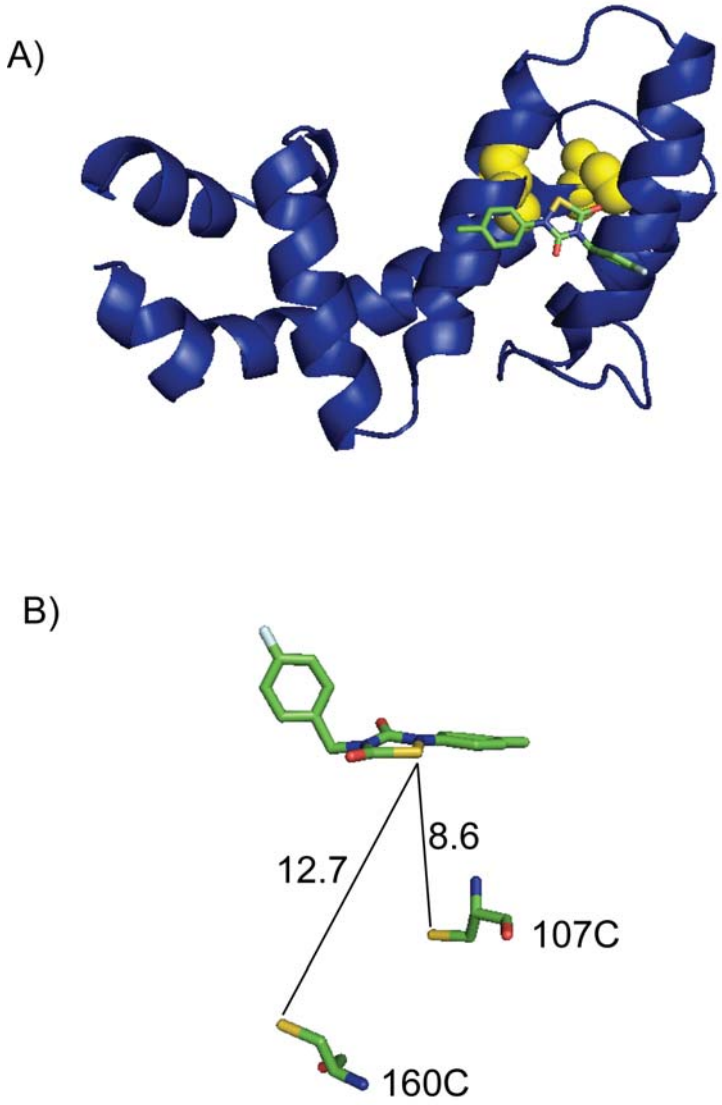


Figure 3.13 Hypothesized binding site of CCG-50014 on RGS8. **A)** This binding site was determined to be the most energetically favorable site for docking of CCG-50014 to RSG8, with an estimated Ki of 18 μM. This site is near the analogous "B" site on RGS4, which is important for RGS regulation by calmodulin and acidic phospholipids. **B)** Assuming a static protein, this binding site places the compound close to the two cysteine residues in RGS8, but not close enough for a covalent reaction to occur at any reasonable rate. A conformational change must occur in the RGS to allow compound intercalation into the helix bundle. Distances are shown in angstroms.

Limiting the reactivity of CCG-50014 diminishes potency:

To probe the chemical space around CCG-50014, Dr. Stephen Husbands, Dr. Benjamin Greedy and Dr. Emma Casey synthesized a series of 76 analogs of the lead compound and we analyzed them in an attempt to identify compounds with optimized physicochemical and pharmacological properties (see **Chapter IV**). While the detailed activity of these compounds is described in the next chapter, there are a few interesting findings that contribute to the understanding of the mechanism of action of this compound class. The first and most prominent trend is that the center heterocycle (the thiadiazolidine dione) is absolutely required for function. This is not particularly surprising, because it is likely to be the site of cysteine thiol reactivity. I hypothesized that the extremely potent IC_{50} value on RGS4 meant that the compound interacted with the protein in a way that was governed by more than simple covalent reactivity. To determine if there was significant non-covalent affinity of this compound, non-reactive, or less-reactive analogs of CCG-50014 were synthesized and tested for activity (**Table 3.2**). These compounds showed limited, if any, activity in the FCPIA assay, suggesting that the main mechanism of action of CCG-50014 is through covalent reactivity with one or more cysteine residues on the RGS. However, since this compound is dramatically more potent than two other general cysteine alkylators, it is likely that there is a non-covalent docking mechanism at play that drives the affinity of CCG-50014 and analogs for the RGS.

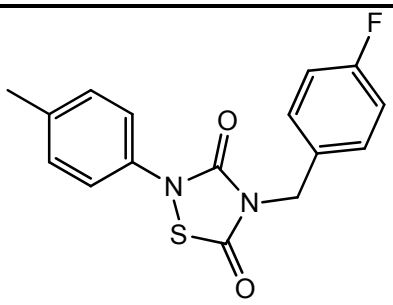
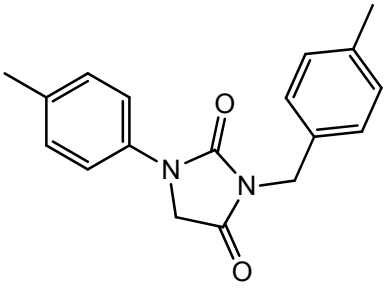
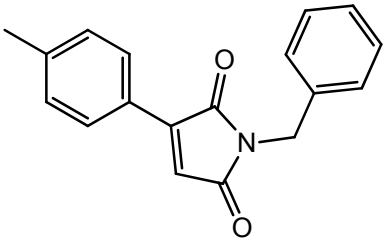
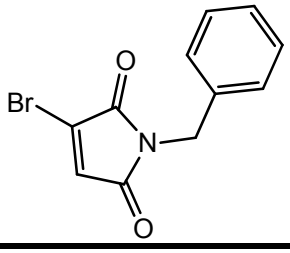
Compound ID	Structure	Putative Reactivity	RGS4 IC_{50} (μM)	RGS8 IC_{50} (μM)
CCG-50014		Reactive	0.030	1.1
CCG-203778		Non-reactive	>100	>100
CCG-203779		Less reactive	93	>100
CCG-203780		Less Reactive	32	>100

Table 3.2. CCG-50014 analogs with limited reactivity. Data are presented as the mean of two independent FCPIA experiments.

Discussion:

Molecules disrupting the RGS/Gα interaction are likely to have significant physiological effects by increasing the magnitude and/or duration of Gα signaling responses. Inhibition of RGS protein activity genetically produces dramatic physiological phenotypes (see **Chapter I**), suggesting that a small molecule RGS inhibitor might provide similar actions *in vivo*.

CCG-50014 is the most potent small molecule RGS inhibitor identified to date. It inhibits the *in vitro* interaction between RGS4 and $G\alpha_o$ with a low nanomolar IC_{50} value. It is nearly 3 orders of magnitude selective for RGS4 over two closely related RGS proteins, RGS8 and RGS16 (**Fig. 3.2**, **Table 3.1**). I also show that CCG-50014 is a covalent modifier of cysteine residues (**Fig. 3.4, 3.6, 3.7**), raising concerns about the therapeutic potential of this class of compounds. Even so, studying this compound has provided significant insight into the mechanism of allosteric RGS inhibition and as shown in **Chapter IV**, it is active in a series of cellular assays, suggesting that we may be close to the physiological modulation of RGS activity by small molecules.

CCG-50014 does not inhibit a mutant RGS4 where all cysteine residues in the RH domain were mutated to alanine (**Table 3.1** and [4]). Furthermore, the compound was inactive on RGS7, an RGS protein that naturally has no cysteine residues in its RH domain (**Table 3.1**). These two pieces of information suggested that the mechanism of action of CCG-50014 requires at least one cysteine residue – a hallmark of a sulfhydryl-reactive irreversible inhibitor. This

hypothesis was confirmed by the FCPIA reversibility experiments (**Fig. 3.4**) and subsequent mass spectral analysis of CCG-50014 treated RGS8 (**Fig. 3.7**). Based on the chemical structure of the compound and the full molecular weight adduct observed in the LC-MS experiments, it is likely that the mechanism of reaction of CCG-50014 with a cysteine residue on an RGS protein is by nucleophilic attack of the cysteine thiol onto the sulfur atom of the central heterocycle causing a ring opening event. Consistent with the DTT-induced reversibility of CCG-50014 inhibition (**Fig. 3.4**), this newly formed disulfide is likely to be sensitive to reductants.

Interestingly, CCG-50014 interacts with cysteine residues in RGS8 that are not near the Gα interaction interface (**Fig 3.13**), suggesting an allosteric mechanism of action. Unbiased computational modeling predicts that CCG-50014 could non-covalently bind to a site on RGS8 that is near to the acidic phospholipid binding site on RGS4. Binding in this site would place the reactive group of CCG-50014 within 8-13 Å of the two cysteines in the RGS8 RH domain. While at this distance it is unlikely that a covalent bond could be formed, I propose that the compound may initially bind to this pocket and a subsequent conformational change in the protein provides access to the cysteine thiol. This conformational change is likely to be the fundamental mechanism by which the allosteric modulation of G protein binding activity is conferred.

The differential sensitivities of the cysteine mutants to CCG-50014 are also explained by this binding modality. The decreased activity and increased reversibility of CCG-50014 on 107C RGS8 (**Fig 3.6**, **3.8**, **3.9**) is in accord with the

fact that Cys 107 is more solvent accessible and is closer to the hypothesized binding site of the compound. Compound reacting with Cys 160 causes drastic protein unfolding (**Fig. 3.9**), which also fits with this model.

The data accumulated in this chapter allow for the development of a reasonable mechanism of action for RGS inhibition by CCG-50014. I propose that the compound originally binds to a surface equivalent to the "B" site on RGS4 in a manner that does not produce an inhibitory effect. This interaction may provide enough interaction energy to keep the compound in close proximity to the protein long enough for the RGS to enter a cysteine-exposed conformation. Assuming that the compound binding site is as modeled (**Fig 3.13**), the first cysteine to become exposed to the compound is likely to be Cys 107. Upon reacting with this cysteine, CCG-50014 can trap the RGS in a conformation that is incapable of binding to Gα. Reversal of this reaction is possible, leading to reactivation of the RGS. If the compound interacts with the more deeply buried cysteine, Cys 160, it causes a dramatic disruption of the hydrophobic core of the protein, leading to protein denaturation. This is shown primarily by the gel filtration data (**Fig. 3.8**). My data also suggest that Cys 107 is labeled more rapidly than Cys 160 in the wild type protein (**Fig. 3.10**). These data are consistent with the hypothesis that the compound initially interacts with Cys 107 to form a weak, DTT- or time/dilution-reversible inhibition of RGS activity. Then, either the Cys 107-bound compound transfers to Cys 160 or a second CCG-50014 molecule binds to Cys 160 to produce the completely irreversible reaction observed the gel filtration experiments (**Fig. 3.8**). The mechanism

behind the irreversible inhibition after labeling of Cys 160 is likely due to a massive destabilization of the hydrophobic core of the RH domain that would occur by the intercalation of CCG-50014.

Selectivity is a significant issue when studying reactive compounds. I undertook a series of experiments to determine if CCG-50014 is a just a general, non-specific cysteine alkylator, or if it has some intrinsic selectivity for RGS proteins. While a comprehensive analysis of CCG-50014 effects upon all cysteine-dependent processes in a cell is clearly intractable, I showed that CCG-50014 does not inhibit the activity of the cysteine protease papain at concentrations over 3000 times higher than that required for RGS inhibition (**Fig. 3.11**). In contrast, the cysteine alkylator iodoacetamide dose-dependently inhibited the activity of this protease but had no effect on RGS4. Furthermore, the cellular activity observed (see **Chapter IV**), also suggests that these compounds do not dramatically affect a large number of cellular processes.

In this chapter I have characterized the mechanism of action of the most potent RGS inhibitor identified to date. This compound irreversibly inhibits RGS4 with nanomolar potency and the mechanism of this inhibition is predominantly through reacting with cysteine residues at an allosteric site on the RGS. As will be shown in the next chapter, this compound and related analogs are also the first examples of RGS inhibitors that are active in living cells.

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Chapter IV:
Cellular and Structure-Activity Studies of the CCG-50014 Compound Class

Introduction:

The work presented thus far focuses solely upon the biochemical mechanism of action of either novel or previously identified small molecule inhibitors of the RGS-$G\alpha_o$ PPI. While this knowledge is academically useful, pharmacologically important chemical entities need to have activity in a physiological (preferably clinical) setting. Prior to the work described in this chapter, there were no examples of RGS inhibitors that functioned in a living, whole-cell system. I present here work performed by myself and collaborators that shows for the first time a class of irreversible small molecule inhibitors that produce substantial RGS-inhibitory effects in several cell-based systems.

There are a number of issues involved with the use of covalent, irreversible inhibitors for research or clinical applications. A major problem is target specificity. If the compound is too reactive, it will bind many different proteins and will likely cause a barrage of undesired side effects However, there are a number of clinically useful irreversible inhibitors (e.g. lactams, cyclophosphamide), some of which function by reacting with thiols (e.g. omeprazole) [1]. A final issue that pertains primarily with the clinical utility of irreversible inhibitors is that their effects are often difficult to quickly reverse. Medicine, like all human endeavors, is prone to mistake. Having a mechanism to

compensate for or to correct dosing errors can play a critical role in patient outcome. The challenging nature of reversing the physiological effects of irreversible inhibitors is likely to diminish their clinical utility.

Irreversible inhibition can however be a benefit in the research setting. With this, I present the following data showing the effects of CCG-50014 and selected analogs in a series of cellular assays. It is shown here that CCG-50014 and related analogs are able to inhibit the RGS4/$G\alpha_o$ PPI in living cells and to potentiate signaling through the δ opioid receptor and the M3 muscarinic receptor. The success of this work required a substantial amount of structure-activity data, both at the biochemical and cellular levels, to minimize off target effects and to improve aqueous solubility. These compounds and the information presented in this chapter should accelerate the development of small molecule modulators of RGS function by providing a fundamental groundwork from which to design future analogs and to judge future novel scaffolds.

The work in this chapter was performed by me with the following exceptions: 1) All compound synthesis was performed by Dr. Benjamin Greedy and/or Dr. Emma Casey in the laboratory of Dr. Stephen Husbands; 2) cAMP experiments with SH-SY5Y cells were performed by Dr. Qin Wang in the laboratory of Dr. John Traynor.

Materials and Methods:

Reagents and Compounds:

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) and were reagent grade or better. Avidin-coated

microspheres were purchased from Luminex (Austin, TX). CCG-50014 (4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione) and analogs were purchased from Thermo-Fisher (Waltham, MA) from the Maybridge compound collection or were provided from complete synthesis in the laboratory of Dr. Stephen Husbands.

Protein expression, purification, and labeling:

All RGS and G proteins were prepared as previously described [2]. G protein activity was determined by [^{35}S]GTPγS binding [3]. In all cases, proteins were purified to >90% homogeneity before use. RGS proteins were biotinylated and $G\alpha_o$ was labeled with AlexaFluor-532 as previously described [4].

FCPIA Dose Response experiments:

FCPIA was performed as previously described using chemically biotinylated RGS proteins and AlexaFluor-532 labeled $G\alpha_o$. ([5, 6])

Single Turnover GTPase Measurements:

Compounds were tested for the ability to inhibit the RGS4 and RGS8-stimulated increase in GTP hydrolysis by $G\alpha_o$ as previously described [7, 8].

Solubility experiments:

Compounds were diluted to 100 mM in DMSO and the further diluted to 500 μM in H_2O, vortexed and centrifuged for 10 minutes at 13,000 x g at ambient

temperature. Solubility was quantified by visual inspection of pellet formation on a scale from 0 – 5, with 0 being no pellet and 5 being a pellet of the same size as that of CCG-50014. Since the key metric was improved solubility, in the rare instance that a compound was deemed to be more insoluble than CCG-50014, it was given a value of 5.

WST-1 Cell Viability Studies:

HEK-293 cells were plated to a density of 30,000/well in 96-well plates and grown for 48 hours in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO_2 at 37^{o}C. Compound treatment was performed overnight in DMEM containing 0.1% bovine serum albumin under 5% CO_2 at 37^{o}C. After compound incubation ten microliters of the WST-1 reagent (Hoffmann-La Roche, Switzerland) was added to every well and the cells were incubated for 1 hour under 5% CO_2 at 37^{o}C. Absorbance was measured at 450 nm using a Victor II plate reader (Perkin-Elmer, Picastaway, NJ).

Cellular Localization Studies:

HEK-293T cells grown to 80-90% confluency in 6-well dishes in DMEM supplemented with 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO_2 at 37 ^{o}C. RGS and $G\alpha_o$ expression was induced by transient co-transfection with either 250 ng of full-length human RGS4 with an N-terminal GFP tag (RGS4pEGFP-C1) or a C-terminal RGS4-GFP

(RGS4pDEST47) and 250 ng of pcDNA3.1 or pcDNA with wildtype human $G\alpha_o$. Cells were split onto poly-D-lysine coated glass coverslips and cultured for 24-48 hours after transfection before live cell imaging. Images were acquired on an Olympus Fluoview 500 confocal microscope with a 60 x 1.40 numerical aperture (N.A) oil objective. Images were obtained by taking a series of stacks every 0.5 μm through the cell and combining the images into a composite stack. The light source for the fluorescent studies was a 488 nm laser with a 505-525 nm bandpass filter. Images were quantified using NIH ImageJ software version 1.43r.

Calcium Mobilization Experiments:

A stable cell line was developed based upon the HEK-293 Flp-In TREx cell line (Invitrogen, Carlsbad, CA) that stably express the muscarinic M3 receptor and have human RGS4 expression under doxycycline control. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 μg/ml) under 5% CO2 at 37°C. For experiments, cells were split into 96-well black, clear bottom, poly-D-lysine coated microtiter plates (Nunc, Cat. # 152037) at a density of 20,000 cells/well in DMEM containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 μg/ml). RGS4 expression was induced by supplementing the medium with 1 μg/mL doxycycline for 24-48 hours before experimentation. Cells were loaded with Fluo-4 No Wash dye in buffer for 30 minutes at 37°C. Compounds and/or carbachol were added to the wells and the fluorescence

intensity was measured using a FlexStation (Molecular Devices, Sunnyvale, CA) plate reader. Data analysis was performed by calculating the area under the curve or maximal fluorescence intensity from a 120 second kinetic measurement.

cAMP Accumulation:

SH-SY5Y cells were grown in DMEM containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO_2 at 37^{o}C. Cells were plated into 24-well plates to reach ~ 90% confluency on the day of assay and washed once with fresh serum-free medium, then the medium was replaced with 1 mM IBMX (3-isobutyl-1-methylxanthine) in serum-free medium for 15 min at 37°C, and changed to the medium containing 1 mM IBMX, 30 µM forskolin, and 100 nM of either morphine or SNC80 with or without compound CCG-50014 for 5 min at 37 °C. Reactions were stopped by replacing the medium with ice-cold 3% perchloric acid and samples were kept at 4 °C for at least 30 min. An aliquot (0.4 ml) from each sample was removed, neutralized with 0.08 ml of 2.5 M $KHCO_3$, vortexed, and centrifuged at 15,000 x *g* for 1 min to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 10-15 µl aliquot of the supernatant from each sample following the manufacturer's instructions (cAMP radioimmunoassay kit from GE Healthcare, Piscataway, NJ). Data are from four separate experiments, each carried out in duplicates and calculated as percent inhibition. The basal cAMP accumulation with forskolin alone with or without compound CCG-50014 did not differ.

Results:

Structure Activity Relationship Studies of the CCG-50014 Family of Compounds:

Biochemical Optimization:

To explore the chemical space around the thiadiazolidine scaffold, we analyzed a series of 76 analogs of CCG-50014 using a variety of cellular and biochemical experiments (**Table 4.1**). As a primary screening methodology, a RGS4/RGS8 duplex FCPIA assay was performed to obtain biochemical IC_{50} values for the inhibition of $G\alpha_o$ binding to both proteins. Secondary screening was performed to assess compound solubility and the ability to induce a calcium transient in HEK cells, an off-target effect that was originally observed with the lead compound CCG-50014. The primary goals of the SAR studies were to optimize compound solubility and potency and to minimize off target effects. A secondary goal of this study was to determine if it was possible to remove or minimize the reactivity of this scaffold and still retain RGS inhibitory activity.

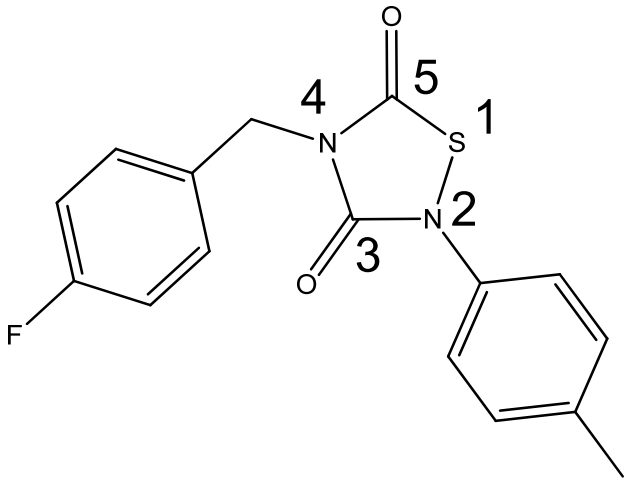
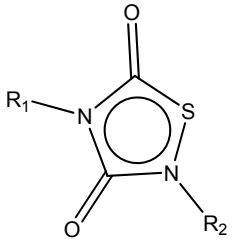


Figure 4.14 The chemical structure of CCG-50014, (4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione)

The majority of the analogs of CCG-50014 that were tested contain the thiadiazolidinedione core structure with varied substituents at the 2 and 4 positions. Because CCG-50014 is only modestly soluble in aqueous solutions, we wanted to identify active compounds with improved solubility for use in cellular and whole-animal studies. Compound solubility was assessed on a 0-5 scale with 5 being as insoluble as the lead compound, CCG-50014 (solubility ~100 μM in aqueous) and 0 being completely soluble at 500 μM in double-distilled H_2O. As expected, it was found that the key determinant of compound solubility was size, whereby large hydrophobic substituents lead to more insoluble compounds. While this was not particularly surprising, I did identify a number of compounds that were much more soluble than CCG-50014 that retained similar potency for RGS4 inhibition (e.g. CCG-203759, CCG-203769).

To attempt to identify a pharmacophore for the CCG-50014 class of compounds, I ranked all of the compounds in order of potency on RGS4 (**Table 4.1**). Unfortunately, no clear trends emerge. I then probed this data for compounds that possessed increased selectivity for RGS4 over RGS8 (**Table 4.2**). This analysis shows that small alkyl groups at the R1/R2 positions provide the greatest RGS4/RGS8 selectivity. However, there are several compounds with phenyl-containing sidechains that show >1000 fold selectivity (e.g. CCG-203742). These compounds all contain a -CH_3-Ph-*p*-Me (or in one instance a -CH_3-Ph-*p*-OMe, CCG-203705) at the R2 position, a moiety that appears to confer selectivity regardless of group at the R1 position. This suggests that this region of

the molecule is important for discriminating between RGS proteins. The most significant result from the SAR analysis was the dependence of the thiadiazolidinedione for activity (see **Chapter III**, **Table 3.2**), whereby compounds that did not contain this reactive center lost substantial (>1000 fold) potency against RGS4.

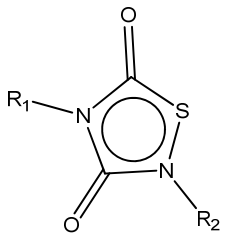


CCG #	R1	R2	Solubility Scale (1-5)	Stock Form	Calcium transient (% 50014)	RGS4 IC_{50} (μM)	RGS4 Hill Slope	RGS8 IC_{50} (μM)	RGS8 Hill Slope	Fold Selectivity (R8/R4)
203731	CH_3-Ph-*p*-Cl	Ph-*p*-Me	5	Solid	-2.6	0.005	-0.70	11.8	-1.50	2360
203734	CH_3-Ph	CH_3-Ph-*p*-Me	5	Solid	591.3	0.007	-0.84	20.4	-0.66	2914
203732	CH_3-Ph-*p*-Me	Ph	3	Solid	12.2	0.009	-1.50	8.3	-0.87	922
203736	CH_3-Ph-*p*-Me	Ph-*p*-Me	4	Solid	4.9	0.009	-1.35	11.6	-1.45	1289
203735	CH_3-Ph-*p*-F	Ph-*p*-OMe	4	Solid	6.2	0.011	-1.33	11.4	-1.27	1036
203741	CH_3-Ph-*p,m*-Cl	Ph-*p*-OMe	3	Solid	202.4	0.013	-0.86	5.9	-0.64	454
203742	CH_3-Ph-*p*-F	CH_3-Ph-*p*-Me	5	Solid	108.5	0.013	-1.19	39.8	-0.22	3062
203724	CH_3-Ph	Ph-*p*-Me	5	Solid	176.8	0.014	-3.63	7.5	-0.94	536
203727	CH_3-Ph-*p*-F	Ph-*p*-Cl	5	Solid	158.5	0.014	-1.66	7.6	-1.06	543
203761	iBu	Ph-*p*-Me	4	Solid	98.9	0.014	-1.47	7.7	-0.92	550
203769	Bu	Et	0	Oil	2.7	0.014	-0.71	83.5	-0.56	5964
203781	CH_3-Ph-*m*-Me	Ph	3	Solid	242.8	0.015	-1.65	8.4	-1.17	560
203726	CH_3-Ph-*p*-F	Ph	2	Solid	20.9	0.016	-1.42	6.2	-0.82	388
203765	CH_3-Ph-*p*-F	Bu	3	Solid	183.8	0.016	-0.81	31.6	-0.59	1975
203733	CH_3-Ph-*p*-Me	Ph-*p*-Cl	5	Solid	20.0	0.017	-1.59	12.8	-1.17	753
203777	CH_3-Ph-*m*-Cl	Ph-*p*-Me	1	Solid	179.0	0.017	-0.96	17.5	-1.00	1029
203759	Me	Ph-*p*-Me	0	Solid	238.0	0.019	-1.56	8.4	-1.06	442
203767	Bu	Ph-*p*-Me	1	Solid	123.0	0.020	-1.46	9.5	-0.96	475
203757	Bu	Me	0	Oil	138.3	0.023	-1.01	28.4	-1.10	1235
203722	CH_3-Ph	Ph	1	Solid	3.8	0.024	-1.89	5.6	-0.80	233

Table 4.8 Structure Activity Relationships of CCG-50014 family. All FCPIA IC_{50} and calcium transient values presented are an average from two independent experiments. The calcium transient data are presented as the effect observed by 10 μM compound expressesed as a percentange of the effect observed by 10 μM CCG-50014. All compounds were synthesized in the laboratory of Dr. Stephen Husbands

CCG #	R1	R2	Solubility Scale (1-5)	Stock Form	Calcium transient (% 50014)	RGS4 IC_{50} (μM)	RGS4 Hill Slope	RGS8 IC_{50} (μM)	RGS8 Hill Slope	Fold Selectivity (R8/R4)
203739	CH_3-Ph-p,*m*-Cl	Ph	3	Solid	47.6	0.024	-0.96	7.5	-0.80	313
203746	CH_3-Ph	Ph-*p*-OMe	3	Solid	74.6	0.024	-1.45	12.3	-1.00	513
203730	CH_3-Ph-*p*-Cl	Ph-*p*-Cl	5	Solid	141.3	0.026	-1.48	12.7	-1.36	488
203762	iBu	Et	0	Oil	38.3	0.026	-0.87	70.6	-0.78	2715
203760	Me	tBu	0	Solid	47.2	0.028	-0.99	56.0	-0.89	2000
203723	CH_3-Ph	Ph-*p*-Cl	3	Solid	119.8	0.029	-1.99	5.2	-0.80	179
203763	iBu	tBu	0	Oil	73.5	0.029	-0.85	193.7	-0.52	6679
203770	Bu	Bu	0	Oil	188.2	0.030	-0.83	122.3	-0.53	4077
203783	CH_3-Ph-*m*-Cl	Ph	5	Solid	57.7	0.031	-1.41	9.4	-1.47	303
203773	CH_3-Ph	Ph-*m*-Cl	4	Solid	198.9	0.033	-1.25	10.9	-0.87	330
203738	CH_3-Ph-*p*-Me	CH_3-Ph-*p*-Me	5	Solid	251.4	0.033	-0.92	94.7	-0.51	2870
203745	CH_3-Ph-*p*-OMe	Ph	4	Solid	18.6	0.034	-1.78	7.2	-0.77	212
203743	CH_3-Ph-*m,p*-Cl	Ph-*p*-Me	3	Solid	45.6	0.034	-1.43	15.7	-0.65	462
203755	CH_3-Ph-*p*-OMe	Ph-*p*-OMe	2	Solid	54.9	0.035	-1.27	17.6	-0.81	503
203728	CH_3-Ph-*p*-F	Ph-*m,p*-Cl	5	Solid	127.6	0.036	-1.15	17.2	-1.78	478
203785	CH_3-Ph	Ph-*m*-Me	2	Solid	144.6	0.038	-1.13	21.3	-0.73	561
203764	iBu	Bu	1	Oil	141.6	0.039	-0.87	98.0	-0.44	2513
203771	CH_3-Ph	Ph-*p*-tBu	4	Solid	123.1	0.044	-1.79	17.4	-0.78	395
203794	CH_3-Ph-*m* –Me	Ph-*m*-Me	3	Solid	63.3	0.046	-1.79	10.4	-0.82	226
203776	CH_3-Ph-*p*-F	Ph-*m*-Cl	4	Solid	222.9	0.052	-1.08	12.8	-1.07	246
203768	Bu	tBu	0	Oil	91.7	0.054	-3.18	119.0	-0.70	2204
203772	CH_3-Ph	Ph-*m*-MeF_3	3	Solid	164.8	0.057	-1.43	16.4	-1.15	288
203786	CH_3-Ph-*m*-Cl	Ph-*m*-Me	3	Solid	141.6	0.064	-1.52	10.1	-0.95	158
203740	CH_3-Ph-*m,p*-Cl	Ph-*p*-Cl	3	Solid	-0.1	0.068	-1.31	16.7	-0.76	246
203750	CH_3-Ph-*p*-OMe	Ph-*p*-Cl	3	Solid	84.6	0.069	-3.26	9.9	-0.73	143
203747	CH_3-Ph-*p*-Cl	CH_3-Ph-*p*-Me	5	Solid	11.4	0.073	-1.23	336.6	-0.60	4611
203775	CH_3-Ph-*p*-F	Ph-*m*-MeF_3	2	Solid	170.1	0.079	-0.88	16.2	-1.14	205
203748	CH_3-Ph-*p*-OMe	Ph-*m,p*-Cl	2	Solid	63.4	0.087	-1.04	36.0	-1.50	414
203725	CH_3-Ph	Ph-*m,p*-Cl	2	Solid	64.2	0.089	-2.06	13.2	-1.62	148
203756	CH_3-Ph-*p*-OMe	CH_3-Ph-*p*-Me	4	Solid	169.0	0.103	-2.42	92.9	-0.64	902
203753	CH_3-Ph-*m,p*-Cl	CH_3-Ph-*p*-Me	2	Solid	51.7	0.113	-1.33	109.1	-0.71	965
203788	CH_3-Ph-*p*-Me	Ph-*m*-Me	4	Solid	134.0	0.121	-1.91	7.1	-1.09	59

CCG #	R1	R2	Solubility Scale (1-5)	Stock Form	Calcium transient (% 50014)	RGS4 IC_{50} (μM)	RGS4 Hill Slope	RGS8 IC_{50} (μM)	RGS8 Hill Slope	Fold Selectivity (R8/R4)
203797	CH_3-Ph-*p*-F	Ph-*p*-$N(CH_3)_2$	5	Oil	111.2	0.146	-1.65	10.3	-1.28	71
203749	CH_3-Ph-*m,p*-Cl	Ph-*m,p*-Cl	5	Solid	49.6	0.155	-0.87	125.0	-0.85	806
203752	CH_3-Ph- *p*-Cl	Ph-*p*-OMe	4	Solid	104.1	0.166	-1.73	40.2	-0.91	242
203790	CH_3-Ph-*p*-tBu	Ph-*p*-Me	5	Solid	18.9	0.178	-1.36	>100	N/A	N/A
203784	Ph-*p*-tBu	Ph-*m*-Cl	2	Solid	31.8	0.224	-2.03	32.2	-0.58	144
203791	CH_3-Ph-*p*-tBu	Ph	5	Solid	14.4	0.245	-1.72	>100	N/A	N/A
203796	CH_3-Ph-*p*-tBu	Ph-*p*-tBu	5	Solid	14.8	0.351	-1.70	>100	N/A	N/A
203787	CH_3-Ph-*o*-Me	Ph-*m*-Cl	4	Solid	78.2	0.381	-2.01	31.0	-1.10	81
203792	CH_3-Ph-*m*-Cl	Ph-*m*-Cl	4	Solid	67.8	0.419	-1.55	21.1	-1.66	50
203793	CH_3-Ph-*m*-Me	Ph-*m*-MeF_3	5	Solid	65.2	N/A	N/A	15.9	-1.03	N/A



CCG Number	R1	R2	Fold Selectivity (RGS8 IC_{50} /RGS4 IC_{50})
203763	iBu	tBu	6679
203769	Bu	Et	5964
203770	Bu	Bu	4077
203742	CH_3-Ph-*p*-F	CH_3-Ph-*p*-Me	3062
203734	CH_3-Ph	CH_3-Ph-*p*-Me	2914
203738	CH_3-Ph-*p*-Me	CH_3-Ph-*p*-Me	2870
203762	iBu	Et	2715
203764	iBu	Bu	2513
203731	CH_3-Ph-*p*-Cl	Ph-*p*-Me	2360
203760	Me	tBu	2000
203765	CH_3-Ph-*p*-F	Bu	1975
203736	CH_3-Ph-*p*-Me	Ph-*p*-Me	1289
203757	Bu	Me	1235
203735	CH_3-Ph-*p*-F	Ph-*p*-OMe	1036
203777	CH_3-Ph-*m*-Cl	Ph-*p*-Me	1029
203732	CH_3-Ph-*p*-Me	Ph	922
203733	CH_3-Ph-*p*-Me	Ph-*p*-Cl	753
203785	CH_3-Ph	Ph-*m*-Me	561
203781	CH_3-Ph-*m*-Me	Ph	560
203761	iBu	Ph-*p*-Me	550
203727	CH_3-Ph-*p*-F	Ph-*p*-Cl	543
203724	CH_3-Ph	Ph-*p*-Me	536
203746	CH_3-Ph	Ph-*p*-OMe	513
203755	CH_3-Ph-*p*-OMe	Ph-*p*-OMe	503
203730	CH_3-Ph-*p*-Cl	Ph-*p*-Cl	488
203728	CH_3-Ph-*p*-F	Ph-*m,p*-Cl	478
203767	Bu	Ph-*p*-Me	475
203743	CH_3-Ph-*m,p*-Cl	Ph-*p*-Me	462
203741	CH_3-Ph-*p,m*-Cl	Ph-*p*-OMe	454
203759	Me	Ph-*p*-Me	442
203771	CH_3-Ph	Ph-*p*-tBu	395
203726	CH_3-Ph-*p*-F	Ph	388
203773	CH_3-Ph	Ph-*m*-Cl	330
203739	CH_3-Ph-p,*m*-Cl	Ph	313
203783	CH_3-Ph-*m*-Cl	Ph	303
203722	CH_3-Ph	Ph	233
203794	CH_3-Ph-*m* –Me	Ph-*m*-Me	226
203745	CH_3-Ph-*p*-OMe	Ph	212
203723	CH_3-Ph	Ph-*p*-Cl	179

Table 4.9 Selectivity of CCG-50014 analogs for RGS4 over RGS8. For clarity, only compounds with IC50 values on RGS4 <50 nM are included in this table. Selectivity was determined by comparing the IC50 values from duplex FCPIA assays. n≥2.

Identification of compounds with minimized off-target effects:

I initially attempted to test CCG-50014 in a Ca^{2+} mobilization assay using a cell line stably expressing the M3 muscarinic receptor and expressing RGS4 under control of a doxycycline promoter. A 30-minute pretreatment of these cells with CCG-50014 completely inhibited the Ca^{2+} response to carbachol. To probe the mechanism of this effect, I reduced the compound preoccupation time so that I could monitor intracellular calcium levels directly after compound addition (**Fig. 4.2A**). I found that CCG-50014 induced a marked calcium transient in these cells that after prolonged treatment would be expected to deplete calcium stores. Since proper calcium handling is crucial for cellular functioning, I tested all of the available CCG-50014 analogs for their ability to induce calcium mobilization at a concentration of 10 μM. As shown in **Table 4.1** and **Fig 4.2B**, there were a number of analogs that did not produce a significant calcium effect in this cell system, several of which still potently inhibit RGS4.

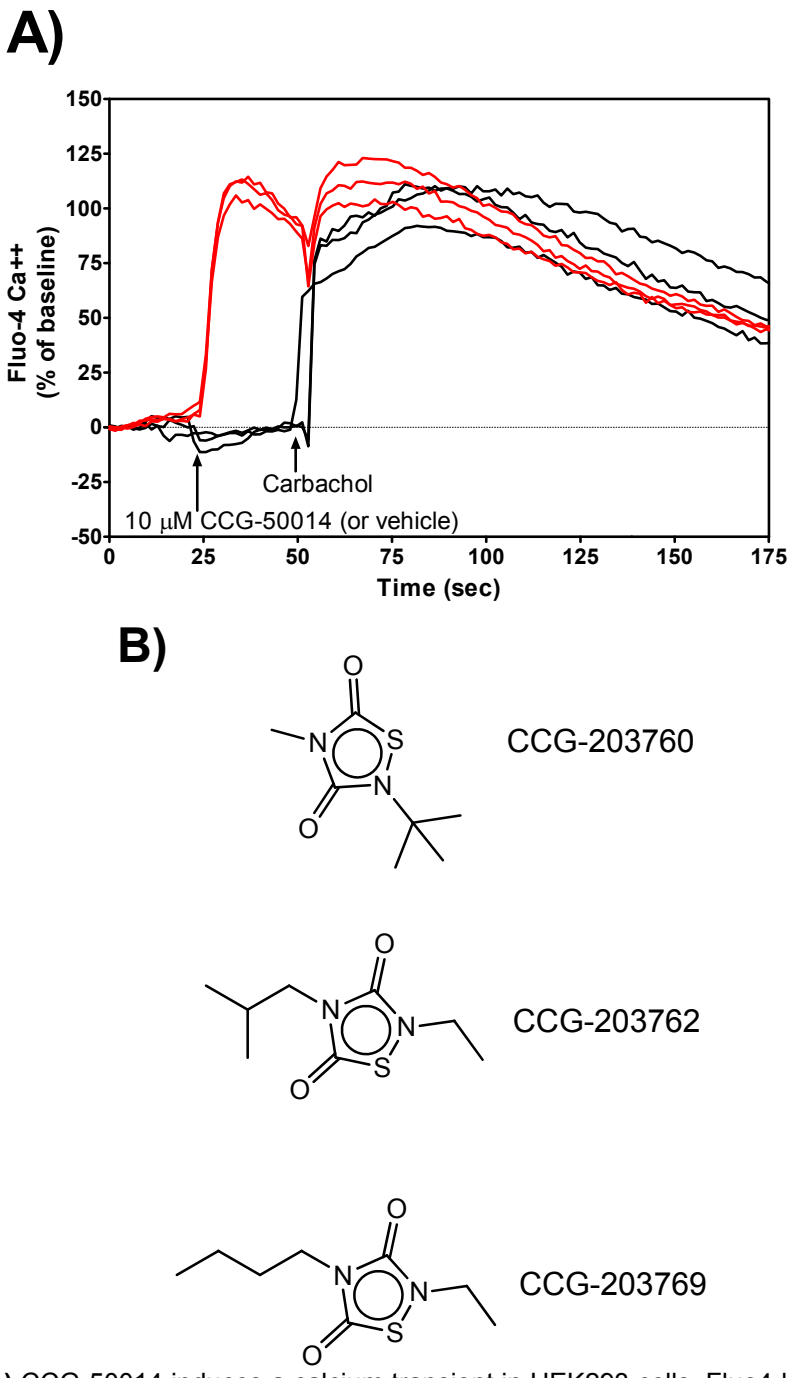


Figure 4.15 A) CCG-50014 induces a calcium transient in HEK293 cells. Fluo4-loaded cells were exposed to 10 μM CCG-50014 (red traces) or DMSO control (black traces) before an injection of 1 nM carbachol. CCG-50014 induced a calcium mobilization event on its own, suggesting that this compound has an as-of-yet unidentified off-target effect. Representative data shown from three wells for each condition. **B)** Chemical structures of the 3 compounds that did not show calcium mobilization effects and were more potent and soluble than CCG-50014.

The SAR analysis identified three compounds that have no ability to induce calcium mobilization that also have improved solubility and potency as compared to CCG-50014. These three compounds are CCG-203760, CCG-203762, and CCG-203769. Of these compounds, CCG-203769 displays most of the desired properties of an RGS inhibitor. The compound is highly soluble (>5mM, aqueous), potently inhibits RGS4 (IC50 14 nM), displays greater selectivity for RGS4 vs RGS8 than did other compounds, and did not induce a calcium transient in HEK-293 cells.

From these SAR studies, we have identified compounds that are more potent and selective than the parent compound (e.g. CCG-203731, CCG-203769) and have identified potential regions of the molecule (R2) that are important in driving RGS selectivity. It appears that an alkyl chain or a phenyl ring with a small substituent at the *para* position provide compounds with selectivity for RGS4. Also, we have identified compounds (e.g. CCG-203769) that have improved physicochemical properties, and minimal off-target calcium effects, thus facilitating future *in vivo* experiments which would have been hampered by solubility and off-target issues of CCG-50014.

CCG-50014 does not affect cell viability:

CCG-50014 is no more toxic than the vehicle (DMSO) control (**Fig. 4.3**). HEK293 cells were treated with CCG-50014 or vehicle controls in DMEM + 0.1% bovine serum albumin. After an overnight incubation at 37°C cells were analyzed for viability using the WST-1 reagent. At concentrations up to 100 μM, CCG-50014 did not reduce cell viability below control levels.

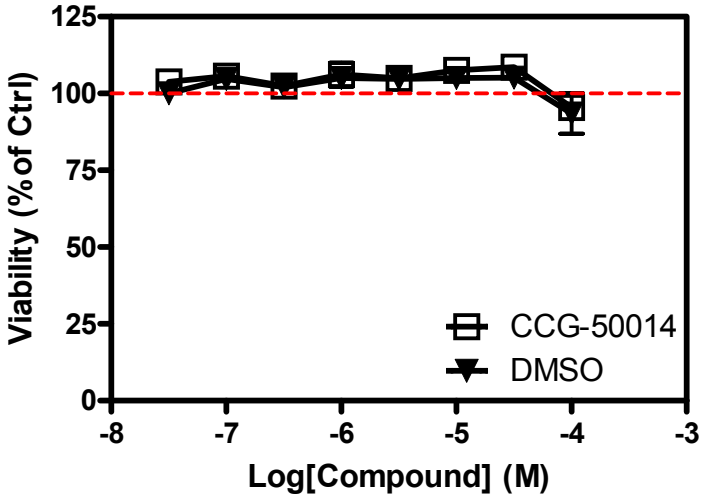


Figure 4.16 CCG-50014 does not affect HEK-293 viability. Cells were treated with CCG-50014 or vehicle control overnight and assayed for viability using WST-1. Data are presented as the mean±SEM from three independent experiments.

CCG-50014 and related analogs inhibit the RGS4/Gα_o interaction in living cells:

The members of the CCG-50014 class of compounds are potent inhibitors of the RGS4/Gα_o PPI with IC_{50} values in the 3-200 nM range *in vitro*. To determine if these compounds can also inhibit this PPI in living cells, I performed a series of experiments designed to monitor the subcellular localization of a green fluorescent protein (GFP)-tagged RGS4 that was transiently overexpressed in HEK-293T cells (**Fig. 4.4**). GFP-RGS4 is primarily expressed as a diffuse cytosolic protein (**Fig. 4.4A**). Upon co-transfection with either wild-type Gα_o or the constitutively active Gα_o (QL) mutant, GFP-RGS4 relocates to the plasma membrane. Co-expression with the RGS insensitive mutant Gα_o (G184S) did not cause membrane localization of GFP-RGS4. At this level of overexpression, the RGS4/Gα_o PPI is constitutive probably due to the rapid GDP exchange rate of Gα_o [9]. If cells expressing Gα_o and GFP-RGS4 are exposed to

CCG-50014, the membrane localization of GFP-RGS4 is reversed and the RGS protein translocates back into a cytosolic expression pattern (**Fig. 4.4D/E**). I also tested several analogs of CCG-50014 in this assay (including CCG-203769 and CCG-203757) and found similar results (**Appendix I**). To ensure that the N-terminal GFP tag was not affecting the assay, I repeated these experiments with a C-terminal GFP fusion of RGS4 and obtained similar results (**Appendix II**). This effect was observed for concentrations down to 1 μM CCG-50014. At high concentrations (100 μM), the effect was observed essentially instantaneously (<1 minute) and occurred within 10 minutes for lower concentrations (e.g. 10 μM). These results show that CCG-50014, CCG-203769, and other analogs can inhibit the RGS4/$G\alpha_o$ interaction in living cells.

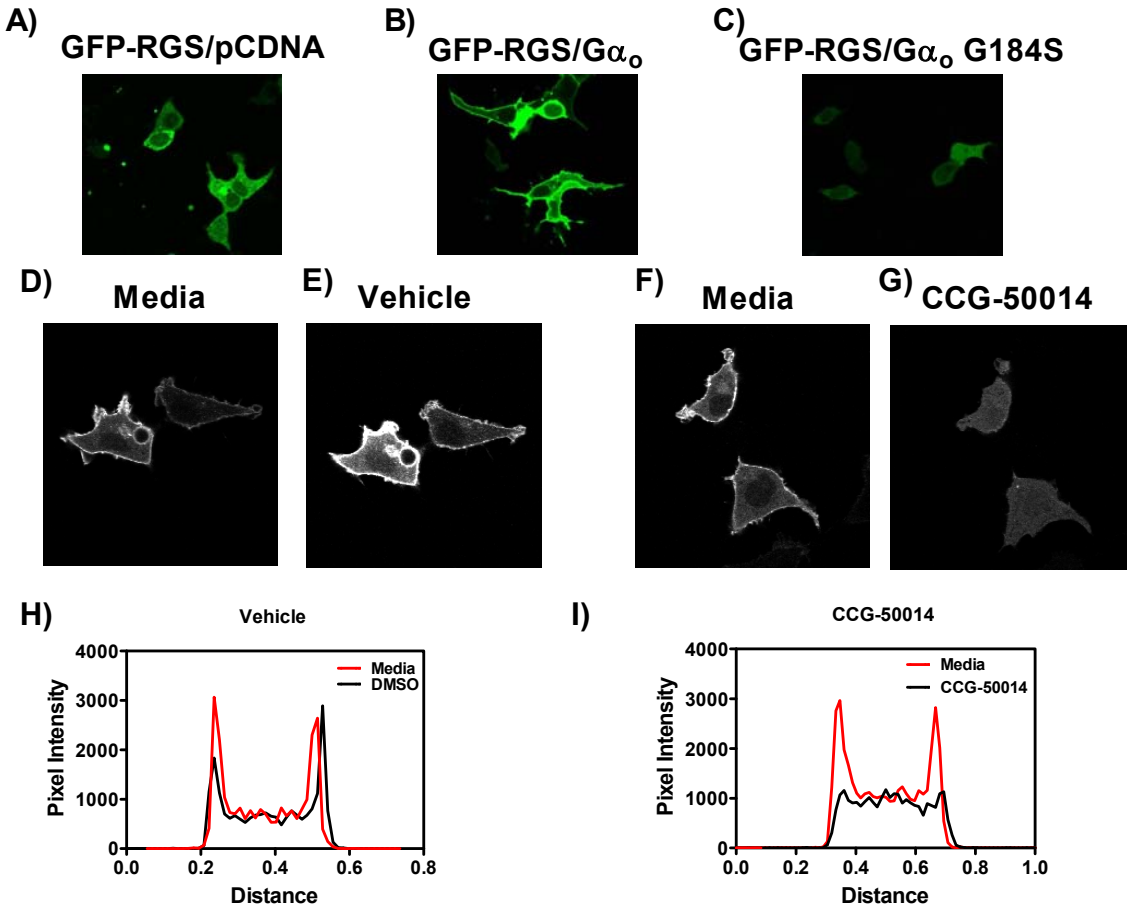


Figure 4.17 RGS4 is recruited to the plasma membrane by coexpression of $G\alpha_o$ and this recruitment is inhibited by CCG-50014. **A)** RGS4-GFP is expressed in a diffuse cytosolic pattern. **B)** When coexpressed with $G\alpha_o$, the RGS translocates to the plasma membrane. **C)** Coexpression with the RGS-insensitive $G\alpha_o$ mutant (G184S) does not induce this translocation. **D/E/H)** Cells coexpressing RGS4-GFP and $G\alpha_o$ show no change in the membrane localization of RGS4 after treatment with vehicle control (DMSO). **F/G/I)** CCG-50014 (100 μM) is able to reverse the $G\alpha_o$- induced RGS membrane translocation. Representative data shown from at least three independent experiments with 5-10 cells imaged per experiment. Line scans shown in H and I were quantified by drawing a line perpendicular to the long axis of the cell at identical sites in both pre/post treatment images and calculating pixel intensity using ImageJ.

CCG-50014 potentiates signaling through the δ-opioid receptor:

Wang et al. [10] previously showed that in the SH-SY5Y neuroblastoma cell line, siRNA knockdown of RGS4 selectively potentiates signaling through the δ-opioid receptor over the μ-opioid receptor. Since both receptors couple to the same G-proteins, the RGS4 selectivity observed in this system is particularly

useful in dissecting the compound's action. A low concentration (100 nM) of either the μ-opioid receptor agonist morphine or the δ-opioid receptor agonist SNC-80 produced a small (10-20%) inhibition of forskolin-stimulated adenylate cyclase activity. CCG-50014 (100 μM) significantly potentiated the effect of the δ-opioid agonist (44% inhibition vs 22% inhibition without the compound, $p<0.001$), as would be expected of an RGS4 inhibitor in this system (**Fig. 4.5**), with only a modest effect upon the μ-receptor signaling. The observed receptor selectivity of compound action is consistent with CCG-50014 acting by inhibiting RGS4.

The non-significant increase in μ-opioid signaling in response to compound should be addressed. It is possible that this effect is due to inhibition of other RGS proteins in the cell or because there is some low-level control of μ-opioid receptor signaling by RGS4. Regardless, there is significant potentiation of the δ-opioid signaling as would be expected of an RGS inhibitor.

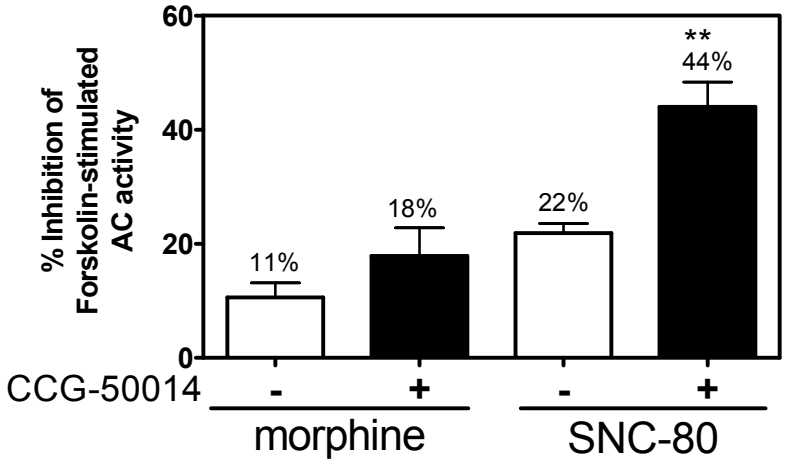


Figure 4.18 CCG-50014 potentiates the activity of the δ-opioid receptor ligand SNC-80 in SH-SY5Y cells. Inhibition of forskolin-activated adenylate cyclase activity by either the μ-opioid receptor or the δ-opioid receptor was measured in the absence and presence of 100 μM CCG-50014. Data are presented as the mean±SEM from three independent experiments. ** $P<0.001$

CCG-203769 potentiates the M3 muscarinic receptor activity via inhibition of RGS4:

To further probe the ability of the CCG-50014 class of compounds to inhibit RGS actions in a cellular setting, I performed a series of experiments using the engineered cell system expressing the M3 muscarinic receptor with RGS4 under doxycycline control. Upon addition of carbachol to these cells, a $G\alpha_q$-dependent intracellular calcium mobilization is initiated by the activation of phospholipase C. After induction of RGS4 expression, calcium signaling is suppressed by ~80%. A compound that inhibited RGS4 should relieve this suppression.

As noted above, when these experiments were performed with CCG-50014 it was found that the compound itself induced a sizable calcium transient. This off-target effect clearly makes it difficult to interpret data from these experiments. To circumvent this issue, I identified CCG-50014 analogs that were unable to induce a calcium flux in HEK-293 cells. One of these compounds, CCG-203769, is more potent at inhibiting RGS4 than CCG-50014 and is more soluble and was therefore chosen for study in this system.

Incubation with CCG-203769 for 15 minutes prior to carbachol stimulation resulted in a partial reversal of the RGS4-mediated inhibition (**Fig. 4.6**) without affecting M3-mediated calcium signal in the absence of RGS4 expression. This result further confirms this class of compounds is acting as an RGS4 inhibitor in living cells. Furthermore, the response occurs at micromolar concentrations, which is more in concordance with what is observed *in vitro*. This assay also

measures G_q signaling (as opposed to $G_{i/o}$ in the opioid studies), proving that the effect of this compound is not G-protein dependent.

The enhanced Ca^{++} signaling induced by CCG-203769 occurs at concentrations as low as 1 μM. While this compound was chosen for study because of its inability to stimulate calcium mobilization by itself, it is possible that a weak effect on calcium handling is starting to play a significant role over the time course of the experiment. It is also possible that at high concentrations of compound other, as of yet unknown off-target effects are occurring that disrupt calcium handling. Regardless, it does appear that CCG-203769 can inhibit RGS4 in living cells at concentrations that do not cause any measurable off-target effects.

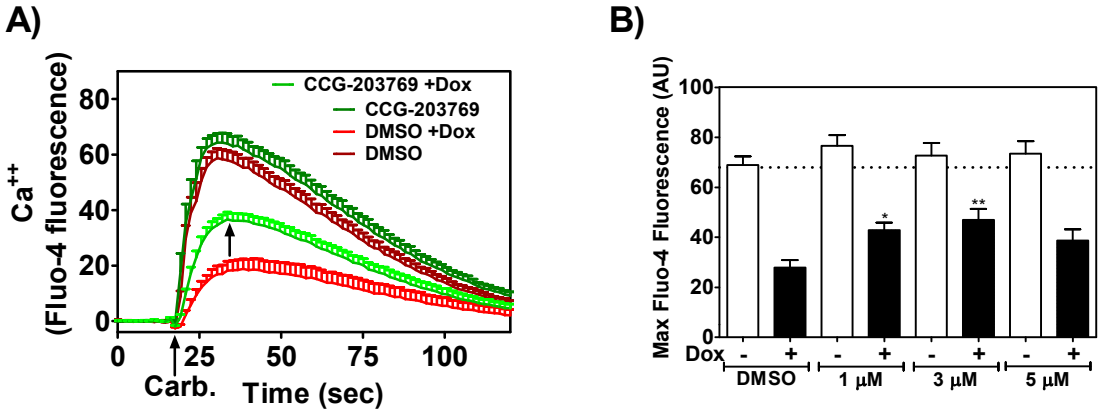


Figure 4.19 CCG-203769 partially reverses the RGS4-mediated suppression of carbachol responsiveness in HEK293 cells expressing the M3 muscarinic receptor. **A)** Representative traces of cells responding to 3 nM carbachol. Doxycycline (+Dox) treated cells express RGS4 and show a significant suppression of Ca^{2+} mobilization. Pretreatment of these cells for 15 minutes with 1 μM CCG-302769 (CCG-203769 +Dox) partially rescues the Ca^{2+} response. Data are presented as the mean±SEM of 12 wells per condition from a single experiment. **B)** Concentration dependence of the effect observed in A. Data are presented as the mean±SEM from three independent experiments. * P< 0.05; ** P < 0.01.

Discussion:

The development of RGS inhibitors has till now been dominated by compounds that lacked efficacy in a cellular environment. The reasons for this lack of cellular activity stem from: permeability issues (e.g. peptides and possibly the CCG-638x family), weak *in vitro* activity (e.g. CCG-638x family and CCG-4986), and inactivation in the presence of reductants (e.g. CCG-4986 and the CCG-50014 family). In this chapter I have presented the first evidence for a family of RGS inhibitors that functions in a cellular environment.

Early biochemical studies of this compound class suggested that it would likely lack cellular activity. As noted in Chapter III, this family of compounds is inactivated by reductants, including physiologically relevant concentrations of the intracellular reductant glutathione. Furthermore, the cysteine-dependent reactivity of the compounds raised concerns about target specificity and potential off-target effects. It was with these issues in mind that we attempted cellular studies with CCG-50014 and some selected analogs.

I developed a method to directly measure the $G\alpha_o$/RGS4 PPI in living cells. This approach allowed me to determine if my compounds can inhibit this PPI in living cells like they do *in vitro* (**Fig. 4.4A**). In this system, the RGS is expressed as a diffuse cytosolic protein until co-expression with $G\alpha_o$, which drives membrane association of RGS4. This co-localization is not induced by the co-expression of a mutant $G\alpha_o$ (G184S) that is insensitive to RGS GAP activity. The lack of interaction between the G184S $G\alpha_o$ mutant and RGS4 confirms the findings in cell and whole animal knock-in models [11-17]. CCG-50014 and CCG-

203769 are both able to inhibit the membrane localization of RGS4 in this assay, strongly suggesting that these compounds can inhibit the $G\alpha_o$/RGS4 interaction in living cells. These data lend considerable credence to the notion that the functional effects observed in the subsequent studies stem from an inhibition of one (or more) RGS proteins.

To further confirm that the compounds could function in a cellular setting, we performed a series of cellular studies to test the ability of these compounds to block the negative regulation of GPCR signaling by RGS proteins. Dr. Qin Wang, working in the laboratory of Dr. John Traynor, was able to show that CCG-50014 could potentiate the signaling through the δ-opioid receptor selectively over the μ-opioid receptor in SH-SY5Y cells. The signaling pathways used in this study are all endogenously expressed in SH-SY5Y cells and this result correlates well with their previous work showing RGS4 selectively inhibits the δ-opioid receptor [10]. These data are important because they show for the first time a small molecule RGS inhibitor having a functional effect on an endogenous signaling pathway.

While it is important to show that the compound can function on endogenous signaling pathways, I wanted to also probe the actions of this compound in a more controlled manner. I tested several compounds using cells stably expressing the muscarinic M3 receptor with RGS4 expression under doxycycline control. By measuring the G_q-dependent calcium mobilization induced by the M3 receptor, I was able to show that CCG-230769 partially inhibits the effects of RGS4. CCG-50014, however, induced a calcium transient

on its own (**Fig 4.2A**). These studies were important for two reasons: 1) it showed that the compound inhibits RGS4 under more controlled conditions than the SH-SY5Y experiments; and 2) these experiments revealed a previously unknown off-target effect elicited by some members of the CCG-50014 family.

I undertook a series of experiments to probe the chemical space around the CCG-50014 scaffold. By analyzing ~80 analogs of CCG-50014 for a variety of parameters including potency, RGS selectivity, and solubility, I was able to identify a number of structure-activity relationships around this scaffold. Potency against RGS proteins can be improved by shortening the side chains (R1/R2 in **Table 4.1**) to small alkyl chains. This also corresponded to an increase in RGS4 selectivity, although some analogs with phenyl rings at the R1/R2 position that have a small substituent in the *para* position on the R2 phenyl ring (e.g. CCG-203702) also display prominent selectivity for RGS4 over RGS8. Modification of the thiadiazolidine ring to a less reactive center drastically reduces activity.

This SAR analysis also provided a means to identify compounds that did not produce the Ca^{2+} mobilization off-target effect noted for CCG-50014. I identified three compounds (**Fig. 4.2B**) that potently inhibit RGS4 yet lack the ability to induce a Ca^{2+} mobilization. Study of the SAR landscape surrounding CCG-50014 has yielded several compounds with improved properties and *in vitro* activity. These compounds, including CCG-203769, are currently being used in isolated organ and whole animal studies to determine their physiological effects on RGS activity and GPCR signaling.

There are at least two advantages that the CCG-50014 class of molecules has over previous generations of RGS inhibitors. The first – and presumably most important – is potency. CCG-50014 is nearly 3 orders of magnitude more potent at inhibiting RGS4 than any of the other small molecule RGS inhibitors described to date. Therefore, even if a substantial fraction of the compound is rapidly metabolized or reacts with glutathione (or other non-target thiols), an active concentration of compound is likely to be present. Another set of advantages this compound has over prior generations of RGS inhibitors (especially CCG-63802) is improved cellular permeability and aqueous solubility. While solubility is variant across this family of compounds, we have identified several that have aqueous solubility >5 mM yet still retain cellular activity. These factors allow us generate pharmacologically relevant concentrations of compound in our assay systems without interfering artifacts such as compound precipitation.

The CCG-50014 class of compounds contains the most potent RGS inhibitors identified to date and the first examples of small molecules that can inhibit the cellular activity of an RGS protein. While this is a significant step forward in the development of small molecule RGS inhibitors, the true value of this compound has yet to be fully elucidated. One major concern that has not been addressed is the pharmacokinetic parameters of these compounds. Furthermore, we have yet to fully determine the physiological effects of systemic administration of CCG-50014 or related analogs. Preliminary data has shown no gross physiological/toxicological effects to low doses of CCG-203769, however,

the appropriate full-scale studies have yet to be performed. Also, the RGS specificity of CCG-203769 has yet to be determined in a cellular setting. Regardless, this family of compounds provides a foothold for development of new RGS inhibitors.

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Chapter V:
Conclusions

Summary of Results:

The work presented in this thesis has significantly advanced our understanding of how to target RGS proteins with small molecules. The major advances that are described in this thesis are 1) the first examples of reversible small molecule RGS inhibitors; 2) characterization of the most potent RGS inhibitors identified to date; and 3) the first example of small molecule RGS inhibitors with cellular activity. The information in this thesis provides both novel methodology and direction for the development of future RGS inhibitors.

In Chapter I, I describe the first set of compounds that can reversibly inhibit RGS4. The prototypical compound from this class, CCG-63802, inhibits RGS4 with an *in vitro* IC_{50} value of 10 micromolar in the FCPIA assay. This family of compounds provided the first proof-of-concept that RGS proteins can be inhibited by small molecules in a reversible fashion. While CCG-63802 may not be the optimal lead for cellular/animal studies, it is nonetheless an important step forward in the development of small molecules targeting RGS proteins.

Chapter II detailed the molecular mechanism of the most potent small molecule RGS inhibitor, CCG-50014. This compound irreversibly inhibits RGS4 with a 30 nM IC_{50} value and has significant selectivity for RGS4 over RGS proteins including RGS7, RGS8, RGS16 and RGS19. While irreversible, this

compound provides several advantages for studying the molecular mechanism of RGS allosteric inhibition.

In Chapter IV I further develop the CCG-50014 class of compounds to show, for the first time, small molecules that can inhibit both the RGS/Gα PPI and RGS activity in a living cell. To our knowledge, this is the first time that a small molecule RGS inhibitor has been shown to directly inhibit the RGS/Gα interaction in a cellular setting. Second generation members from this family, including CCG-203769, are also described. These compounds retain the RGS inhibitory of CCG-50014, but have improved aqueous solubility and were selected for the inability to invoke a calcium transient. The data presented in Chapter IV clearly show that this class of small molecules can inhibit the RGS/Gα PPI in a manner that has a measurable effect upon GPCR signaling. This is a dramatic step forward in the development of small molecule RGS inhibitors and it is hoped that, with continued development, descendents from this class of compounds will become useful research tools and potentially even clinically relevant therapeutics.

Future Research Directions:

The work presented in this thesis opens many potential avenues for further research and development of small molecule RGS inhibitors. The novel methodology described throughout this thesis (TR-FRET high throughput screening, RGS/Gα thermal stability assays, RGS membrane translocation, etc.) provides a framework against which novel RGS inhibitor scaffolds can be tested. Furthermore, the compound classes that have been expounded upon in this

thesis provide the opportunity to both learn more about small molecule binding sites on RGS and also two novel scaffolds for the development of future generations of RGS inhibitors.

The CCG-63802 class of molecules may be able to provide structural information regarding the non-covalent binding of small molecules to RGS. It is possible that many of the small fragments of CCG-63802 that lack RGS inhibitory activity might weakly bind to the protein in a manner that does not produce a functional effect. By performing nuclear magnetic resonance experiments with isotopically labeled RGS4, we should be able to map the binding sites of these fragments on the RGS. Similar studies have been performed before using a variety of different proteins [19-24]. Indeed, experiments along these lines are already in progress by a graduate student in our lab, Andrew Storaska.

Based upon mutagenesis data, it is expected that the mechanism of action of CCG-63802 will primarily be through a conformational change induced by compound binding to the allosteric “B” site on RGS4. The B site is the location on RGS4 that is important for the binding of calmodulin and acidic phospholipids. Previous work by Ishii and colleagues has shown that these two molecules can reciprocally regulate RGS GAP activity, whereby phospholipid binding inhibit the RGS function and this effect can be displaced by calmodulin binding [25, 26]. It is possible that CCG-638002 mimics the effects of acidic phospholipids on RGS4 GAP activity. While technically challenging, it would be very interesting to test this hypothesis by using a series of protection or displacement studies to determine if calmodulin or acidic phospholipids can affect CCG-63802 binding.

Similar experiments could test any RGS inhibitors that are expected to bind via that “B” site (e.g. CCG-50014).

One of the main motivations for the development of small molecule modulators of RGS activity is so that we can better understand the molecular mechanisms behind RGS activity and regulation. While the “B” site hypothesis has been studied from a variety of functional angles, to date there are no data that specifically address how this allosteric binding results in altered GAP activity. This is likely due in part to the challenging nature of performing biophysical studies on proteins in the presence of phospholipids. It is possible that a small molecule that binds to the “B” site to inhibit RGS function could provide insights into the conformational changes that occur during this allosteric regulation.

While initial studies have not been particularly promising, it might also be useful to test selected analogs of CCG-63802 in cellular assays. For these compounds to have significant cellular activity, it is likely that a new analog with the following properties will have to be generated. First, the compound needs to have a greater potency at RGS4. Most compounds identified in this class possess micromolar IC_{50} values *in vitro*, which is likely not potent enough for *in vivo* studies. Ideally, the candidate compound would be >2 Log more potent than the CCG-63802. It would also be beneficial if the compound possessed a smaller polar surface area than CCG-63802 to improve membrane permeability. If we can identify a soluble, stable compound that meets these two conditions, it is likely that it would have cellular activity.

The CCG-50014 class of compounds has already progressed to the point of containing members with documented cellular activity. For this class of compounds, it is most important to focus upon transitioning from biochemical/cellular assays to isolated organ/whole animal experimentation. Several of these studies are currently underway. Through collaboration, we have started testing CCG50014 and CCG-203769 in the langendorff isolated heart model to determine if the compounds can potentiate carbachol-stimulated bradycardia. It is known that RGS4 is expressed in sinoatrial nodal cells, where parasympathetic M2 muscarinic receptor activation slows the heart. We are also approaching this same system in a whole-rat model. Sprague-Dawley rats are implanted with indwelling venous catheters and telemetry probes that monitor heart rate, blood pressure and body temperature. After recovery, these animals are infused with a dose of CCG-203769 and then given a small dose of carbachol to induce a transient bradycardia. It is expected that if the compounds inhibit RGS in the SA node, the carbachol effect will be potentiated. Currently, we have not observed any significant physiological effects with CCG-203769, but the studies are not complete and due to solubilization issues, we have not been able to inject the animals with as large of doses as we would like. Currently, Dr. Stephen Husbands is producing analogs of CCG-50014 that will likely be much more soluble in aqueous environments.

Also through collaboration, we are testing the ability of these compounds to potentiate the activity of the M3 muscarinic receptor in pancreatic islet β cells. In β cells, M3 activation potentiates glucose-stimulated insulin release [27]. Wess

and colleagues showed that RGS4 potently regulates the M3 activity in β cells [28]. Using isolated mouse islets, we have been attempting to determine if CCG-50014 or CCG-203769 can accentuate the M3 activity on glucose-stimulated insulin release. Preliminary data from these experiments have been promising and we are in the process of developing protocols to test the glucose tolerance of animals that were treated with CCG-50014 analogs.

While important, the animal studies need to be accompanied by a full pharmacokinetic profile of the compound before any conclusions regarding its usefulness as a potential therapeutic may be drawn. We, in collaboration, are currently in the process of developing a method to detect the CCG-50014 analogs in whole blood and isolated tissues. With this methodology in hand, we will be able to perform single dose pharmacokinetic profiles of important CCG-50014 analogs. These data will be critical for the animal studies to ensure that we are reaching and maintaining appropriate blood and tissue concentrations.

The CCG-63802 and CCG-50014 classes of molecules have provided significant advances to the field of RGS inhibitors. Both of these families also have the potential to provide further knowledge into the location and geometry of small molecule binding sites on RGS4. Furthermore, they may function as pharmacological tools to study the effects of RGS proteins *in vivo*. It is anticipated that these molecules will provide many new discoveries in the fields of RGS biology and small molecule protein-protein interaction inhibitors.

Therapeutic applications of RGS modulation:

Modulating RGS activity could have a variety of potential therapeutic applications. While clearly much work needs to be done to generate a drug candidate targeting an RGS protein, the potential benefit of such a drug in several disease states could be significant.

Recent work has shown that RGS4 negatively modulates the parasympathetic stimulation of insulin release [28]. The cholinergic stimulation of β cells does not directly cause a release of insulin, but it does potentiate the responsiveness of these cells to high blood glucose. In this setting, a selective RGS4 inhibitor would be expected to potentiate the autonomic stimulation of insulin release in a glucose-dependent manner. This could be a very beneficial mechanism for treatment of early-stage type II diabetes and could require much less monitoring than conventional insulin replacement therapies.

Another potential application for an RGS inhibitor is in the treatment of depression. Traynor and colleagues have shown that the pan-inhibition of RGS activity on $G\alpha_{i2}$ produces a striking antidepressant-like effect [18]. While we still do not know which RGS protein(s) to target for antidepression therapy, these results suggest that subtle modulation of endogenous neurotransmitter signaling might provide an effective treatment for depression and potentially other neurological disorders. I find this approach to be particularly elegant, especially when compared to the commonly used drugs to treat neurological disorders – many of which affect many different signaling systems in ways that we still do not fully understand [29-31].

Another potential target is RGS9-2, the brain specific isoform of RGS9. This molecule, is strongly and specifically expressed in certain parts of the brain, including the striatum and nucleus accumbens [32]. Knockout of RGS9 increases supra-spinal and spinal cord mediated analgesia and delays the development of tolerance to morphine [33]. Small molecules specifically targeting RGS9 may provide a mechanism to potentiate morphine analgesia under conditions that may slow the development of tolerance.

Unfortunately, the application of RGS9 inhibitors might be expected to have a variety of undesired side effects. An isoform of RGS9, RGS9-1, is the major RGS controlling phototransduction [34]. Unless the compound is pharmacokinetically excluded from the eyes or is exquisitely specific for RGS9-2 over RGS9-1, vision problems (likely trailing images) may occur. Furthermore, RGS9-2 knockout mice are sensitized to the dyskinesias associated with activation of D2 dopamine receptor after prolonged pharmacological dopamine receptor antagonism [35]. These potential issues will have to be addressed before inhibitors of RGS9 will be adopted as viable drug candidates.

While the work in this thesis has focused upon developing inhibitors of RGS function, there are several situations in which an activator of RGS activity could provide significant therapeutic benefit. Gold and colleagues have shown that overexpression of RGS9-2 in the striatum of non-human primates can suppress the dyskinesias associated with chronic L-dopa treatment [36]. These data suggest that potentiating RGS9-2 expression in the striatum or

pharmacological activation of RGS9-2 in patients with Parkinson's disease may provide a level of relief from the side effects of L-dopa treatment.

Modulating RGS proteins with small molecules could provide significant therapeutic benefit. RGS modulation could provide a mechanism to increase the efficacy of endogenous signaling in the body, something that current GPCR-targeted approaches often cannot attain. Furthermore, the unique expression patterns of RGS proteins may provide a level of tissue specificity not achievable with GPCR agonists/antagonists. It is hoped that future generations of RGS inhibitors will provide therapeutic benefit to mankind.

The Future of Small Molecule Protein-Protein Interaction Inhibitors:

It is clear that substantial challenges lie ahead in the development of PPI modulators. Continued progress is being made in the cancer arena where the requirements of oral absorption and/or blood-brain-barrier penetration are less critical. Indeed, the affinity of PPIIs in the cancer field has increased (to IC_{50} values <10 nM *in vitro* and 10s-100s of nM in cells) and the molecular weight of these PPIIs has decreased over the last 10 years [37, 38]. That work and continued academic efforts to develop tool compounds blocking PPIs should advance this field substantially. The need to maintain small size and appropriate physicochemical properties of compounds may require novel approaches. Rather than targeting the immediate PPI site, the identifying and targeting of allosteric sites on the target protein may permit the use of more suitable chemical structures. In the cancer drug design arena, fragment-based design of PPI

inhibitors has proven useful so applications to other targets should be pursued. Also, identifying compounds that could make use of cellular uptake mechanisms could permit compounds to achieve higher intracellular concentrations and result in additional cell-type specificity of action.

There are a large number of potential PPI targets that could provide subtle modulation of cellular processes if a successful drug could be developed. As with the history of protein kinase inhibitors, overcoming initial reluctance to embrace the concept will likely require a success story. At present, it is hard to predict a major breakthrough in this field but continued refinement of existing approaches and further development of existing targets is likely to reach a threshold of success in the not too distant future. In the meantime, substantial genetic and biological studies will continue to define novel PPI targets. With the rapid advances in target identification, an increased pace of chemical discovery related to PPIs will be critical to exploit the potential of this novel field.

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Appendix I:
CCG-50014 Analogs Inhibit The RGS4-Gα$_o$ Protein-Protein Interaction in Living Cells

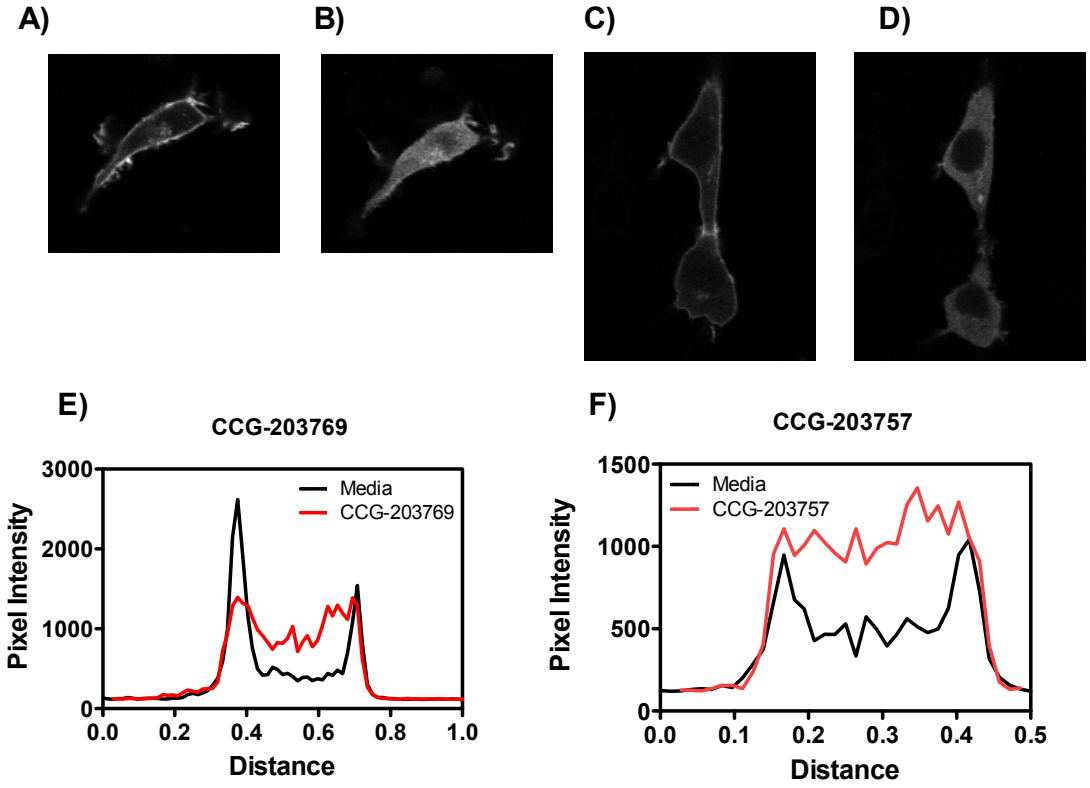

Figure A.1 CCG-203769 and CCG-203757 can inhibit the Gα$_o$-dependent RGS4 membrane translocation. **A)** A representative image of an HEK293T cell expressing RGS4-GFP and Gα$_o$. **B)** The same cell, after addition of 100 μM of CCG-203769. **C)** A representative image of HEK293T cells expressing RGS4-GFP and Gα$_o$. **D)** The same cells, after incubation with CCG-203757. **E)** Quantification (line scans) of the images in A/B. **F)** Quantification (line scans) of the images in C/D.

Appendix II:
Tag Localization on RGS4 Does Not Effect Translocation or Sensitivity to CCG-203769

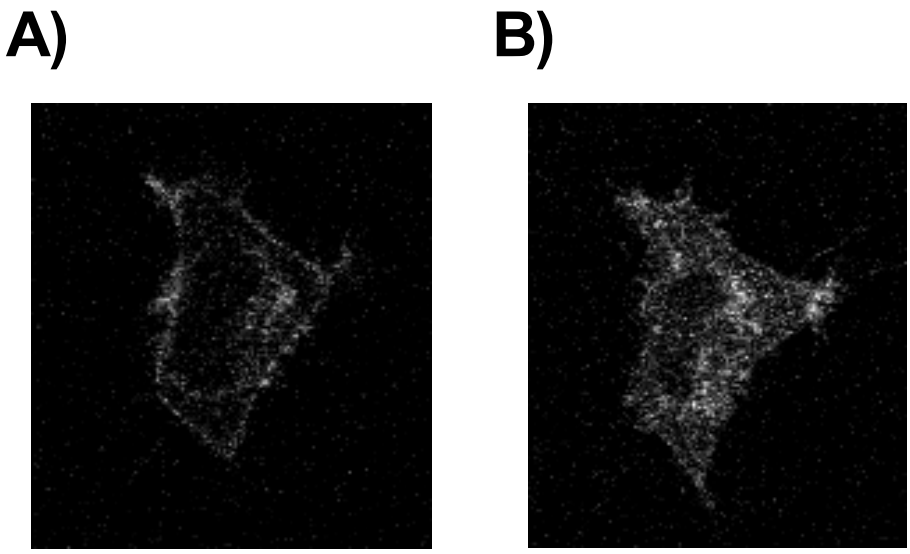

Figure A.2 Location of the GFP tag on RGS4 does not affect its sensitivity to CCG-203769. **A)** RGS4 was expressed with a C-terminal GFP tag and is membrane localized when co-expressed with $G\alpha_o$. **B)** After incubation with 100 μM CCG-203769, RGS4-GFP translocates to the cytosol. Images here are of poorer quality than the other confocal experiments shown because this particular construct was weakly expressed in HEK-293T cells. Representative data shown.