Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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Evaluation of the Selectivity and Cysteine-Dependence of Inhibitors Across the Regulator of G Protein Signaling Family

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Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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Running title: Selectivity and Cysteine-Dependence of RGS Inhibitors

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Nonstandard Abbreviations: CCG (Center for Chemical Genomics); CID (Compound identifier); Gα (Guanine nucleotide binding protein, α subunit); GoLoco ($G\alpha_{i/o}$-Loco domain); GAP (GTPase Accelerating Proteins); GDI (Guanine nucleotide dissociation inhibitor); GPCR (G Protein coupled receptor); IMAC (Immobilized metal affinity chromatography); NEM (N-ethyl maleimide); PPI (Protein-protein interaction); RGS (Regulator of G Protein Signaling Protein); RH (RGS homology domain)

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Abstract

Since their discovery over 20 years ago, Regulators of G Protein Signaling (RGS) proteins have received considerable attention as potential drug targets due to their ability to modulate Gα activity. Efforts to identify small molecules capable of inhibiting the protein-protein interaction between activated Gα subunits and RGS proteins have yielded a substantial number of inhibitors, especially towards the well-studied RGS4. These efforts also identified that many of these small molecules inhibit the protein-protein interaction through covalent modification of cysteine residues within the RGS domain that are located distal to the Gα-binding interface. As some of these cysteine residues are highly conserved within the RGS family, many of these inhibitors display activity towards multiple RGS family members. In this work we sought to determine the selectivity of these small molecule inhibitors against 12 RGS proteins, as well as against the cysteine-null mutants for 10 of these proteins. Using both biochemical and cell-based methods to assess Gα-RGS complex formation and Gα enzymatic activity, we found that a number of previously identified RGS4 inhibitors were active against other RGS members, such as RGS14, with comparable or greater potency. Additionally, for every compound tested, activity was dependent upon the presence of cysteine residues. This work defines the selectivity of commercially available RGS inhibitors and provides insight into the RGS family members for which drug discovery efforts may be most likely to succeed.

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Introduction

The Regulators of G Protein Signaling (RGS) family, comprised of approximately 20 members, terminates Gα signal transduction through their activity as GTPase accelerating proteins (GAP) for activated Gα subunits. This GAP activity is attributed to an approximately 120 amino acid region common to all RGS proteins, aptly termed the RGS Homology (RH) domain, which directly engages GTP-bound Gα. Since their discovery over 20 years ago, RGS proteins have been considered attractive, but challenging, drug targets due to their ability to modulate signaling cascades occurring through a myriad of G protein-coupled receptors (GPCR), including D2 dopamine (Ghavami et al., 2004; Mao et al., 2004; Rahman et al., 2003), μ opioid (Clark et al., 2003; Psifogeorgou et al., 2011; Talbot et al., 2010b; Wang et al., 2009), 5-HT1A serotonin (Ghavami et al., 2004; Talbot et al., 2010a; Wang et al., 2014), and AT1 angiotensin (Matsuzaki et al., 2011; Wang et al., 2002), to name just a few. Because of their effects on signaling downstream of various GPCRs, RGS proteins have been implicated in a number of disease states, ranging from cancer to disorders of the CNS. Several reviews have focused on disease states in which modulation of RGS function could be therapeutically advantageous (Hurst and Hooks, 2009; Roman and Traynor, 2011; Sjogren, 2011).

Due to the potentially beneficial effects of modulating RGS activity, considerable effort has been devoted to identifying inhibitors, particularly of RGS4, one of the most-studied and perhaps best understood member of this protein family. Some of the earliest efforts focused on designing cyclic peptides that mimic the regions of $G\alpha_i$ that bind RGS4, leading to the discovery of the competitive peptide inhibitor YJ34 (Jin et al.,

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2004). This work further led to the discovery of 5nd, a peptide that covalently modified RGS4 cys residues (Roof et al., 2009), a theme that would become common among RGS4 inhibitors.

As the development of peptides into bona fide drugs is challenging, subsequent efforts focused on using high throughput biochemical screens to identify small molecule inhibitors of RGS4, the first of which was CCG-4986 (Roman et al., 2007). Follow-up work identified that this compound also was a covalent cys modifier (Kimple et al., 2007), albeit through binding to an allosteric site located distally to the Gα binding face of RGS4 (Roman et al., 2010). In fact, four other RGS4 inhibitors, CCG-63802, CCG-63808, CCG-50014, CCG-55919 were found to either interact with cys residues in this same region or exhibit cys -dependent inhibition (Blazer et al., 2010; Blazer et al., 2011; Roman et al., 2009). Previous work by our group identified UI-5 and UI-1590 as RGS4 inhibitors, both of which show lower potency against cys -null RGS4, pointing to a cys -dependent mechanism (Monroy et al., 2013). In addition to the biochemical screening methods that generally measure Gα-RGS binding or RGS-mediated GAP activity, a recent cell-based screen that measured RGS4's ability to regulate M3 muscarinic receptor activity identified a number of inhibitors, including 6018993, 1777233, 1911669, 6386479, 5428579, and 1472216 (compound identifier, CID) (Storaska et al., 2013). Though none of these compounds showed activity against cys -null RGS4, 177233 and 5428579 exhibited reversible binding. The chemical structures of all small molecules described above and the references to their discoveries are shown in Table 1.

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The RH domain is composed of nine α-helices arranged into two sub-domains. The terminal sub-domain in made up of helices 1-3, 8, and 9, and contains both the N- and C-termini. The bundle subdomain contains helices 4-7, arranged in an anti-parallel bundle. The RH domains tested here contain between zero (RGS6 and RGS7) and four (RGS2 and RGS4) cys residues, and a sequence alignment of the RH domains with cys residues highlighted is shown in Figure 1. Notably the cys residue located at position 95 (Highlighted red, Figure 1B) in RGS4 is conserved except for RGS6 and RGS7, where a Val residue is found. The cys residue at RGS4 position 148 (highlighted purple, Figure 1B) is somewhat conserved, as it is found in members RGS1, RGS2, RGS4, RGS8, and RGS16.

As different RGS proteins are involved in different signaling pathways and therefore different physiological processes as well as potential disease states, we explored the RGS RH domain selectivity profile of previously identified RGS4 inhibitors and the reactivity of the cys residues located in this domain. Given the knowledge that nearly every known small molecule inhibitor of RGS4 either covalently modifies cys residues or displays cys -dependent activity, we also tested each compound against a cys -null (cys to ala) mutant for every RGS tested.

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Materials and Methods

Compounds

RGS inhibiting compounds were obtained from commercial sources at indicated purity as follows: 6018993 and 1777223, both ≥95% purity (Vitas M Labs, Champaign, IL); 6383479, ≥90% purity (Enamine, Kiev, Ukraine); CCG-50014, >97% (Selleck, Houston, TX); CCG-63802, ≥98% (Sigma-Aldrich, St. Louis, MO); UI-5 and UI-1590, both ≥95% (Microsource, Gaylordsville, CT), 5428579 and CCG-4986, both ≥95% (Chembridge, San Diego, CA); CCG-63808, ≥97% (MedChem Express, Monmouth Junction, NJ); N-ethyl maleimide, ≥98% (MP Biomedicals, Santa Ana, CA); DACM, ≥95% (Anaspec, Fremont, CA); 1472216, 98% (Key Organics, Bedford, MA); 1911669, ≥92% (Pharmeks, Moscow, Russia); CCG-55919, ≥90% (Maybridge, Thermo Fisher,Waltham, MA).

Plasmid Construction

WT-RGS protein constructs were a gift from Nicola Burgess-Brown [Addgene plasmid # 39143 (RGS1), 38812 (RGS2), 38932 (RGS6) 38813 (RGS7), 38805 (RGS8), 39138 (RGS10), 39139 (RGS14), 39140 (RGS16), 39141 (RGS17), 39142 (RGS18)]. Human RGS5 residues 52-185 and rat RGS4 residues 51-179 were cloned into pNIC-Bsa4 (Addgene #26103, gift from Opher Gileadi), as previously described (Savitsky et al., 2010). Cys-null mutants were obtained by purchasing G Blocks (Integrated DNA Technologies, Coralville, IA) containing RGS coding sequences with all cys mutated to Ala (GCG codon), which were then cloned into pNIC-Bsa4 (Addgene #26103), as previously described (Savitsky et al., 2010). All RGS constructs were designed to code for the RGS RH domains with an N-terminal TEV protease-cleavable 6X-His tag.

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Protein Purification

All RGS proteins were transformed into BL21-CodonPlus(DE3)-RIPL cells, and colonies were selected and grown at 37°C in Terrific Broth until an OD_{600} of 2.0, induced with 1mM IPTG, and grown for 16 hours at 18°C while shaking at 275-300 rpm. Cells were pelleted, resusupended in 50 mM HEPES, 500 mM NaCl, 1 mM β-ME, 10 mM imidazole pH 8 (Buffer A), lysed with lysozyme and DNAse I, and subjected to multiple freeze-thaw cycles in liquid N_2. Lysates were clarified by centrifugation at 100,000*g*, supernatant separated from insoluble pellet, and supernatant subjected to immobilized metal affinity chromatography (IMAC) (Ni Sepharose 6 Fast Flow, GE Healthcare, Chicago, IL). Fractions containing RGS protein were then treated with His-tagged TEV protease and dialyzed overnight at 4°C against Buffer A to cleave 6X-His tag. Samples were again subjected to IMAC and flow through collected, resulting in >90-95% purity as determined using SDS-PAGE (Supplemental Figure S1). Rate-altered mutant $hG\alpha_{i1}$ (R178M, A326S) and $rG\alpha_o$-GST were purified as previously described (Monroy et al., 2013).

Chemical Biotinylation of RGS RH domains

RGS proteins were biotinylated using EZ-link NHS-Biotin (Thermo Scientific, Waltham, MA) per manufacturer protocol with either 10:1, 5:1, or 3:1 molar excess biotin reagent and incubated on ice for two hours. Coupling reaction was quenched with the addition of 5X molar excess glycine, and RGS proteins were dialyzed overnight against Buffer A to remove excess glycine and biotin.

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Alpha Screen PPI assay

Biotinylated RGS was conjugated to streptavidin coated donor bead at 9X desired protein concentration and 135 ng/μL bead in Assay Buffer (20 mM HEPES, 100 mM NaCl, 1% BSA, 1% Lubrol, pH 8) on ice. After 30 minutes of conjugation the RGS/bead mixture was diluted to 3X desired concentration and added to 384-well plates (Corning 3824, Corning, NY) containing 3X concentration compound and incubated at RT. Separately, GST-$G\alpha_o$ was conjugated to anti-GST acceptor beads at 90 nM protein and 135 ng/μl bead in assay buffer on ice. After 20 minutes of conjugation, a portion of the G protein/bead mixture was removed and diluted to 30 nM protein in assay buffer. This represents the negative control. The remaining G protein/bead mixture was diluted to 30 nM in assay buffer supplemented with 5 mM NaF, 5 mM $MgCl_2$, 5 μM $AlCl_3$, and 2.5 mM GDP. This mixture was incubated for an additional 10 minutes on ice before being added to the appropriate wells of the assay plate. Final concentrations were 1X desired for RGS, 10 nM for $G\alpha_o$, and 15 ng/μl for each bead. Assay was incubated for one hour at room temp prior to measurement on an Envision plate reader (Perkin Elmer, Waltham, MA). All WT-RGS proteins were tested at 10 nM final. Cys-null RGS1 and RGS14 were tested at 31.6 and 100 nM, respectively, as these concentrations were necessary to achieve an acceptable difference between positive and negative control wells (data not shown).

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RGS-Gα_{i1} GAP Activity Assay (Malachite Green Phosphate Detection)

Malachite Green free phosphate detection assay was performed largely as described previously (Monroy et al., 2013). For assessment of RGS protein activity, RGS protein was diluted in half log serial dilutions concentrations ranging from 3.16 μM to 3.16 nM. For compound dose response experiments, RGS protein concentration was normalized to each RGS protein's respective EC_{80}, as determined above.

WT-RGS Cysteine DACM Reactivity

Proteins were exhaustively dialyzed against 50 mM HEPES 500 mM NaCl at the indicated pH in order to remove β-ME, which can directly interact with DACM. 20 μL of 2 μM RGS protein was then added to black 384-well plate (Corning 3575, Corning, NY), followed by 20 μL of 2 μM DACM (Anaspec, Fremont, CA), and fluorescence intensity continuously monitored on Envision plate reader (Perkin Elmer, Waltham, MA) for 1500 seconds with excitation and emission wavelengths of 385 and 440 nm, respectively.

NanoBit protein complementation assay

The NanoBit complementation assay was performed as previously described, with minor modifications as described here. (Bodle et al., 2017). NanoGlo Live Cell Reagent was prepared as a 5X stock and added to wells of the assay plate at 10 μl per well. Baseline was established for 30 minutes, after which vehicle or AlF_4 (stock solution: 40 mM NaF, 500 μM $AlCl_3$) was added to wells at 10 μl per well and assay plate was read for an additional 30 minutes. A 4X stock of compound or DMSO was added to

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appropriate wells and plate was then read for an additional hour. All reads on a Synergy 2 plate reader (BioTek, Winooski, VT) at 37°C.

Data Analysis

All data were analyzed using Prism 7 (GraphPad, San Diego, CA). For Alpha Screen and malachite green experiments, IC_{50} values were generated on the combined data set. The mean value of duplicate wells for each independent experiment was determined, and these values from $n \geq 3$ independent experiments were combined in a single table to generate the mean ± SD values reported. Concentration-response curves were generated by a single fit on the combined mean ± SD data using log(inhibitor) vs. response, variable slope (four parameter) fit. Data are normalized such that the absence of inhibitor was set to 100% in each assay. For AlphaScreen, 0% was normalized to the absence of GDP-AlF_4, which is the negative control for PPI formation. Absence of RGS represented the negative control and the 0% normalization for malachite green GAP activity. Following normalization, the top and bottom of each curve was constrained to 100% and 0% respectively. IC_{50} values were calculated in Prism software, such that the IC_{50} is the concentration at which the curve crosses the 50% plane on the graph. Any calculated IC_{50} value outside the range of concentrations (up to 100 μM) tested was identified as being >100 μM. 95% confidence intervals were calculated by GraphPad Prism 7 using the asymmetrical (likelihood) method on the single concentration response curve fit of the combined data set (mean ± SD data) described above. Calculation of statistical significance was performed via one way ANOVA with a Dunnett's multiple comparisons post hoc analysis.

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Results

WT and Cys-null RGS RH domains have GAP activity

To ensure that both the previously described and novel WT and cys-null RGS constructs produced active protein following purification (Supplemental Figure S1), the GAP activity of each RGS protein for $G\alpha_{i1}$ was assessed using the previously described malachite green colorimetric assay (Figure 2 A-E) (Monroy et al., 2013). Briefly, this assay measures the GTPase activity of Gα, by monitoring cleavage of the γ phosphate group of GTP by $G\alpha_{i1}$ (R178M, A326S). R178M mutation leads to decreased intrinsic GTPase activity of $G\alpha_{i1}$ without effecting RGS sensitivity, making phosphate release RGS-dependent (Berman et al., 1996). A326S mutation increases the rate of GDP release following cleavage of the γ phosphate, the rate limiting step in the Gα cycle, leading to receptor-independent GTP binding by Gα (Posner et al., 1998). For WT proteins, all RH domains increased free phosphate concentration above baseline to an appreciable degree, though RGS2 and RGS6, only reached 21% and 46% of the maximal observed activity (1μM RGS1), respectively. For RGS2, this was expected, as it couples preferentially with $G\alpha_q$ in biochemical assays (Soundararajan et al., 2008). Interestingly, the RGS2 cys-null mutant exhibited higher potency (WT= 1100 nM, cys-null= 390 nM) and higher maximal GAP activity (WT= 21%, cys-null= 47%). For RGS1, RGS4, RGS16, RGS17, and RGS18, WT and cys-null mutants had EC_{50} values overlapping at the 95% confidence interval, and no appreciable difference in the maximal GAP activity observed. RGS5 WT and cys -null had overlapping EC_{50} values, but a slight decrease in maximal activity was observed in the cys-null mutant (92% WT

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versus 74% cys-null). RGS8 WT exhibited more potent GAP activity than cys-null, as the observed EC_{50} increased from 40 nM to 95 nM, though no differences were observed in the maximal activity. This was also true for RGS10, as the cys-null mutant exhibited an EC_{50} value (560 nM) that was over two-fold greater than WT (220 nM). The RGS14 mutant's maximal GAP activity was only half that of WT RGS14 (33% versus 64%), but overlapping EC_{50} values were observed.

RGS-Gα_o PPI Inhibition by Compounds using AlphaScreen

To assess the selectivity of known RGS inhibitors against a panel of 12 WT and 10 cys-null mutant RGS proteins, we used the AlphaScreen PPI platform. Each WT RGS exhibited a signal window of at least 9-fold or better over negative control, while each mutant exhibited a signal window of 4-fold or better over negative control. WT and mutant RGS2 exhibited no binding to $G\alpha_o$ (data not shown). A panel of 13 known small molecule RGS inhibitors and one known cys reactive molecule were tested for the ability to inhibit the panel of 22 RGS proteins using AlphaScreen. All compounds used in this study were first discovered as inhibitors of RGS4. Compounds and reference in which they were first discovered are shown in Table 1. N-ethylmaleimide (NEM) was also included, as it is a known cys reactive compound with inhibitory activity towards RGS4 (Blazer et al., 2011). IC_{50} values with the 95% CI range for each compound against each RGS are shown in Table 2 (with corresponding concentration response curves in Supplemental Figure S2). Interestingly, 6018993 did not demonstrate inhibition of any RGS tested, and NEM demonstrated only minimal inhibition RGS4 and RGS8. These results are excluded from Table 2 for clarity, but are presented in

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Supplemental Table 1. Additionally, some WT RGS proteins were refractory to inhibition by any compound tested. For RGS6, RGS7, no appreciable inhibition was observed with any compound, and RGS18 was only inhibited by a single compound (CCG-50014). All other WT RGS proteins investigated resulted in at least two compounds producing a calculable $IC_{50,}$ defined as both the upper and lower limit of the 95% CI were below the max concentration tested (100 μM). Interestingly, NEM only produced calculable IC_{50} values for RGS8 and RGS4. This aligns with previous reports that RGS4 is sensitive to inhibition by NEM at high concentrations (IC_{50} 30 μM) but not iodoacetamide (Blazer et al., 2011). This indicates that there is a degree of substructure specificity or adduct size requirement involved in the inhibition of RGS proteins by small molecules, and that simple covalent modification of cys is not sufficient to impart inhibition.

Furthermore, there were some differences between inhibition observed in our paradigm and previous reports in the literature. Our results differ from the reports by Storaska et al. with respect to inhibition and selectivity of RGS7 and RGS8. Compound 1777233, which did not inhibit RGS8 in an FCPIA assay, resulted in inhibition in our assay. Compounds 1911669 and 5428579 were reported to inhibit RGS7, but not RGS8 (Storaska et al., 2013), but our results indicate that these compounds inhibit RGS8 but not RGS7. Compound 6383479 did not inhibit RGS8 or RGS16 using FCPIA (Storaska et al., 2013), however we report inhibition of both proteins using AlphaScreen. It is possible these differences can be attributed to differences in methodologies between FCPIA and AlphaScreen, such as incubation time, buffering conditions, RGS

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protein concentrations, or the amount of "active" RGS protein in the protein batch used for a given experiment.

The panel of compounds was also tested for activity against the cys-null mutant of each RGS (Table 3, Figure S2). With the exception of compounds CCG-55919 and compounds CCG-50014, every compound was found to be inactive (IC50 > 100 μM) against cys-null mutants (values excluded from Table 3 for clarity). Only cys-null RGS14 was somewhat inhibited by CCG-55919 and CCG-50014, albeit at a drastically reduced potency (39 fold reduced potency CCG-55919, 5000 fold reduced potency CCG-50014). This was as expected given the published reports detailing the activity of these compounds against cys-null RGS4.

RGS14 is more sensitive to small molecule inhibition than RGS4

One of the more striking results of this investigation is that although each small molecule investigated (NEM excluded) were discovered as inhibitors of RGS4, 8 of the 13 were selective for RGS14 RH at the 95% CI, while another (CCG-62808) was equipotent for RGS4 and RGS14 at the 95% CI. One of these compounds, CCG-50014, was previously identified as one of the most potent RGS4 inhibitors to date with an IC_{50} of 30 nM (Blazer et al., 2011). However, the result of this investigation demonstrates CCG-50014 inhibits RGS14 with an IC_{50} of 8 nM and is selective for RGS14 at the 95% CI.

Several other compounds demonstrated moderate selectivity for RGS14 over other RGS proteins. For example, considering the UI series compounds, UI-5 was 10 times more potent for RGS14 over the next RGS, and UI-1590 inhibited RGS14 with

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sub-micromolar potency while the next closest RGS was inhibited with low μM potency. In fact, of the 11 compounds that demonstrated inhibition of RGS14 only three (CCG-63808, CCG-4986, and CCG-55919) were not selective for RGS14 over all other RGS proteins. The apparent selectivity of these tested compounds for RGS14 is not a trait that is shared by fellow R12 family member RGS10, as only 3 compounds had calculable IC_{50} values for RGS10. This holds true for the R4 family as well, as 11 of 13 compounds resulted in calculable IC_{50} values against RGS4, while only one (CCG-50014) resulted in a calculable IC_{50} for R4 family member RGS18.

RGS GAP Activity Inhibition by Compounds

Having established a biochemical pharmacological profile for known RGS inhibitors in a protein-protein interaction assay, we assessed whether the same trends would hold true in a secondary, functional biochemical assay. RGS6, RGS7, RGS10, and RGS18 were excluded due to minimal inhibition in AlphaScreen assay. Though RGS14 was readily inhibited by many of the compounds, it was excluded because 80% maximal activity could not be observed at up to 10 μM final protein concentration, (Figure 2C). As no cys-null mutants were sensitive to small molecule inhibition in AlphaScreen, none were included.

Trends observed in the free phosphate detection assay were comparable to those observed in the protein-protein interaction assay (Table 4, Figure 3A-G). Compound 5428579 is not included in Table 4, as no RGS proteins inhibition was observed in this assay. RGS1 and RGS4 were the most susceptible to inhibition in this assay, with six compounds resulting in calculable IC_{50} values each protein. Additionally,

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CCG-63808 and CCG-50014 resulted in overlapping IC_{50} values for RGS1 and 4 at the 95% CI in this assay, where they were not overlapping in the AlphaScreen assay (Table 2). In general, for RGS1 and RGS4 the potencies of the compounds were comparable between the two assays.

This was not the case for the other four RGS proteins tested. CCG-50014 exhibited high nM potencies for RGS5, RGS8, RGS16, and RGS17 using AlphaScreen, but demonstrated reduced potencies in the free phosphate detection assay. Similar results were obtained for CCG-55919, as this compound resulted in low μM potencies using AlphaScreen yet resulted in IC_{50}s of greater than 100 μM in this GAP activity assay. This is possibly due to higher protein concentrations (μM) required to measure GAP activity, whereas much lower concentrations (nM) are needed to measure RGS: Gα interaction using AlphaScreen. Alternatively, this could be caused by each assay employing different Gα subunits, as AlphaScreen uses $G\alpha_o$ and the GAP assay a mutant of $G\alpha_{i1}$ with accelerated GTP-binding activity. Additionally, this could be a phenomenon dependent on which RGS family member is being evaluated.

RGS RH Domains Show Marked Differences in Cysteine Reactivity

Due to the fact that RGS inhibitors display very little activity towards cys-null mutants, we sought determine if the relative potencies of these inhibitors were directly related to the intrinsic reactivity of their cys residues. In order to assess this we used the cys reactive dye DACM, which only has appreciable fluorescence once it has covalently labeled cys residues in proteins (Figure 4, Table 5). By treating the WT-RGS proteins with a 1:1 molar ratio of DACM, we observed drastically different reaction rates across

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the RGS family, which could be grossly categorized as those who readily react with DACM, those that are moderately reactive, and those that are not. Independent of pH tested, RGS1, RGS2, RGS4, RGS16, and RGS18 were modified by DACM to a level that was appreciably higher than either buffer alone or the cys devoid RGS6 and RGS7. An intermediate level of reactivity was observed for RGS14 at each pH tested and RGS10 at pH 7.0 and 6.5, but not 7.5. Alternatively, RGS5, RGS8, and RGS17 interacted with DACM minimally. At every pH tested, RGS18, which contains two cys, was the fastest to react, though it did not display the highest relative intensity value. The next fastest rate observed was for RGS4 followed by RGS1, with RGS4 displaying lower $t_{1/2}$ and higher relative fluorescence at every pH tested, possibly due to four cys in RGS4 versus three in RGS1. The next most reactive RH domain belonged to RGS16, which reacted approximately four times slower than RGS4 at pH 7.5 and 7.0. RGS2 reacted similarly to RGS16 at pH 7.5, but was approximately two fold slower at pH 7.0. Though the rate of reaction of RGS14's two cys residues was slow (displaying an approximately linear rate), it was able to produce appreciable fluorescence at every pH, as was RGS10 at pH 6.5 and 7.0. Alternatively, RGS5, RGS8, and RGS17 only showed minimal interaction with DACM. RGS5 (one cys) reacted nearly half as fast as RGS8 (two cys), though paradoxically, the $t_{1/2}$ values for both proteins did not decrease with increasing pH, as would be predicted. RGS10 and RGS17 both only have calculable $t_{1/2}$ values at pH 7.5 due to curve shape, though the magnitude of these reactions (fluorescence intensity) is so low, that they may be of limited relevance.

Compounds 6383479 and CCG-63802 inhibit RGS proteins in cells

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Using a cell-based split NanoLuciferase (NanoLuc) system, we assessed the ability of several compounds to inhibit the interaction of $G\alpha_{i1}$ with RGS proteins in cells. In this assay one PPI binding partner is tagged with an 18 kD fragment of NanoLuc and the other binding partner a 1.3 kD NanoLuc peptide. Formation of PPI results in formation of a competent NanoLuc and luminescence is recorded.

This system was used to test 6383479, CCG-4986, CCG-50014, and CCG-63802 against RGS1, RGS4, RGS8, RGS14, RGS16, and cys-null RGS4 with $G\alpha_{i1}$. These compounds were chosen based on the combined criteria of potency, known activity in cells and/or *in vivo*, and apparent RGS selectivity based on AlphaScreen and/or GAP activity results. While CCG-4986 and CCG-50014 did demonstrate inhibition of several RGS proteins in this assay, this inhibition was not significantly from the inhibition of the assay itself, as determined using a control PPI (Supplemental Figure S3), such that no conclusions about the activity of these compounds in cells can be made. Compound 6383479 demonstrated a significant difference in signal means as determined via one way ANOVA ($F(7,16)=10.37$, $p < 0.001$). Dunnett's post hoc multiple comparisons analysis revealed that RGS1, RGS4, RGS14, and cys-null RGS4 were inhibited by 6383479 to a significant degree compared to PPI control ($p = 0.03$, < 0.001, < 0.001, and 0.023, respectively) while RGS8 and RGS16 were not. The most robust inhibition was observed for RGS4.

Compound CCG-63802 also demonstrated a significant difference in signal means as determined via one way ANOVA ($F(7,16)=13.4$, $p < 0.001$). Dunnett's multiple comparisons analysis revealed RGS1, RGS4, and RGS14 were inhibited by CCG-63802 to a significant degree compared to PPI control ($p = 0.031$, < 0.001, and <

Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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0.001, respectively) while RGS8, RGS16, and cys-null RGS4 were not. For CCG-63802 the most robust inhibition was observed for RGS4, then RGS14, and then RGS1. Slight inhibition was also observed for RGS8, RGS16, and cys-null RGS4, though not to a statistically significant degree.

This cell-based system was also amenable to the RGS2-$G\alpha_q$ PPI. Of the four compounds tested, two were found to inhibit RGS2 in cells (Figure 5A-B). Compounds 6383479 and CCG-63802 reduced the signal of the RGS2- $G\alpha_q$ interaction to roughly 35% and 19% of DMSO treated controls, respectively, representing a statistically significant deviation from the control PPI (determined via one way ANOVA as above, Dunnett's multiple comparison *p* vales for each as follows, compound 6383479 $p = 0.001$, compound CCG-63802 $p < 0.001$). CCG-4986 and CCG-50014 did not result in inhibition of RGS2-$G\alpha_q$ that was discernable from their effects on the assay as was observed with $G\alpha_{i1}$ (Supplemental Figure S3A-C).

Discussion

Here we present an investigation of a panel of known RGS4 inhibitors against a panel of 12 WT and 10 cys-null mutant RGS proteins, establishing the most comprehensive pharmacological profile of these inhibitors for RGS proteins to date. Additionally, we investigated the intrinsic RGS property of cys reactivity to potentially elucidate why certain compounds are more potent against certain RGS proteins.

Investigation of 13 known RGS4 inhibitors resulted in the identification of at least one inhibitor for 10 of the 12 WT RGS proteins, with only RGS6 and RGS7 demonstrating a lack of inhibition. For RGS1, RGS2, RGS5, RGS10, RGS14 and

RGS18 this represents the first identification of small molecules that inhibit these RGS proteins. Each of these RGS proteins have been directly implicated in specific pathologies (reviewed in (Hurst and Hooks, 2009)) or have been suggested to play a yet unverified role in pathologies (Evans et al., 2015). Therefore, through the identification of small molecule inhibitors of these proteins, this work has provided tool compounds that may be used to probe the consequence of pharmacological inhibition of these RGS proteins in their respective disease states. Perhaps the most powerful such tool will be CCG-50014, which we demonstrated inhibits RGS1, RGS5, RGS8, RGS14, RGS1 , and RGS17 with sub micro-molar potency and has reported activity in mouse models *in vivo* in the investigation of RGS4 (Yoon et al., 2015). Additionally, our data greatly expands the RGS pharmacological profile for these compounds and establishes a more comprehensive examination of compound: RGS selectivity.

The revelation that many of the small molecule inhibitors tested were selective for the RH domain of RGS14 over that of RGS4 was surprising, as previous reports detailing the discovery of these compounds all demonstrated selectivity for RGS4. Perhaps one of the most surprising of these was CCG-50014, which was previously identified as one of the most potent small molecule inhibitors of RGS proteins to date with an IC_{50} of 30 nM against RGS4 (Blazer et al., 2011; Roman et al., 2009). This compound was found to be a more potent inhibitor of RGS14, with an IC_{50} of 8 nM and no overlap in IC_{50} with RGS4 at the 95% CI. This, along with the result that many of the compounds were selective for RGS14, establishes the RH domain of RGS14 as highly sensitive to small molecule inhibition compared to the other RGS proteins tested, and warranting further investigation as a target.

Given that the interrogated compounds exhibited cys dependence in the reports characterizing their initial discoveries, it was not surprising that all the compounds exhibited cys dependence for all the RGS proteins. This led us to investigate whether the newly established pharmacological profile with respect to these RGS proteins could be attributed to the number of cys in the RH domain of these RGS proteins. Aside from the complete absence of cys in the RH domain, the number of cys residues in the RH domain does not correlate to the potency of inhibition. There are several examples of this. RGS14 was often inhibited with the greatest potency, and yet only contains two cys residues compared to RGS4, which contains four. Additionally, RGS18 contains two cys, one of which is highly conserved among RGS proteins, and yet was only susceptible to inhibition by CCG-50014. Compare that to RGS17, which only contains one cys residue (also residing in this highly-conserved region) and is inhibited by eight of the compounds tested. This led us to hypothesize that it is not the number of cys but rather distinct structural characteristics of the RGS proteins that alter the accessibility and thus reactivity of the cys residues, and that cys accessibility would correlate to observed potency of these compounds. However, our assessment of the accessibility of cys residues via use of the dye DACM demonstrates that this is not the case. In these experiments, neither the magnitude of fluorescence nor the rate of signal increase correlated to the newly established pharmacological profile of inhibition of RGS proteins via these small molecules. This is likely because these small molecules are interacting with specific cys residues, or a specific subset of cys residues in the RGS protein. Therefore, identification of the critical cys residues required for inhibition and assessment of those resides for cys accessibility may yield more interesting results. For

example, RGS10 and RGS14 are closely related, as both reside in the R12 family and share >52% identity within the RH domain. RGS14 contains an additional cys residue near its C-terminus in α9 and is readily inhibited by many of the compounds tested, whereas RGS10 lacks this residue and is only inhibited by three of the compounds tested, each with IC_{50} >7 μM. This provides some preliminary evidence that this C-terminal cys residue may be an important determinant for inhibitor specificity. An RGS14 construct in which the only cys present is the highly conserved cys residue in α4 could be a useful tool in future studies aimed at further understanding the molecular determinants of RGS inhibition.

Additionally, it is possible that some other structural features of the RGS RH domains confer the small molecule selectivity established here, such as protein dynamics or altered pK_a values of critical cys residues. It should be noted that this work utilized the RH domains of all RGS proteins tested, yet many RGS proteins are larger and more complex, containing additional domains that are sites of post translational modification or are responsible for G protein independent signaling cascades. The work here is critical in the investigation of variation within the RH domain that result in susceptibility to small molecule inhibition among RGS proteins. However, investigation using the full-length constructs would shed light on the selectivity of these compounds in a more physiologically representative system, particularly for RGS family members that possess more complex multi-domain and multi-functional properties. For example, many of the small molecule inhibitors tested here were found to be more selective for RGS14 over other RGS proteins. However, RGS14 contains a $G\alpha_{i/o}$ – Loco interaction (GoLoco) domain that acts as a guanosine nucleotide dissociation inhibitor (GDI) and

Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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prevents dissociation of GDP from the Gα subunit. Thus, the GoLoco motif adds another level of regulation of $G\alpha_{i/o}$ signaling for RGS14 (Kimple et al., 2001; Willard et al., 2004). As such, the consequence of RGS14 RH inhibition on the net regulation of Gα signaling in a physiological system (i.e. utilizing full length RGS14) needs to be assessed.

Finally, we established a cell based pharmacological profile for a subset of compounds using our previously characterized protein complementation assay (Bodle et al., 2017). The differential inhibition of RGS proteins observed suggests that this system may be utilized for the establishment of a more detailed cell based pharmacological profile for both these and future RGS small molecule inhibitors.

In conclusion, we have established a more comprehensive RGS pharmacological profile for 13 published RGS4 inhibitors against a panel of 12 RGS proteins, which represents roughly 60% of the RGS family. Ten RGS proteins now have at least one identified small molecule inhibitor that may be used as a tool compound to probe RGS function. The revelation that many small molecules inhibited RGS14 with greater potency than RGS4 demonstrates the need for more comprehensive examination of small molecule promiscuity amongst RGS proteins in future discovery efforts. This work, combined with future structural interrogation, will be the foundation for establishment of RGS models that allow for design of RGS specific small molecule inhibitors.

Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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Acknowledgments

We would like to thank the University of Iowa High Throughput Screening facility for providing instrumentation and Joseph P. O'Brien for assistance in cloning and helpful discussion.

Authorship Contributions

Participated in research design: Bodle, Hayes, Roman

Conducted experiments: Bodle, Hayes

Contributed new regents or analytical tools: Bodle, Hayes, Roman

Performed data analysis: Bodle, Hayes, Roman

Wrote or contributed to writing of manuscript: Bodle, Hayes, Roman

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Footnotes

This work was supported by the National Institute of Health [Grants 5R01CA160470, T32GM067795], University of Iowa College of Pharmacy, and the American Foundation for Pharmaceutical Education.

Authors contributed equally to this work: MPH, CRB

Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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Figure Legends

Figure 1. Conservation of Cysteine Residues among RGS Family Members Tested

A. Sequence alignment of the RGS RH domain constructs tested in this study with conserved residues indicated in black, similar residues in grey, and cys residues highlighted in yellow. Residue numbers for full-length RGS proteins are indicated at the beginning of each row with α Helix numbers located below, based on $G\alpha_{i1}$-bound RGS4 (1AGR). B. Conservation of cys residues as spheres mapped to RGS structure with RGS family members containing relevant residue indicated. Blue represents not or very weakly conserved residues, purple somewhat conserved (RGS4 cys148), and red highly conserved (RGS4 cys 95).

Figure 2. GAP activity of recombinant WT and Cys-null RH domains

A-D. Concentration response curve assessment of GAP activity of both WT and mutant RH domains of indicated RGS family members using malachite green colorimetric assay to measure GTP hydrolysis. A. WT and Cys-null RGS1, RGS2, and RGS4. B. WT and Cys-null RGS5, RGS6, RGS7, and RGS8. C. WT and Cys-null RGS10, RGS14, and RGS16. D. WT and Cys-null RGS17 and RGS18. Data were normalized to no RGS (0%) and the maximal GAP activity of RGS1 (100%). Data represent mean ± SD from n=3 experiments.

Figure 3. Inhibition of WT RGS RH domain GAP activity by small molecules

A-G. Concentration-response assessment of indicated inhibitor's effect on WT RGS RH domain GAP activity using malachite green colorimetric assay to measure GTP

hydrolysis. Data represent mean ± SD from n=3 independent experiments and are summarized in Table 4.

Figure 4. Reactivity of WT-RGS RH domains with DACM

Kinetic traces of the reactivity of 1 μM DACM with 1 μM indicated RGS protein normalized to RGS16 maximum. RH domains that showed appreciable reactivity are shown in A, B, and C, at pH 7.5, 7, and 6.5, respectively. RH domains that showed moderate to minimal reactivity are shown in D, E, and F at pH 7.5, 7, and 6.5, respectively with grey-boxed regions expanded in G, H, and I. Data represent mean ± SD from n=3 independent experiments. Time to half maximal reactivity ($t_{1/2}$) is summarized in Table 5.

Figure 5. Inhibition of RGS: Gα Interaction in Cells

NanoBiT luminescence complementation assay in HEK293T cells expressing indicated RGS and $G\alpha_{i1}$ (except RGS2 with $G\alpha_q$). Decrease in signal in response to compound treatment as a percent of vehicle treatment for 31.6 μM 6383479 (A) and 31.6 μM CCG-63802 (B). Data represent n=3 independent experiments in at least duplicate ± SD. Statistical significance determined via one ANOVA with Dunnett's multiple comparisons test with respect to Control PPI. *P* values on graph are reflective of multiple comparisons results, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

Molecular Pharmacology Fast Forward. Published on October 19, 2017 as DOI: 10.1124/mol.117.109843
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Tables

Table 1. Chemical structures of previously identified inhibitors of RGS Protein family members.

Given are compound names and structures of commercially available RGS inhibitors. Compounds with numbers only refer to their PubChem identifier, and CCG or UI indicates Center for Chemical Genomics (University of Michigan) or University of Iowa, respectively, nomenclature. Ref. is the reference in which the indicated compound was first identified. NEM was included due to its reactive nature towards cys residues.

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Table 1. Previously identified RGS Inhibitors

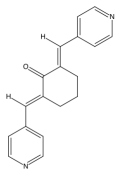
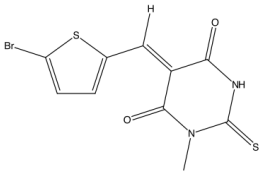
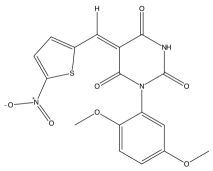
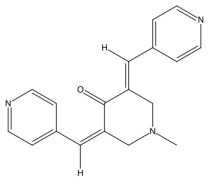
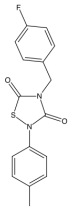
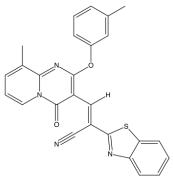
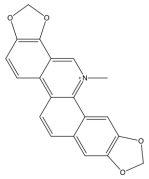
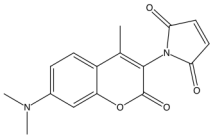
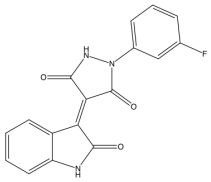
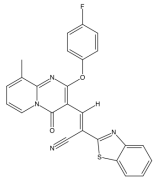
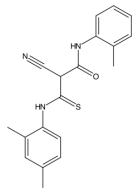
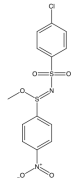
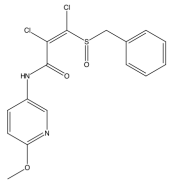
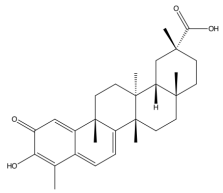
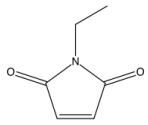
Compound	Structure	Ref.
6018993		(Storaska et al., 2013)
1777233		(Storaska et al., 2013)
1911669		(Storaska et al., 2013)
6383479		(Storaska et al., 2013)
CCG-50014		(Roman et al., 2009)
CCG-63802		(Blazer et al., 2010)
UI-5		(Monroy et al., 2013)
DACM		-
5428579		(Storaska et al., 2013)
CCG-63808		(Blazer et al., 2010)
CCG-55919		(Roman et al., 2009)
CCG-4986		(Roman et al., 2007)
1472216		(Storaska et al., 2013)
UI-1590		(Monroy et al., 2013)
NEM		-

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Table 2. Inhibition of WT-RGS: $G\alpha_o$ Interaction by RGS Inhibitors

Biochemical characterization of RGS inhibitors for their ability to disrupt the WT-RGS: $G\alpha_o$ PPI using AlphaScreen assay. Data represent the IC_{50} with the 95% CI in parentheses from n=3 independent experiments, with the average of duplicate wells. Concentration response curves for each value given are shown in Supplementary Figure S2. NC indicates that an IC_{50} value was not calculable, due to an altogether lack of inhibition.

Table 2. Inhibition of WT-RGS: $G\alpha_o$ Interaction by Previously Described Inhibitors

	IC_{50}, μM											
RGS	**1777233**	**1911669**	**6383479**	**CCG-63808**	**CCG-55919**	**5428579**	**CCG-4986**	**CCG-50014**	**CCG-63802**	**1472216**	**UI-5**	**UI-1590**
1	16 (12- 21)	62 (31->100)	90 (58->100)	37 (28-51)	18 (10-34)	18 (13-25)	38 (30-50)	0.12 (0.10-0.14)	23 (19-29)	24 (18-33)	20 (17-24)	1.8 (1.3-2.4)
4	17 (13-24)	82 (46->100)	7.4 (5.6-9.7)	15 (10-23)	0.97 (0.58-1.6)	9.9 (7.6-13)	3.4 (2.4-4.9)	0.029 (0.023-0.038)	19 (15-25)	31 (21-50)	15 (9.7-25)	1.2 (0.80-1.7)
5	>100	>100	>100	>100	17 (10-30)	58 (31->100)	50 (37-72)	0.6 (0.46-0.80)	>100	70 (35->100)	>100	18 (12-28)
6	NC	>100	>100	NC	>100	>100	>100	>100	NC	>100	>100	>100
7	NC	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
8	40 (30-57)	76 (49->100)	50 (35-78)	59 (43-89)	16 (11-25)	12 (9.2-15)	61 (45-90)	0.65 (0.29-1.5)	44 (34-58)	92 (68->100)	80 (55->100)	6.4 (5.4-7.7)
10	>100	>100	>100	>100	68 (46->100)	>100	>100	7.6 (6.1-9.6)	>100	>100	>100	51 (41-65)
14	5.0 (4.2-5.9)	7.5 (6.0-9.3)	>100	13 (11-15)	2.4 (1.9-3.1)	5.9 (5.2-6.7)	22 (19-27)	0.008 (0.006-0.010)	11 (9.3-13)	3.2 (2.6-3.9)	1.5 (1.3-1.8)	0.46 (0.39-0.54)
16	76 (56->100)	>100	56 (36->100)	88 (62->100)	13 (10-17)	51 (34-84)	54 (43-70)	0.44 (0.30-0.65)	62 (46-92)	>100	>100	21 (15-30)
17	28 (16-57)	>100	>100	>100	11 (7.7-17)	41 (31-56)	71 (50->100)	0.78 (0.58-1.0)	>100	22 (14-36)	86 (49->100) [a]	7.3 (5.6-9.7) [a]
18	>100	>100	>100	>100	>100	>100	>100	28 (14-67)	>100	>100	NC	>100

[a] Data previously reported in Bodle et al., 2017b.

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Table 3. Inhibition of Cys-Null RGS: $G\alpha_o$ Interaction by RGS Inhibitors

Biochemical characterization of RGS inhibitors for their ability to disrupt the cys-null RGS: $G\alpha_o$ PPI using AlphaScreen assay. Data represent the IC_{50} with the 95% CI in parentheses from n=3 independent experiments, each with duplicate wells. Concentration response curves are shown in Supplementary Figure S2. All compounds from Table 2 were tested, and any compounds for which all cys-null RGS proteins exhibited >100 μM IC_{50} values were excluded from this Table.

Table 3. Inhibition of Cys null-RGS:$G\alpha_o$ Interaction by Inhibitors		
	IC_{50}, μM (95% CI)	
RGS	CCG-55919	CCG-50014
1	>100	>100
4	>100	>100
5	>100	>100
8	>100	>100
10	>100	>100
14	94 (60->100)	40 (22-92)
16	>100	>100
17	>100	>100
18	>100	>100

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Table 4. Inhibition of WT-RGS GAP Activity at hGα_{i1} (R178M, A326S)

Biochemical characterization of RGS inhibitors for their ability to disrupt the GAP activity of WT-RGS RH domains using phosphate detection assay to monitor Gα GTPase activity using hGα_{i1} (R178M, A326S) rapid turnover mutant. Data represent the IC_{50} with the 95% CI in parentheses from n=3 independent experiments, each with duplicate wells. Concentration response curves are shown in Figure 3. NC indicates that an IC_{50} value was not calculable, due to an altogether lack of inhibition.

Table 4. Inhibition of WT-RGS GAP Activity at hGα_{i1} (R178M, A326S)

	IC_{50}, μM (95% CI)					
RGS	**CCG-63808**	**CCG-55919**	**CCG-4986**	**CCG-50014**	**CCG-63802**	**UI-1590**
1	7.2 (5.7-9.1)	1.9 (1.5-2.4)	64 (43->100)	0.16 (0.14-0.19)	15 (12-18)	1.8 (1.3-2.5)
4	3.9 (2.4-6.0)	0.54 (0.41-0.71)	3.6 (2.6-4.9)	0.22 (0.18-0.27)	5.3 (4.3-6.5)	1.4 (1.2-1.7)
5	20 (15-28)	>100	NC	17 (14-22)	34 (25-48)	27 (16-58)
8	22 (16-30)	>100	NC	22 (17-28)	38 (28-52)	39 (21->100)
16	19 (15-25)	>100	>100	6.8 (5.2-8.9)	32 (25-40)	40 (25-74)
17	31 (21-46)	NC	NC	55 (35->100)	49 (32-84)	>100

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Table 5. Cysteine Reactivity of RGS RH Domains with DACM

Summary of RGS RH domain reactions with the environmentally sensitive maleimide dye DACM from kinetic traces shown in Figure 4. Data represent mean time to half max. ($t_{1/2}$) as determined via fit to one phase association with the 95% CI in parentheses from n=3 independent experiments. n.d. indicates reactivity that was undistinguishable from RGS6 and RGS7, which lack cys residues.

Table 5. Cysteine Reactivity of RGS RH Domains

RGS	# Cys Residues	$t_{1/2}$, sec (95% CI)		
		pH 7.5	7.0	6.5
1	3	74 (69-80)	225 (218-232)	762 (742-781)
2	4	275 (269-282)	1170 (1140-1200)	>1000
4	4	61 (55-68)	150 (139-162)	316 (302-331)
5	1	830 (591-1200)	614 (508-746)	n.d.
8	2	531 (355-799)	476 (390-583)	>1000
10	1	n.d.	472 (414-539)	>1000
14	2	>1000	>1000	>1000
16	3	224 (216-233)	623 (605-641)	>1000
17	1	n.d.	361 (263-495)	n.d.
18	2	10 (9-12)	59 (56-63)	244 (237-251)

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Figures

Figure 1.

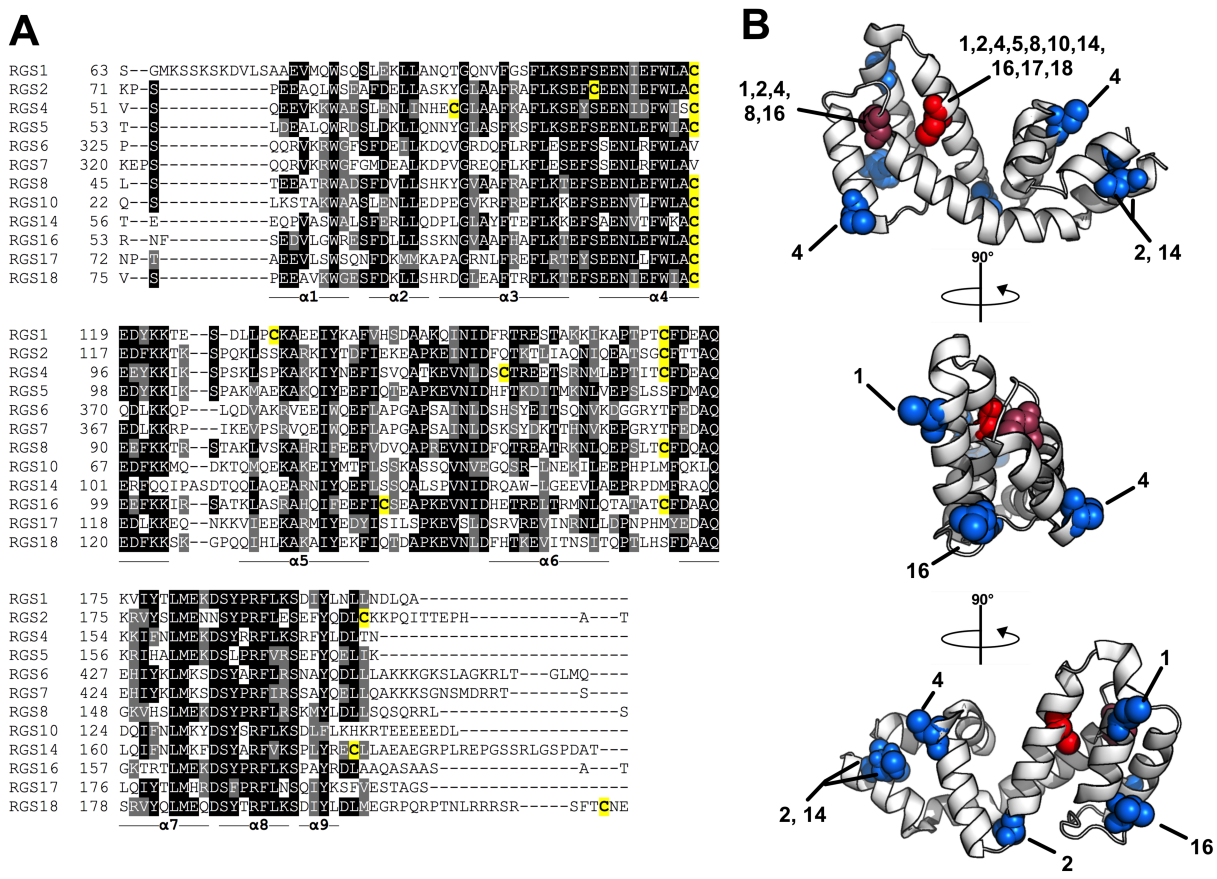

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Figure 2

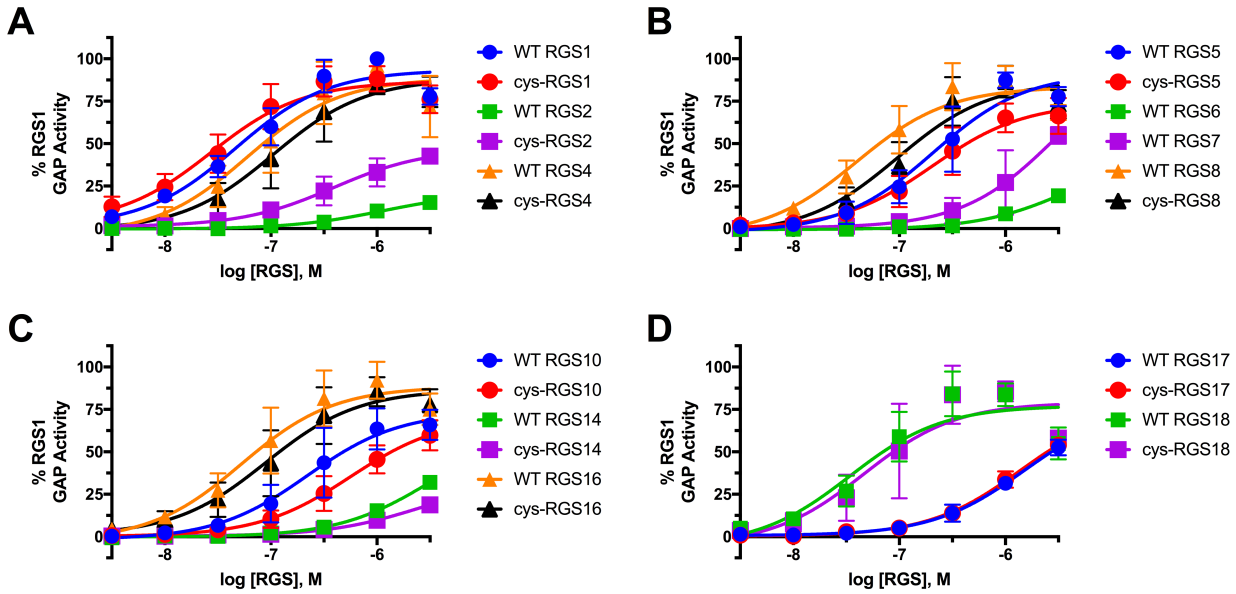

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Figure 3

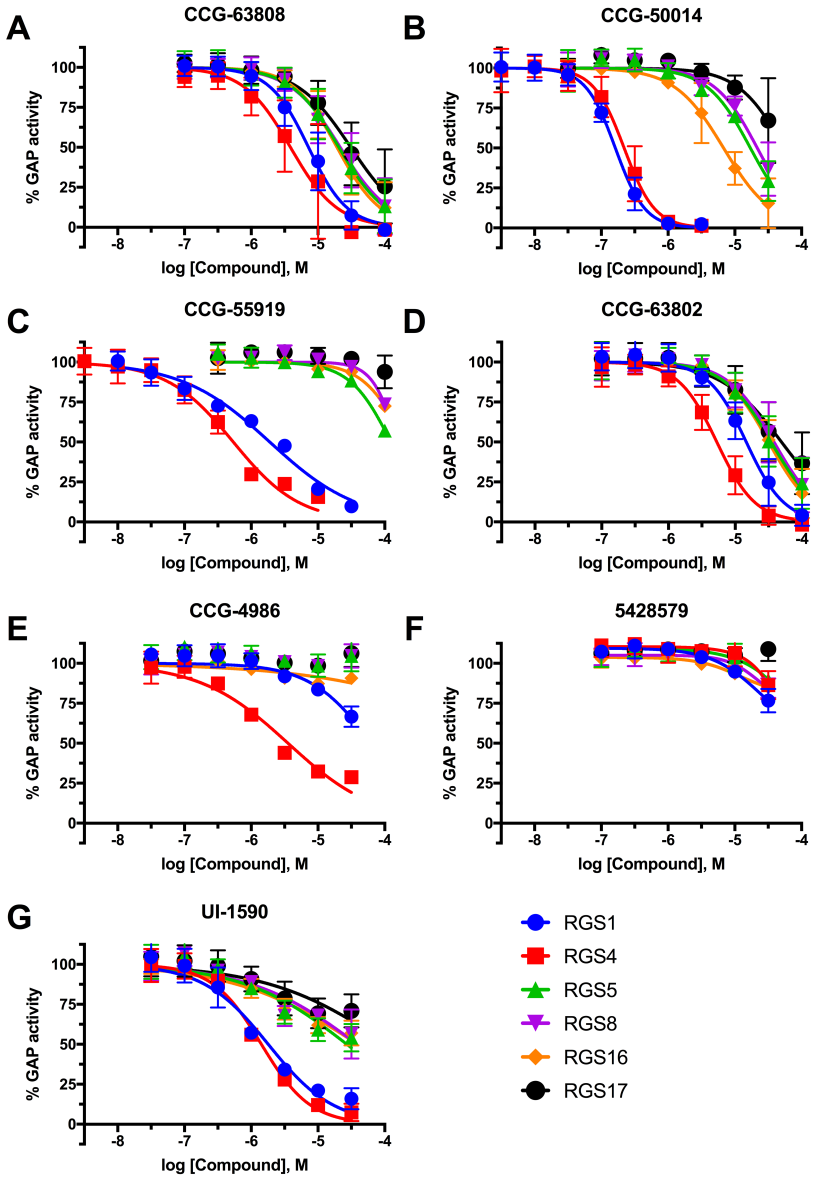

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Figure 4

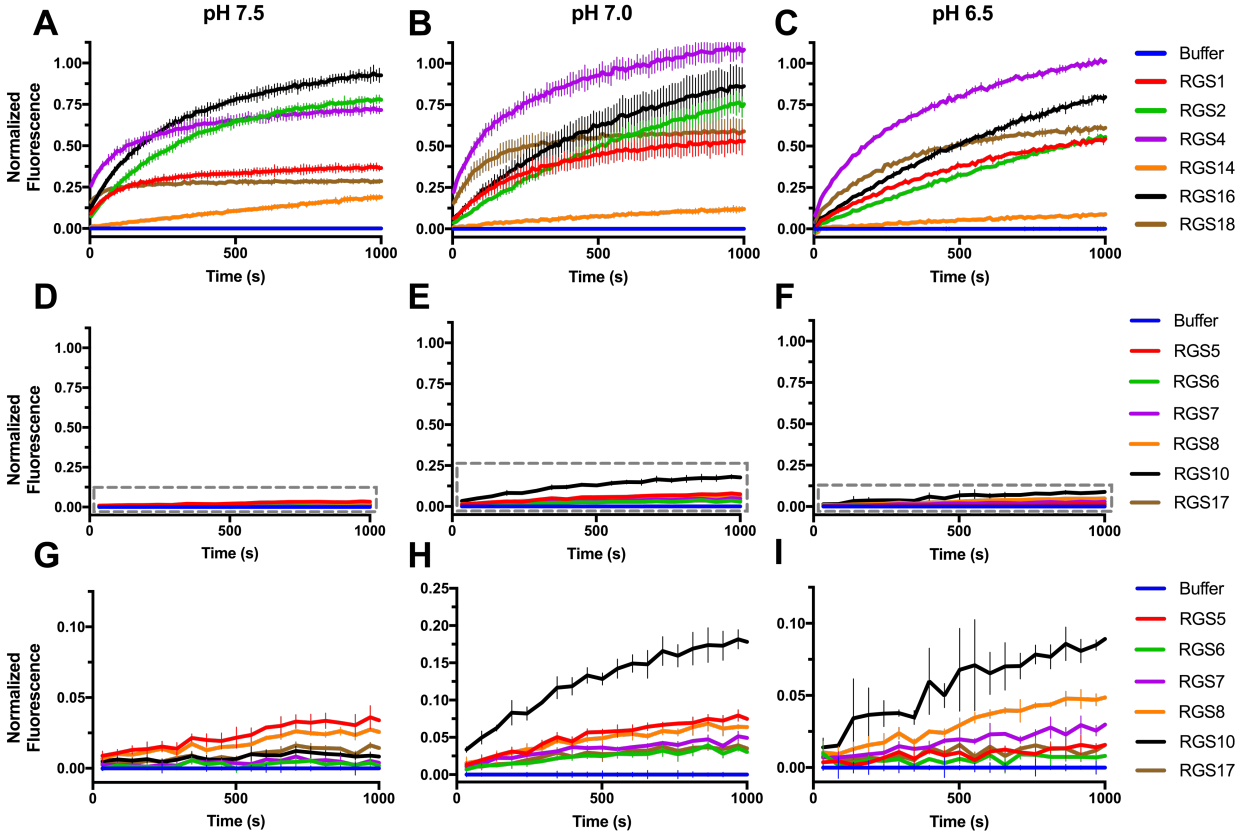

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Figure 5.