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Reversible, allosteric, small-molecule inhibitors of RGS proteins

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Running Title: First Reversible Small Molecule RGS Inhibitors

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List of Abbreviations:
TR-FRET: Time-Resolved Fluorescence Resonance Energy Transfer
RGS: Regulator of G Protein Signaling
FCPIA: Flow Cytometry Protein Interaction Assay
SMPPII: Small Molecule Protein-Protein Interaction Inhibitor
PPI: Protein-Protein interaction
GAP: GTPase Accelerating Protein

Abstract:

Regulators of G protein Signaling (RGS) proteins are potent negative modulators of G protein signaling and have been proposed as potential targets for small molecule inhibitor development. We report a high throughput time-resolved fluorescence resonance energy transfer (TR-FRET) screen to identify inhibitors of RGS4 and describe the first reversible, small-molecule inhibitors of an RGS protein. Two closely-related compounds, typified by CCG-63802, inhibit the interaction between RGS4 and $G\alpha_o$ with an IC_{50} value in the low micromolar range. They show selectivity among RGS proteins with a potency order of RGS 4>19=16>8>>7. The compounds inhibit the GTPase Accelerating Protein activity of RGS4 and thermal stability studies demonstrate binding to the RGS but not to $G\alpha_o$. On RGS4, they depend on an interaction with one or more cysteines in a pocket that has previously been identified as an allosteric site for RGS regulation by acidic phospholipids. Unlike previous small molecule RGS inhibitors identified to date, these compounds retain substantial activity under reducing conditions and are fully reversible on the ten minute time scale. CCG-63802 and related analogs represent a useful step towards the development of chemical tools for the study of RGS physiology.

Introduction:

Networks of protein-protein interactions are crucial for efficient cellular function. 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 (Arkin and Wells, 2004; Berg, 2003; Berg, 2008; Blazer and Neubig, 2008; Gadek and Nicholas, 2003). 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, increasingly, there has been solid progress in SMPPII development (Arkin and Wells, 2004; Arkin and Whitty, 2009; Berg, 2003; Berg, 2008; Blazer and Neubig, 2008; Busschots et al., 2009; Niu and Chen, 2009).

RGS proteins are GTPase accelerating proteins (GAPs) for heterotrimeric G protein α subunits (Berman et al., 1996). They increase the intrinsic rate of GTP hydrolysis by the Gα, thus reconciling the paradox of the sub-second regulation of G protein signaling *in vivo* vs. the relatively long half life of GTP bound to purified Gα *in vitro*. In mammals, there are over 20 known RGS proteins that interact with limited selectivity to most Gα subtypes (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002).

There is substantial interest in the therapeutic potential of small molecule modulators of RGS proteins (Blazer and Neubig, 2008; Neubig and Siderovski, 2002; Riddle et al., 2005; Traynor et al., 2009; Zhong and Neubig, 2001). 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 (Bueno and Fioramonti, 1988)).

To understand the physiological ramifications of inhibiting RGS protein GAP activity, our laboratory has developed two lines of mice that express mutant $G\alpha_o$ or $G\alpha_{i2}$ that are insensitive to RGS effects (G184S). These mice show dramatic phenotypes, including resistance to diet-induced obesity and antidepressant-like behavioral effects ((Huang et al., 2008; Huang et al., 2006), and Talbot et al submitted). RGS4 is upregulated in the dorsal horn of spinal cord during the development of neuropathic pain (Garnier et al., 2003) and RGS4 can inhibit several pain modulating receptors (e.g. μ-opioid receptor) (Garnier et al., 2003; Traynor and Neubig, 2005). Consequently, small molecule modulators of RGS function should have utility as research tools and potentially as therapeutics. Due to the wealth of information on the structure and function of 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 (Roof et al., 2006; Roof et al., 2008; Wang et al., 2008) and one disclosed small molecule inhibitor (Roman et al., 2007). 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 (Roof et al., 2006)). The small molecule compound CCG-4986 irreversibly inhibits RGS4 by reacting with one or more cysteine residues (Kimple et al., 2007) and (Roman, Blazer & Neubig, in revision)) and its activity is lost in the presence of free thiols. This mechanism of action makes CCG-4986 less desirable as a potential lead compound for small molecule probe development. 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 paper describes the identification and characterization of the first class of reversible small molecule inhibitors of an RGS protein. They were found in a biochemical high-throughput screen carried out in the presence of dithiothreitol (DTT). They inhibit the binding and GAP activity of RGS4 with $G\alpha_o$ in a reversible manner through an interaction at an allosteric regulatory site 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.

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). $\gamma[^{32}P]$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 Figure 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).

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 (Ingalls EA, 1967). 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-4Hpyrido[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 (Supplemental Fig. 1). 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).

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 (Roman et al., 2009). For the mutagenesis studies, ΔN51RGS4 (rat) wild type and cysteine → alanine mutants were expressed from the pMALC2H10 vector. Mutagenesis was performed as described (Roman, Blazer & Neubig, in revision) 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 (Lan et al., 1998; Lan et al., 2000; Lee et al., 1994; Roman et al., 2007; Roof et al., 2008). 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 (Lee et al., 1994). G protein activity was determined by $[^{35}S]GTP\gamma S$ binding (Sternweis and Robishaw, 1984). 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 (Sternweis and Robishaw, 1984).

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 Gα_o: Gα_o labeling was performed as previously described (Roman et al., 2007). 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 (Leifert et al., 2006). For the saturation experiments, Tb-Gα_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 MgCl2, 5 µM $AlCl_3$ and allowed to activate for 10

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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-Gα_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 520nm 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 Gα_o was added to the mixture. For this assay, the positive inhibition control (i.e.

no RGS4/Gα_o binding) was Tb-labeled Gα_o in the inactive GDP-bound state and the negative control (i.e. full RGS4/Gα_o binding) utilized Gα_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 Gα_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

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described (Roman et al., 2007) 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 Gα_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 Gα_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 Gα_o as described previously (Roman et al., 2007; Roof et al., 2006).

Thermal Stability Measurements: Untagged ΔN19RGS4 or His$_6$-Gα_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 (Eqn. 1):

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

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-Gα$_o$ interaction screen: We developed a biochemical TR-FRET assay using purified human RGS4 labeled with the AlexaFluor-488 acceptor fluorophore and purified Gα$_o$ labeled with the LanthaScreen Tb probe donor fluorophore (Fig. 1A). Using this system, we observed a saturable, aluminum fluoride-dependent interaction between RGS4 and Gα that has an affinity consistent with other reports of this PPI in the literature (Fig. 1B) (Roman et al., 2007). 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/Gαo binding in the presence of a thiol reducing agent (Table 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 2SD 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.

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 Gα$_o$ to an RGS protein on beads (Roman et al., 2007). Of the 11 compounds tested, 2 showed similar activity on RGS4 in both the TR-FRET dose response and FCPIA experiments (Fig. 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 CCG-63808 and CCG-63802 (Fig. 1D). These compounds differ solely by the substituents on the phenyl moiety and have similar IC_{50} values in TR-FRET and FCPIA. The compounds also contain a vinyl cyanide moiety that may function as a reversible Michael acceptor.

CCG-63802 & CCG-63808 selectively inhibit $G\alpha_o$-RGS interactions: Using TR-FRET to assess the RGS4-$G\alpha_o$ interaction, CCG-63802 and CCG-63808 had IC_{50} values of 1.9 and 1.4 µM, respectively (Fig. 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, Table 2). The compounds are 6- to 7-fold less potent in blocking $G\alpha_o$/RGS4 interactions when tested using FCPIA (IC_{50} ~10 µM) 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). They are also fairly active (IC_{50} 20-50 µM) on the one RZ family member tested, RGS19.

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

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binding. The two compounds inhibit the GAP activity of RGS4 as shown by measurements using the [^{32}P]GTP single turnover GAP assay (Fig. 3). Under these conditions, GTP hydrolysis by Gα_o is accelerated ~10-fold by the addition of wtRGS4 and this effect can be inhibited by the previously described (Roman et al., 2007) RGS4 inhibitor CCG-4986. At a concentration of 50 µM, CCG-63802 and CCG-63808 fully inhibit the RGS activity without affecting basal Gα_o GTPase activity.

CCG-63802 & CCG-63808 bind to RGS4 but not to Gα_o: Since 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 Gα_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 principal that the stability of a protein is often altered upon ligand binding (Lo et al., 2004; Senisterra et al., 2008). 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, Gα_o experiences a >5°C increase in melting temperature (T_m) when compared to nucleotide-free protein (Supplemental Fig. 2). This increases to a >20°C increase in T_m for Gα_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. 4A). 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 Gα_o (Fig. 4B).

CCG-63802 and CCG-63808 are reversible inhibitors of the Gα_o-RGS interaction: The effects of CCG-4986, our previously described RGS4 inhibitor (Roman et al., 2007), could not be reversed by dilution and washing away the compound, showing that it acts irreversibly to inhibit the function of RGS4 ((Kimple et al., 2007), (Roman, Blazer & Neubig, in revision), and Fig. 5). 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 ((Kimple et al., 2007) and (Roman, Blazer & Neubig, in revision)). 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 Gα_o binding (Fig. 5). In contrast to the effects of CCCG-4986, full binding was restored to compound-treated RGS-beads after washing (Fig. 5), 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.

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 (Supplemental Fig. 3, Table 3). Consequently, we tested CCG-63808 and CCG-63802 with a panel of RGS4 RGS domain cysteine mutants using FCPIA (Table 3). The G protein binding affinity of these RGS mutants

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has been previously described (Roof et al., 2009) 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 rather 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.

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. 6). 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. 6).

Interestingly, CCG-63802 and CCG-63808 inhibit the GAP activity of RGS4c (Fig. 7) mutant despite their much lower potency to inhibit $G\alpha_o$/RGS4c binding in FCPIA (Supplemental Fig. 3).

Thus these 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 Gα_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 (data not shown), which generally blocks the activity of promiscuous small molecule aggregators (Feng et al., 2007).

Discussion:

RGS proteins play a strong modulatory role in GPCR signaling leading to substantial interest in small molecule inhibitors targeting this class of proteins (Blazer and Neubig, 2009; Neubig and Siderovski, 2002; Zhong and Neubig, 2001); (Riddle et al., 2005; Traynor et al., 2009). The localized expression of RGS proteins (Kurrasch et al., 2004) suggested that RGS inhibitors could provide enhanced tissue specificity for GPCR agonist actions (Blazer and Neubig, 2009; Neubig and Siderovski, 2002; Zhong and Neubig, 2001). Furthermore, up-regulation of RGS proteins in various disease states, for example RGS4 in neuropathic pain models (Garnier et al., 2003), 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 (Roman et al., 2007) which is irreversible and loses function in the presence of reducing agents ((Kimple et al., 2007) and (Roman, Blazer & Neubig, in revision)), the new compounds identified here, act reversibly and 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.

Similar to our original compound, 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 some dependence on cysteine residues since they very weakly inhibit the cysteine-null (C→A) mutant of RGS4 (RGS4c) in the FCPIA assay. However, both compounds at 50 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 $G\alpha$-GTP than for the GDP-AlF_4^- conformation of the $G\alpha$ subunits. Indeed, it is the effects of compounds on RGS GAP activity and not on $G\alpha$ binding that are most relevant in cellular or animal models.

The partial cysteine-dependence of the actions of these compounds suggests a tethering model in which a reactive group binds to RGS cysteine residue(s). 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 (Arkin et al., 2003; Erlanson et al., 2000). Our ability to detect these compounds 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 (Ettari et al., 2008; Jin et al., 2007). 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 (Ocana and Amir, 2009; Sachs et al., 1994). 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 (Erlanson et al., 2003). The activity of CCG-63802 and CCG-63808 to inhibit the GAP activity of RGS4c suggests that this may be a reasonable approach.

The compounds described here require three cysteines for full potency of RGS4 inhibition: cys 95, cys 148 and cys 132. Cysteines 95 and 148 are positioned in the “B site” of RGS proteins (Zhong and Neubig, 2001) which is proposed to participate in the allosteric modulation of RGS4 by acidic phospholipids and calmodulin (Ishii et al., 2005a; Popov et al., 2000). Cysteine 132 is located on the outer edge of the Gα interaction interface and, at high concentrations, may react with CCG-63802 in a reversible Michael reaction to provide modest steric occlusion of the protein-protein interaction. Consequently, these compounds appear to have both allosteric and steric elements in their mechanism.

The binding of CCG-63802 induces a destabilizing effect on RGS4 in the thermal stability studies. This contrasts with the stabilizing effect observed (Grasberger et al., 2005; Wan et al., 2009) for small molecule ligand binding to many proteins (e.g. $G\alpha_o$, Supplemental Fig 2). We observed that this family of compounds causes a left shift in the melting curve to lower temperatures. 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 (Grasberger et al., 2005; Wan et al., 2009). This notion was recently borne out by the recent crystallization of several GPCRs (Cherezov et al., 2007; Jaakola et al., 2008; Rasmussen et al., 2007; Scheerer et al., 2008; Warne et al., 2008). In all cases (the notable exception being opsin), crystals were only obtained in the presence of – among other reagents – a small molecule ligand. This strongly suggests that these ligands are important for the structural stability of this class receptor in solution. Furthermore, our data (Supplemental Fig. 2) and others (Abad et al., 2008; Matulis et al., 2005) 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. This increased protein rigidity is likely to provide a level of protection against the increasingly intense thermally-induced conformational fluctuations as the temperature of the sample is raised.

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. 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.

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

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action at the “B” site on the RGS (Zhong and Neubig, 2001), which has been implicated in the physiological allosteric modulation of RGS proteins by acidic phospholipids and calmodulin (Ishii et al., 2005a; Ishii et al., 2005b). Further studies of the mechanism and structure-activity relationships for this compound class and translation to cellular and animal models of RGS function are currently underway.

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Figure Legends:

Figure 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. 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. D) The chemical structures of CCG-63802 and CCG-63808. n=3 for all data. Data are presented as mean±SEM or mean (95% CI) in B and C, respectively.

Figure 2. RGS specificity of A) CCG-63802 and B) CCG-63808 determined by multiplex FCPIA analysis (n≥3). RGS coated beads were treated with the indicated concentration of compound for 15 minutes at room temperature, after which GDP/AlF_4 bound Gα_o-AF532 was added and allowed to incubate with the RGS/compound mixture for 30 minutes prior to analysis. 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.

Figure 3. 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 Gα_o. Representative GAP data shown, however all experiments were performed a minimum of 3 times. B) Rate constants of GTP hydrolysis. Rate constants are

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presented as mean ± SEM from at least 3 independent experiments. **$p<0.01$, ***$p<0.001$ vs. the DMSO treated RGS control.

Figure 4. CCG-63802 specifically binds to RGS4 and not to Gα_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 (100µM) does not affect the melting temperature of Gα_o. Data are presented as mean±SEM of 3 separate experiments.

Figure 5. 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 Gα_o-AF532 in the presence or absence of 50 µM Compound. Data shown are the mean±SEM of three separate experiments.

Figure 6. 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: $LogIC_{50}$ -5.25±0.07 to -4.39±0.07; CCG-63808: $LogIC_{50}$ -5.39±0.06 to -4.68±0.03). In contrast, C) CCG-4986 loses over two logs of potency ($LogIC_{50}$ -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.

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Figure 7. 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 Gα_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, ***p<0.001 vs. the DMSO treated RGS control.

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Table 1. 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.

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. 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**	
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

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Table 3. RGS4 cysteine mutant sensitivity to CCG-63802. Data are presented as mean ±SEM.

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

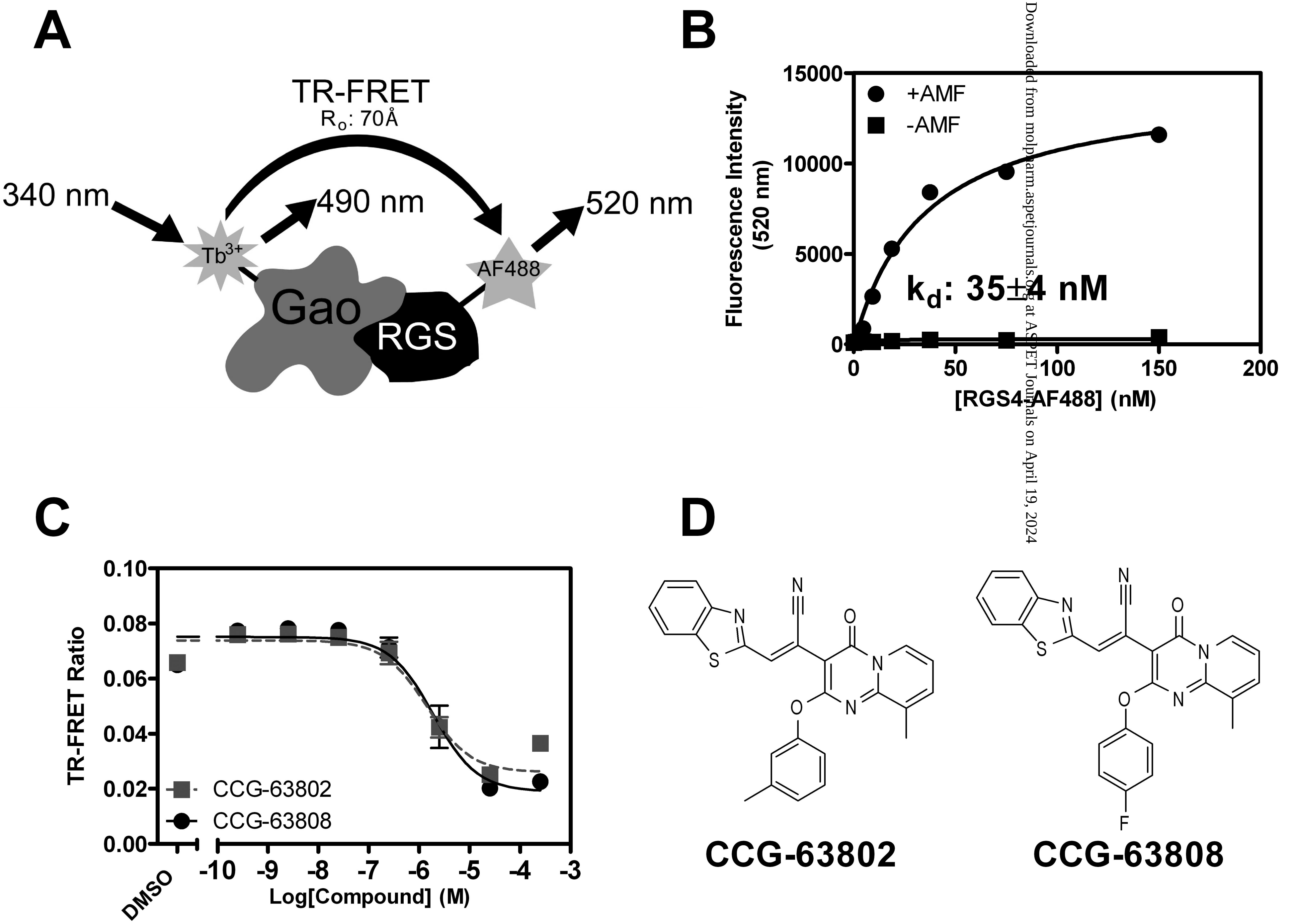
A
TR-FRET
R$_o$: 70Å
340 nm
490 nm
520 nm
Tb^{3+}
AF488
Gao
RGS
B
Fluorescence Intensity (520 nm)
15000
10000
5000
0
+AMF
-AMF
k$_d$: 35±4 nM
0
50
100
150
200
[RGS4-AF488] (nM)
C
TR-FRET Ratio
0.10
0.08
0.06
0.04
0.02
0.00
CCG-63802
CCG-63808
DMSO
-10
-9
-8
-7
-6
-5
-4
-3
Log[Compound] (M)
D
N
S
N
O
N
N
O
F
CCG-63802
CCG-63808

Figure 1.

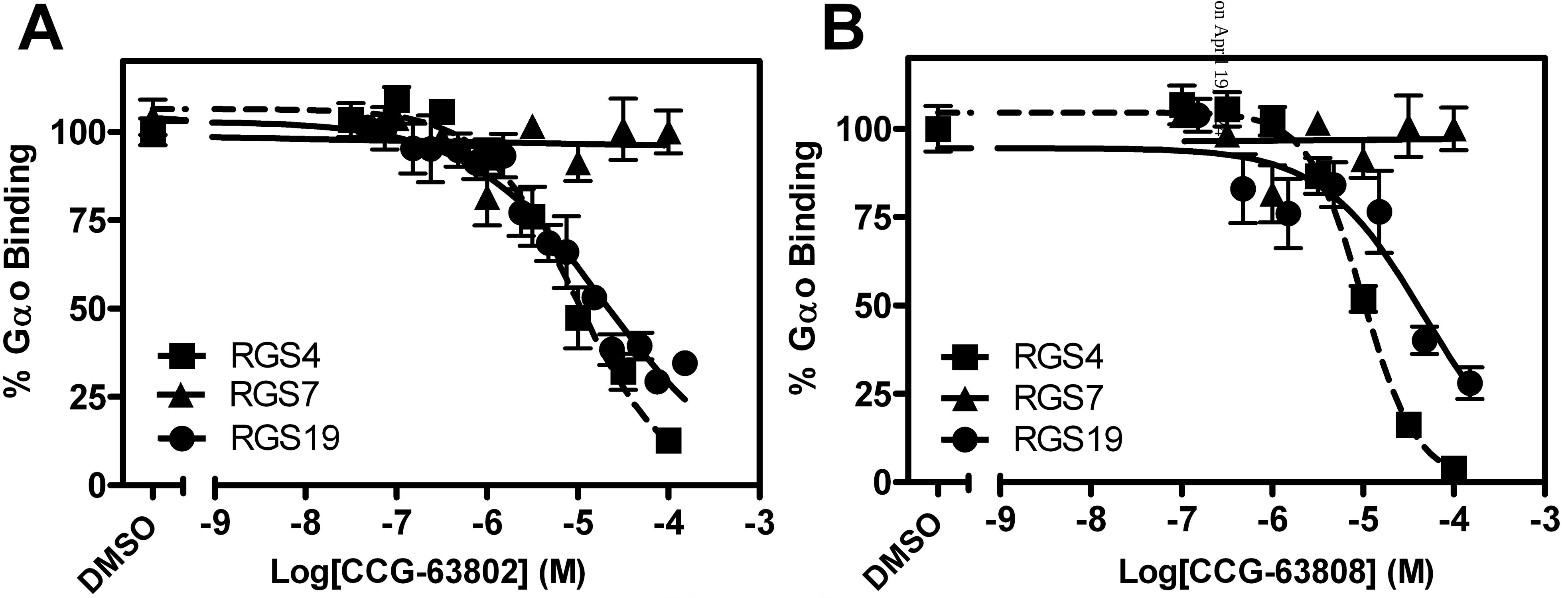

A
% Gαo Binding
0
25
50
75
100
DMSO
-9
-8
-7
-6
-5
-4
-3
Log[CCG-63802] (M)
RGS4
RGS7
RGS19
B
% Gαo Binding
0
25
50
75
100
DMSO
-9
-8
-7
-6
-5
-4
-3
Log[CCG-63808] (M)
RGS4
RGS7
RGS19

Figure 2.

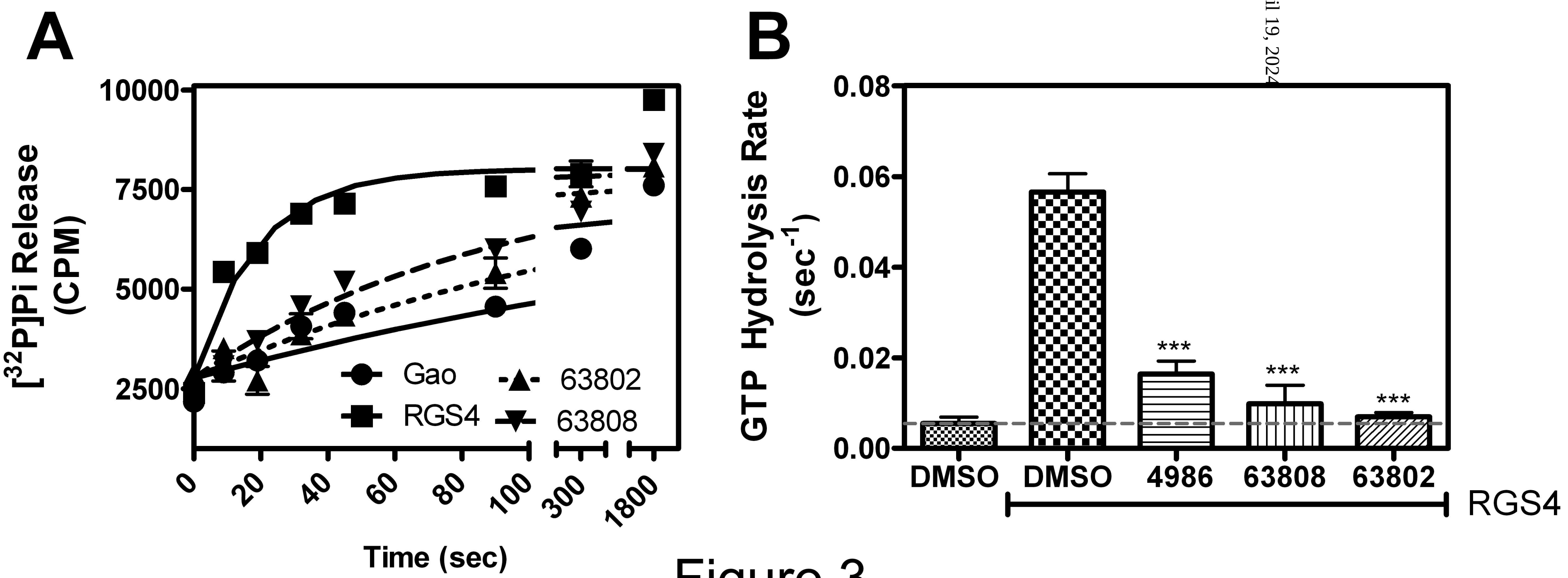

A
$[^{32}P]$Pi Release (CPM)
10000
7500
5000
2500
0
20
40
60
80
100
300
1800
Time (sec)
Gao
RGS4
63802
63808
B
GTP Hydrolysis Rate (sec^{-1})
0.08
0.06
0.04
0.02
0.00
***
***
***
DMSO
DMSO
4986
63808
63802
RGS4

Figure 3.

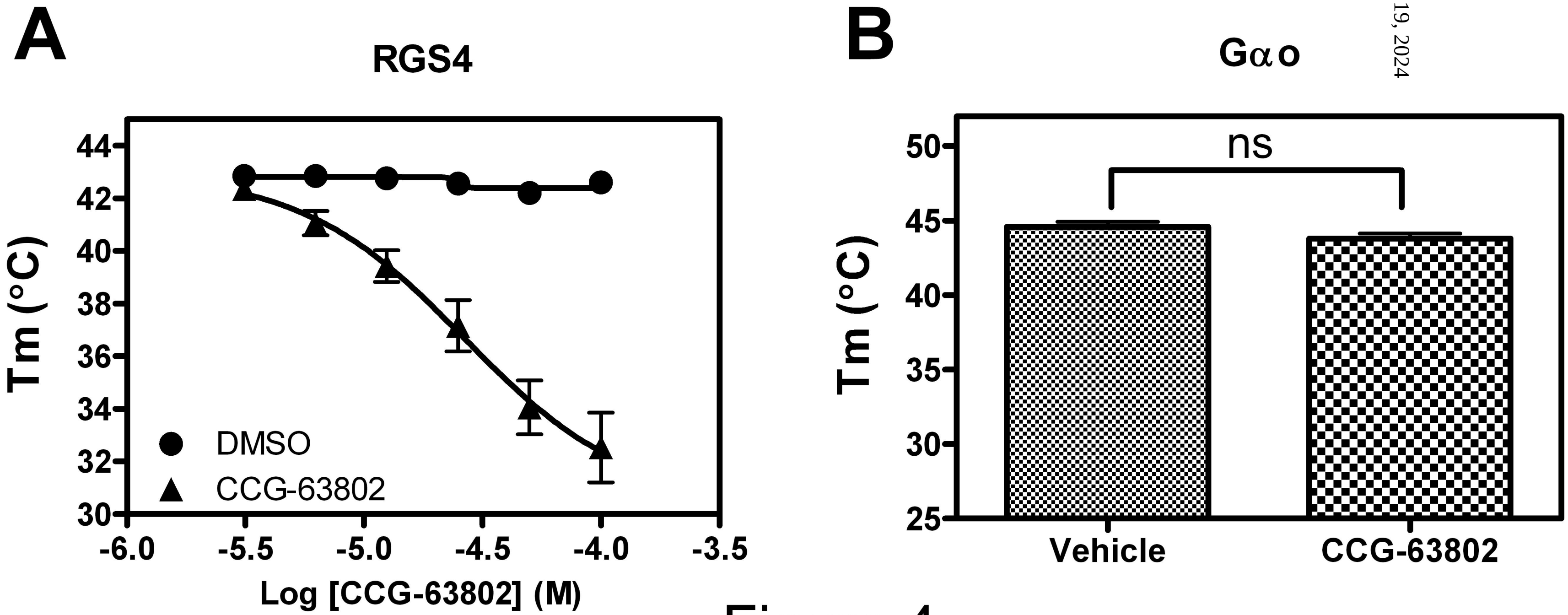

A
RGS4
Tm (°C)
44
42
40
38
36
34
32
30
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
Log [CCG-63802] (M)
DMSO
CCG-63802
B
Gαo
ns
50
45
40
35
30
25
Vehicle
CCG-63802

Figure 4.

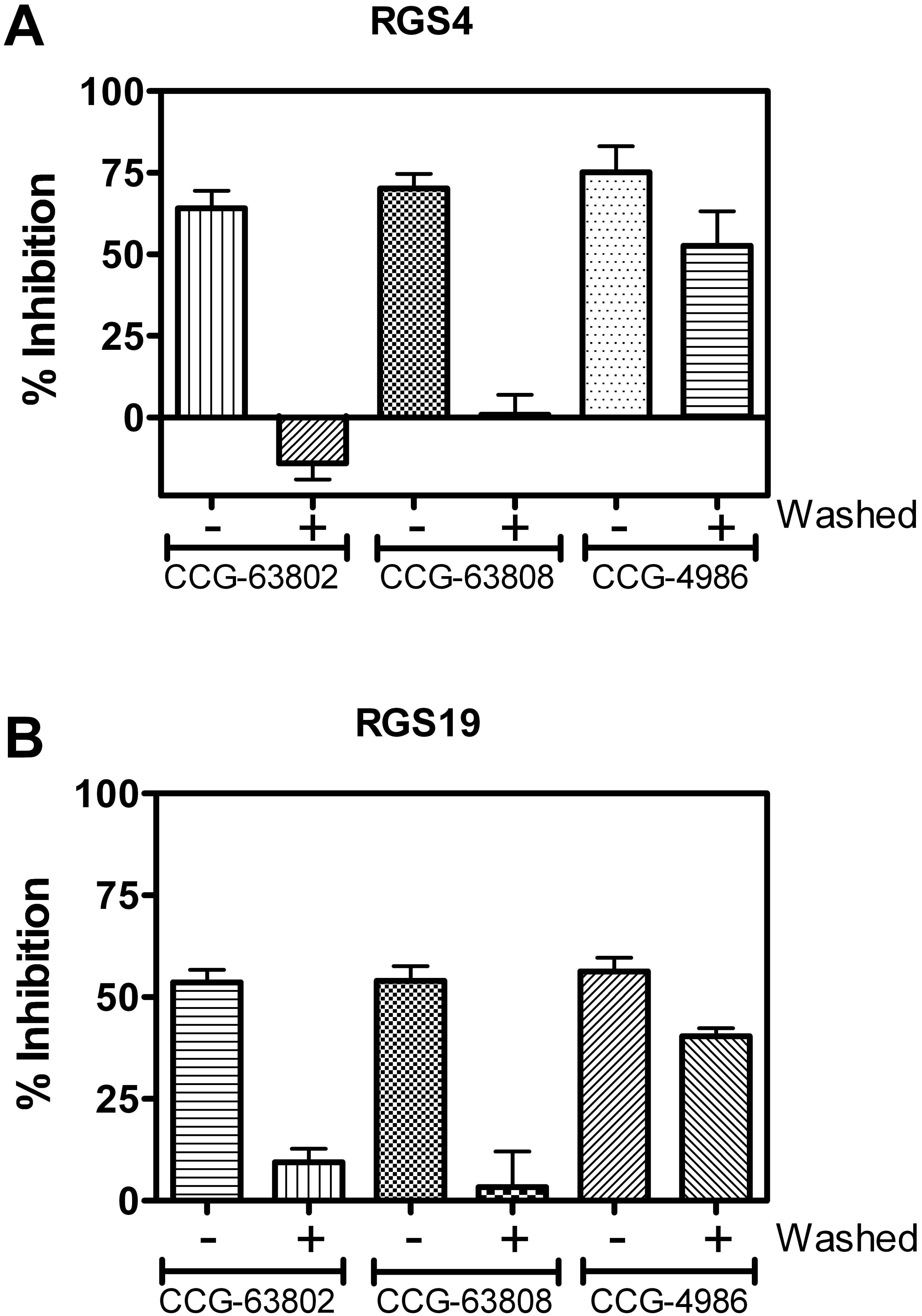

A
RGS4
% Inhibition
100
75
50
25
0
-
+
-
+
-
+
Washed
CCG-63802
CCG-63808
CCG-4986
B
RGS19
% Inhibition
100
75
50
25
0
-
+
-
+
-
+
Washed
CCG-63802
CCG-63808
CCG-4986

Figure 5.

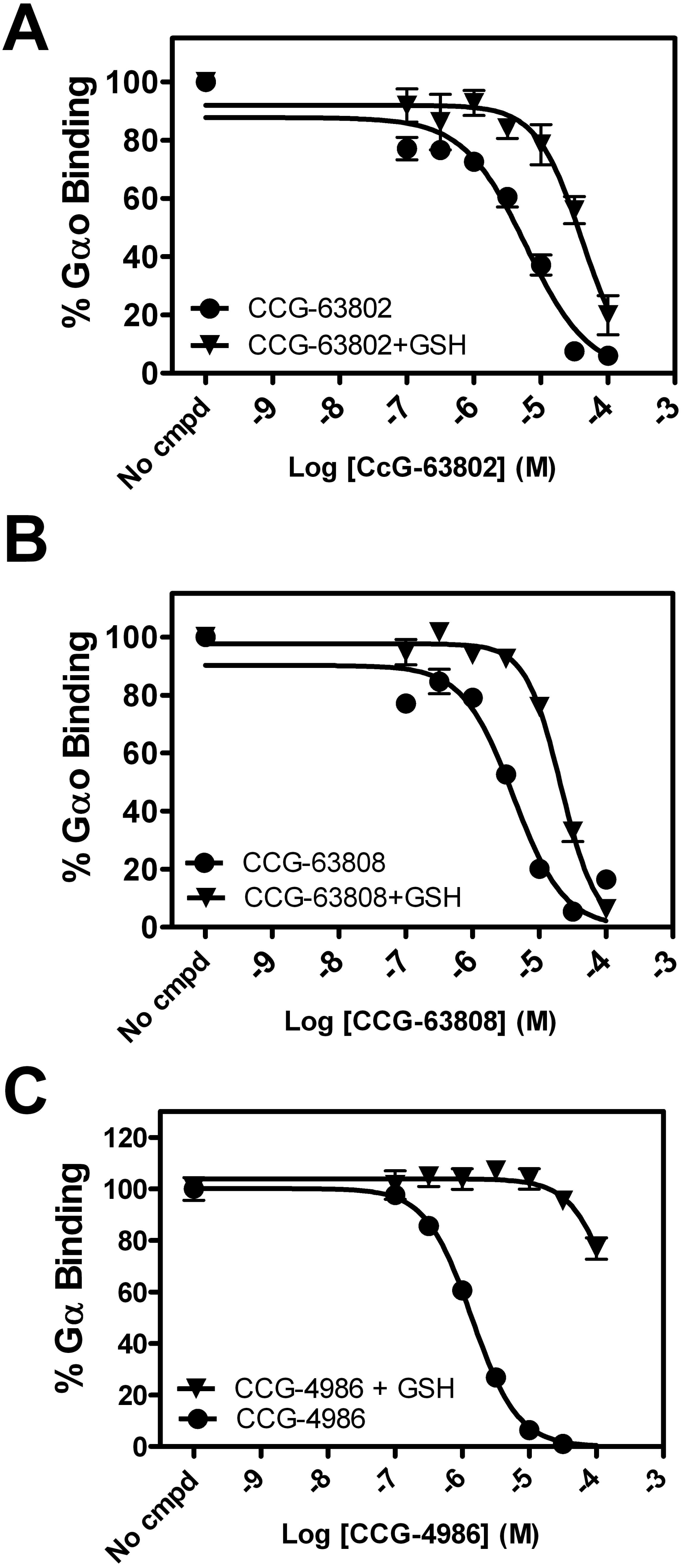

A
% Gαo Binding
100
80
60
40
20
0
CCG-63802
CCG-63802+GSH
No cmpd
-9
-8
-7
-6
-5
-4
-3
Log [CcG-63802] (M)
B
% Gαo Binding
100
80
60
40
20
0
CCG-63808
CCG-63808+GSH
No cmpd
-9
-8
-7
-6
-5
-4
-3
Log [CCG-63808] (M)
C
% Gα Binding
120
100
80
60
40
20
0
CCG-4986 + GSH
CCG-4986
No cmpd
-9
-8
-7
-6
-5
-4
-3
Log [CCG-4986] (M)

Figure 6.

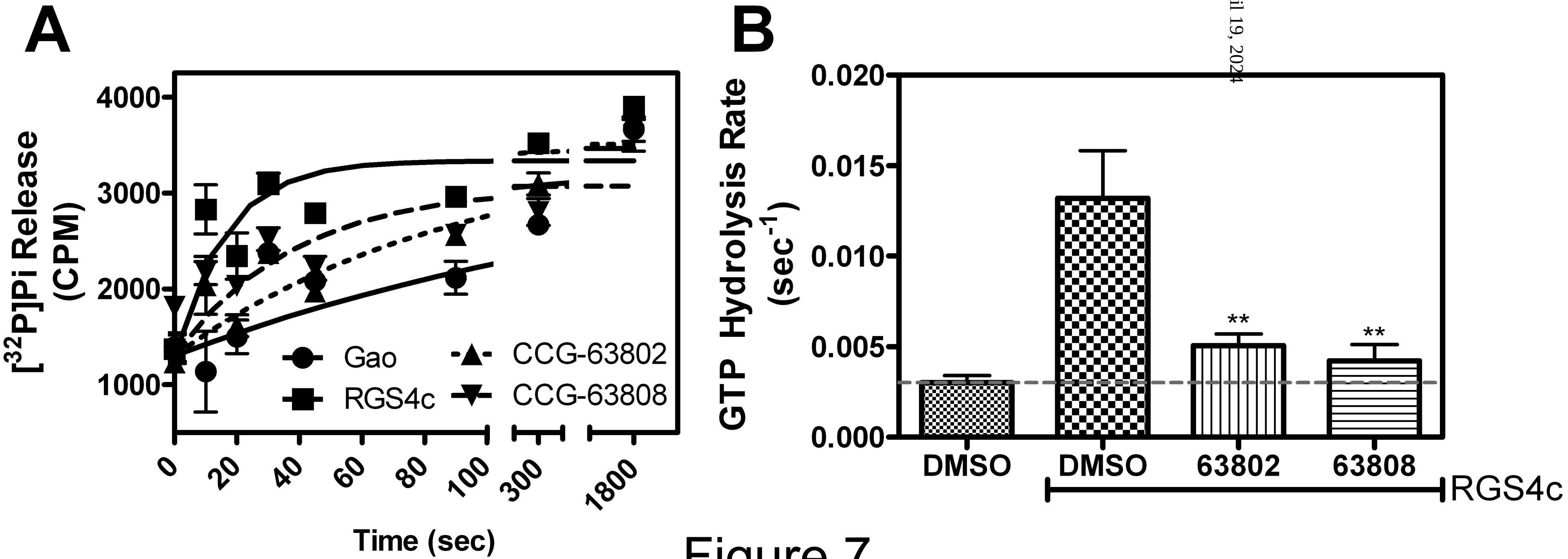
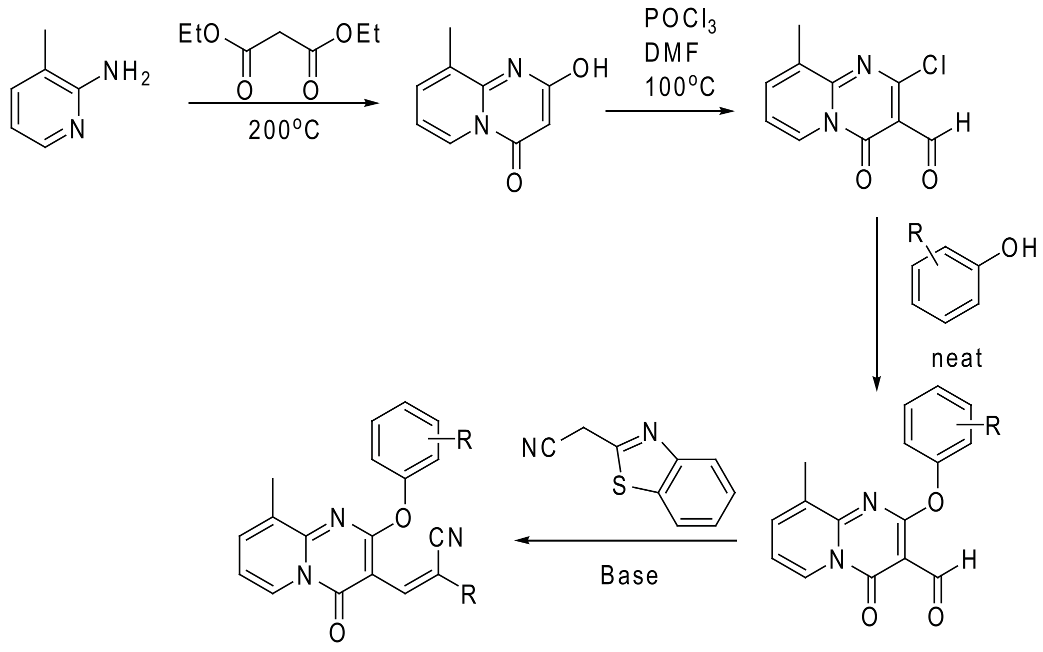

A
[32P]Pi Release (CPM)
4000
3000
2000
1000
0
20
40
60
80
100
300
1800
Time (sec)
Gao
RGS4c
CCG-63802
CCG-63808
B
GTP Hydrolysis Rate (sec-1)
0.020
0.015
0.010
0.005
0.000
DMSO
DMSO
63802
63808
**
**
RGS4c

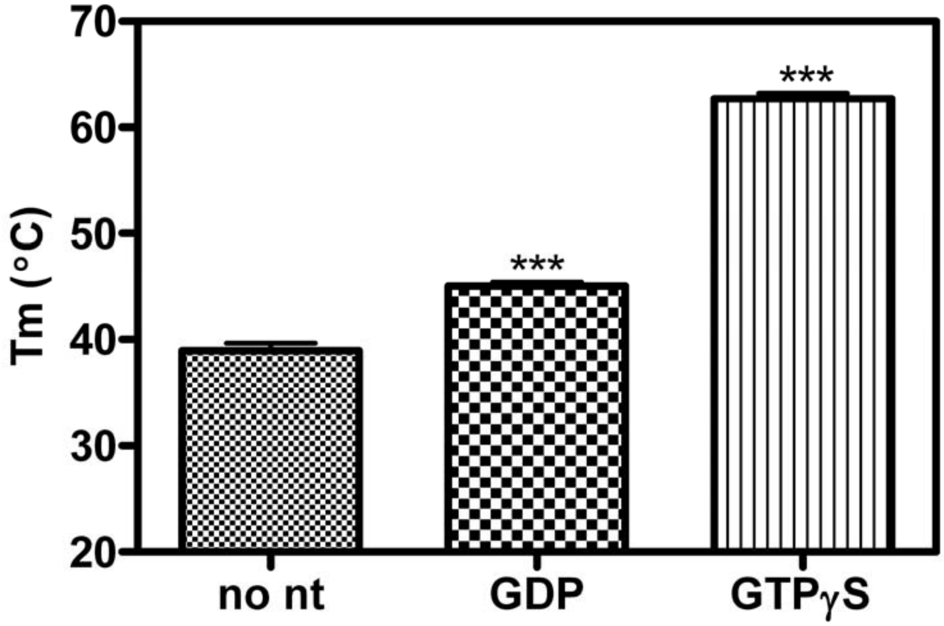
Figure 7.

Supplemental Figures:

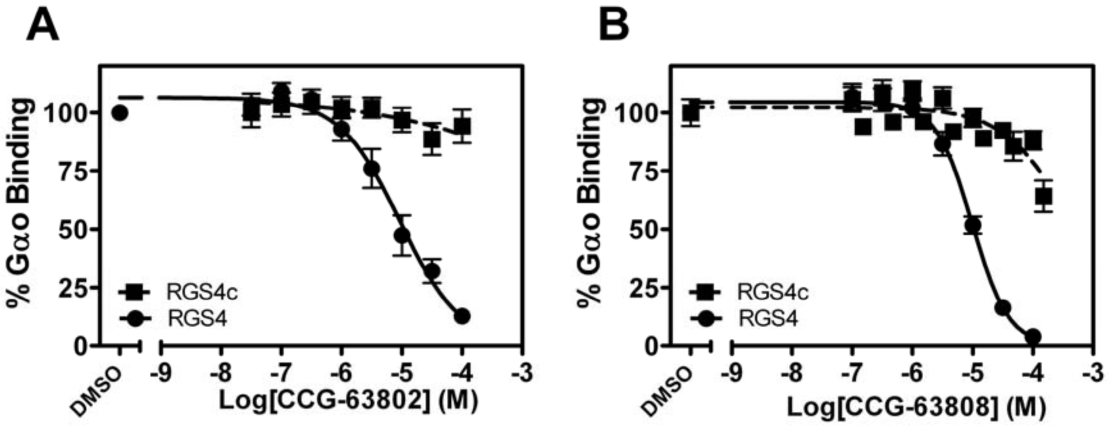
Blazer LL, Roman DL, Chung A, Larsen MJ, Greedy M, Husbands SM, Neubig RR. Reversible, allosteric, small-molecule inhibitors of RGS proteins. Molecular Pharmacology



Supplemental Figure 1. Reaction scheme for the synthesis of CCG-63802 and CCG-63808.



Supplemental Figure 2. Gα$_o$ is thermally stabilized in presence of nucleotide. Purified Gα$_o$ 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 Gα$_o$ (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) Gα$_o$ 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.



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