

Identification of small molecule inhibitors of Regulator of G-protein Signaling 4 (RGS4) using a high throughput flow cytometry protein interaction assay (FCPIA)

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Identification of small molecule inhibitors of RGS4

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Non-standard abbreviations:

FCPIA	Flow Cytometry Protein Interaction Assay
RGS	Regulator of G-protein Signaling
GAP	GTPase Accelerating Protein
CCG-4986	(methyl N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfonimidoate)

Abstract

Regulators of G-protein signaling (RGS) proteins are important components of signal transduction pathways initiated through G-protein coupled receptors (GPCRs). RGS proteins accelerate the intrinsic GTPase activity of G-protein α -subunits ($G\alpha$) and thus shorten the time course and reduce the magnitude of G-protein α and $\beta\gamma$ subunit signaling. Inhibiting RGS action has been proposed as a means to enhance the activity and specificity of GPCR agonist drugs but pharmacological targeting of protein-protein interactions has typically been difficult. The aim of this project was to identify inhibitors of RGS4. Using a Luminex® 96-well-plate bead analyzer and a novel flow-cytometric protein interaction assay (FCPIA) to assess $G\alpha$ -RGS interactions in a high-throughput screen, we identified the first small molecule inhibitor of an RGS protein. Of 3028 compounds screened, one, CCG-4986, inhibited RGS4/ $G\alpha_o$ binding with 3-5 μ M potency. It binds to RGS4, inhibits RGS4 stimulation of $G\alpha_o$ GTPase activity *in vitro*, and prevents RGS4 regulation of μ -opioid inhibited adenylyl cyclase activity in permeabilized cells. Furthermore, CCG-4986 is selective for RGS4 and does not inhibit RGS8. Thus we demonstrate the feasibility of targeting RGS/ $G\alpha$ protein-protein interactions with small molecules as a novel means to modulate GPCR-mediated signaling processes.

Inhibition of signaling networks through disruption of protein-protein interactions presents unique new targets for development of chemical tools and for possible therapeutic drug discovery (Arkin and Wells, 2004; Pagliaro et al., 2004). While extracellular ligand binding domains, ion channels, and enzyme active sites are considered more “druggable”, blockade of signaling *per se* generally stops all function. In contrast, inhibition of regulatory protein-protein interactions allows manipulation of distinct steps of a signaling cascade or modifies subcellular targeting which should permit more subtle pharmacological effects.

Activated G protein coupled receptors (GPCRs) turn on signaling by inducing GTP exchange for GDP on G α subunits which then undergo an activating conformational change releasing free α and $\beta\gamma$ subunits to cause downstream events, such as channel opening, control of adenylyl cyclase, or hydrolysis of phosphoinositides (Gilman, 1987). Upon hydrolysis of the bound GTP, the G α subunit returns to its inactive, GDP-liganded form and its signaling functions are terminated. The hydrolysis of GTP due to the G α subunit’s intrinsic GTPase activity is very slow but is accelerated over 1000-fold by Regulators of G protein Signaling (RGS) proteins (Lan et al., 2000; Mukhopadhyay and Ross, 1999). The GTPase Accelerating Protein (GAP) activity of RGS proteins shortens the lifetime and, in general, reduces the magnitude of signaling by both the active G α and the free G $\beta\gamma$ as G $\alpha/\beta\gamma$ reassociation occurs rapidly upon formation of the G α -GDP complex (Neubig et al., 1994). In addition to their GAP activity RGS proteins can competitively inhibit effector coupling by G α subunits (Hepler et al., 1997). Of the 20 classical RGS proteins, 19 act on G α_i family G proteins while at least 12 act on G α_q family signaling (Traynor and Neubig, 2005). Loss of RGS function *in vivo* leads to markedly altered GPCR signaling (Fu et al., 2006; Heximer et al., 2003b; Huang et al., 2006; Rahman et al., 2003).

The RGS proteins have been proposed as novel drug targets (Cho et al., 2004; Liebmann, 2004; Neubig and Siderovski, 2002; Riddle et al., 2005; Zhong and Neubig, 2001) since the utility of targeting GPCRs directly is the basis for the development of many therapeutics. Furthermore, evidence is mounting that RGS proteins may play a role in disease states such as schizophrenia (Erdely et al., 2006; Mirnics et al., 2001; Williams et al., 2004), Parkinson’s disease (Ding et al., 2006; Kovoor et al., 2005; Tekumalla et al.,

2001), hypertension (Heximer et al., 2003a; Riddle et al., 2006), and addiction (for review, see Traynor and Neubig, 2005). Inhibiting a specific RGS would potentiate the action of natural or exogenous agonist ligands. Moreover, it could enhance the tissue specificity of broadly active agonist ligands by selectively increasing receptor action in tissues that express that particular RGS protein (Zhong and Neubig, 2001). RGS specificity for discrete G α subunits could also permit a pathway-specific enhancement of agonist action (Neubig and Siderovski, 2002; Zhong and Neubig, 2001).

Small molecule inhibitors of RGS proteins would be useful tools for dissecting the role of RGS proteins and may provide a first-step toward the development of therapeutics. Previous work from our lab identified a cyclic octapeptide derived from the RGS interaction site of G α_i subunits that inhibits RGS4 and alters GIRK current kinetics in atrial cells (Jin et al., 2004; Roof et al., 2006). Another group performed a small molecule RGS inhibitor screen using a yeast 2-hybrid readout (Young et al., 2004) that yielded some low affinity inhibitors but no structures or follow-up information were reported.

Therefore, our aim was to identify small molecules that inhibit RGS4 activity. However, the standard biochemical assay for RGS protein function is cumbersome and not appropriate for high-throughput screening (HTS), as it involves a single-turnover assay of [³²P]GTP hydrolysis using a charcoal precipitation step. We chose to measure the high affinity protein-protein interaction between purified G α subunits and RGS proteins when the G α subunit is in the transition-state conformation induced by GDP and AlF₄⁻. We used a flow cytometry protein interaction (FCPIA) approach based on previous studies measuring G α and $\beta\gamma$ subunits interactions (Sarvazyan et al., 1998) and ligand-receptor-G-protein interactions (Simons et al., 2003; Sklar et al., 2002).

Using FCPIA, we screened a 3028 compound library from the Chembridge screening collection to identify inhibitors of the RGS4/G α_o protein-protein interaction. This screen resulted in five potential RGS4 inhibitors, two of which had IC₅₀ values less than 10 μ M. One compound, CCG-4986, inhibited RGS4 GAP activity, as well as RGS4 activity on μ -opioid receptor-mediated signaling. Also, CCG-4986 is selective for RGS4 as compared to RGS8, the most closely related RGS family member (Neubig, 2002).

Thus we report the first small molecule RGS inhibitor which should prove a useful tool for studying their role in GPCR signaling.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Hampton, NH) or Acros Organics (Geel, Belgium) and were reagent grade or better. Avidin-coated microspheres for flow cytometry were purchased from Luminex (Austin, TX). [³²P]GTP and GTPγ[³⁵S] were purchased from Perkin Elmer (Boston, MA). The screening compound library was from the Chembridge screening collection (hit2lead.com) and the identity and purity (>96%) of CCG-4986 (methyl N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate) was confirmed by LC-MS through the University of Michigan Dept. of Pharmacology Mass Spec Facility. Three separate batches of CCG-4986 were obtained to complete these studies, each with NMR analysis through Chembridge that verified its structure. Tissue culture media, Geneticin, fetal bovine serum and trypsin were purchased from Invitrogen (Carlsbad, CA). DAMGO was obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). Digitonin was obtained from Gallard-Schlesinger (Plainview, NY). cAMP kits were purchased from Diagnostic Products (Los Angeles, CA). Data were analyzed using Graphpad Prism 4.0 (Graphpad Software, San Diego CA).

RGS protein Expression and Purification. N-terminally truncated (initiating at Met 19) rat RGS4, designated pQE60RGS4short (Krumins et al., 2004), was used for the experiments in this study¹. The ΔN-variant was selected because it expresses higher levels of soluble protein in *E. coli* as compared to full length RGS4. The protein was prepared essentially as described in (Krumins et al., 2004). After lysis and centrifugation, the supernatant was applied to a 70mL S Sepharose Fast Flow (Amersham Biosciences) FPLC column. The column was washed with 3 column volumes (cv) of Buffer A (50mM HEPES, 1mM DTT, pH 7). A two-stage linear gradient was run, with 0-20% Buffer B (Buffer A + 1M NaCl) in 3 cv and 20-55% in 13 cv. RGS4 eluted in a single broad peak between 290-380mM NaCl. Purified RGS4 was isolated using a Superdex 75 PG gel filtration column. A GST-tagged rat full-length RGS8 was purified as per Lan et al (Lan et al., 1998).

Expression and Purification of $G\alpha_o$. 6xHis-tagged $G\alpha_o$ was expressed and purified from transformed BL21-DE3 E. coli as previously described (Lee et al., 1994). Activity of purified $G\alpha_o$ was determined by [35 S] GTP γ S binding (Sternweis and Robishaw, 1984).

Chemical Biotinylation of Purified RGS Proteins. RGS proteins were biotinylated with amine-reactive biotinamidohexanoic acid N-hydroxysuccinimide ester (NHS) (Sigma, B2643, MW 454.54) in a 2:1 (biotin:RGS) stoichiometry. Biotin-NHS (1mg) was resuspended, with sonication, in 1mL ddH₂O. 40 μ L (100nmol) was added to 1 mg of Δ N-RGS4 (~50nmol) in a total volume of 300 μ L H₅₀E₁N₁₀₀ (50mM HEPES, 1mM EDTA, 100mM NaCl pH 8), and incubated, rotating, at 4C. The reaction was terminated with 10 μ L 1M glycine and excess biotin removed by applying the sample to a 1mL Sephadex G25 spin column and eluting with HEN buffer.

Fluorescent Labeling of $G\alpha_o$. Purified $G\alpha_o$ was chemically labeled with Alexa Fluor® 532 carboxylic acid succinimidyl ester (Invitrogen, Carlsbad CA) at a 3:1 fluorophore:protein ratio. Purified $G\alpha_o$ (500 μ g, 12.5 nmol) was diluted in 250 μ L H₅₀E₁₀₀N₁₀₀ (50mM HEPES, 100mM EDTA, 100mM NaCl, pH8) buffer supplemented with 10 μ M GDP. Then, 2.8 μ L (~38 nmol) of an AF532 solution (1 mg /100 μ L DMSO) was added, and the solution was incubated at 4C in the dark for 1.5 hours. The reaction was quenched with 20 μ L of 1 M glycine and for 30 min. Excess fluorophore was removed via a 1 mL Sephadex G25 spin column and elution with HEN buffer supplemented with GDP. The activity and effective concentration of $G\alpha_o$ was determined post-labeling using [35 S] GTP γ S binding (Sternweis and Robishaw, 1984).

Flow Cytometry Protein Interaction Assay (FCPIA) and High Throughput Screen. Each test compound was spotted in 1 μ L of DMSO into individual wells of 96-well Axygen PCR plates using a Beckman BioMek FX robot. The plates were frozen at -20C and thawed immediately prior to screening. Luminox LumAvidin beads (500 per well, ~50K per plate) were vortexed, briefly sonicated and diluted into 1mL of Bead Coupling Buffer (BCB; PBS, pH 8.0 supplemented with 1% BSA). The beads were pelleted (60 s

at 7K rpm), supernatant was removed, and beads resuspended in 1 mL BCB. This process was repeated for 3 washes. The beads were then resuspended in 500 μ L BCB and biotinylated RGS protein was added to yield a concentration of 40 nM. The beads were incubated for 30 min at rt. After the bead coupling was complete, the beads were spun down, washed with 1mL BCB 3 times and finally resuspended in 5 mL of Flow Buffer (50mM HEPES, 100mM NaCl, 0.1% Lubrol, 1% BSA pH 8.0). Beads (50 μ l) were dispensed into each compound-containing well of the 96 well screening plate and incubated at rt for 10 min. Blank beads (i.e. mock-coupled in the absence of RGS) were used as controls in some experiments to determine nonspecific binding. During the 10 min incubation of RGS with compound, the AF532-labeled $G\alpha_o$ solution was prepared. GDP (5 μ L 10mM), $MgCl_2$ (500 μ l, 50mM), $AlCl_3$ (500 μ L, 50 μ M), and NaF (500 μ L, 50mM) were added to 3.5mL flow buffer and AF532- $G\alpha_o$ was added to form the activated $G\alpha_o$ -GDP- AlF_4^- complex (at 10nM final concentration), which binds RGS with high affinity. After 10 min to ensure activation of the $G\alpha_o$, 50 μ L was aliquoted to each RGS-bead and compound containing well of the 96 well screening plate. The proteins were incubated for 30 min at room temperature in the dark before being read on a Luminex 100IS 96-well plate reading flow cytometer. The gate was set for the appropriate bead number and 100 events were counted per well. Median fluorescence intensity values for bead-bound AF532 $G\alpha_o$ were calculated and used for data analysis. The effective concentration of screening compounds in DMSO was between 10 and 30 μ M, as the library was prepared based on compound mass. The RGS concentration in the assay was 2nM, and the $G\alpha_o$ concentration 5nM. Signal to background ratio was robust at approximately a 50-100:1. Compounds were considered hits if they inhibited the RGS4- $G\alpha_o$ interaction at least 50% as compared to controls.

For FCPIA saturation experiments, the RGS4 beads and AF532 $G\alpha_o$ were prepared as above, except that AF532- $G\alpha_o$ was prepared at 200 nM. This solution was then diluted serially down the 96-well plate to yield final $G\alpha_o$ concentrations between 0.78 and 100 nM. The incubation and plate reading were performed as in the high-throughput screen. FCPIA compound dose-response experiments were done similarly to the flow cytometry screening assay, except that the total assay volume was 150 μ L, with 50 μ L of RGS4-

beads (coupled at 3x final concentration, 6nM), 50 μ L $G\alpha_o$ -AF532 (15nM) and varying concentrations of CCG compounds to give a final range of 100 to .01 μ M.

GAP Assay. Single turnover GTP hydrolysis measurements in 96 well plates were performed as previously described (Roof et al., 2006). Compounds were preincubated with the RGS protein for 10-15 mins on ice before initiating the GAP assay.

cAMP Accumulation Assay. Rat C6 glioma cells expressing the mu opioid receptor were grown to confluency, suspended, and permeabilized by treatment with digitonin, essentially as described previously (Alt et al., 2001). Cells were resuspended in assay buffer (128mM NaCl, 2.4mM KCl, 2mM NaHCO_3 , 3mM MgSO_4 , 10mM Na_2HPO_4 , and 10mM glucose; pH 7.45) with 1 mM IBMX and 100 μ M ATP and then combined with drugs (forskolin, DAMGO) and purified RGS proteins. Assay mixtures were incubated for 15 min at 37C and reactions stopped by the addition of cold perchloric acid. cAMP concentration was measured by radioimmunoassay as described previously (Clark et al., 2003). Inhibition of cAMP formation was determined as a percentage of forskolin-stimulated cAMP accumulation in the absence of DAMGO. Compounds were preincubated with the RGS protein for 10 min at room temperature before initiating the cAMP accumulation.

Intrinsic fluorescence measurements. Fluorescence experiments were performed on a Photon Technology International AlphaScan Spectrofluorometer (PTI, New Jersey USA) with 2.5nm slits using wavelengths of 285 nm and 340 nm for excitation and emission, respectively. Δ N-RGS4 (2 μ M) in 600 μ L of buffer (20mM HEPES, 100mM NaCl, 500 μ M tris(2-carboxyethyl)phosphine) was added to a 5 mm cylindrical quartz cell with micro stir bar. The baseline reading was allowed to stabilize for 2 min, after which 1 μ L injections of a 1.2 mM solution of CCG-4986 in 50% DMSO/50% buffer were added every 30 s from a Hamilton syringe. Dose-response measurements were carried out over 10 min, with final concentrations of CCG-4986 between 1.9 and 31 μ M. For control experiments, the DMSO solvent for CCG-4986 was injected with RGS4 or CCG-4986

was added to a solution of L-tryptophan, following the same procedure and concentration steps.

RESULTS

FCPIA measurements of the RGS4/G α o protein-protein interaction.

FCPIA consists of three key components (Figure 1a-b): 1) avidin-coated microspheres 2) biotinylated RGS4, and 3) activated G α o labeled with Alexafluor 532. For the experiment, biotinylated RGS4 coupled to avidin-coated beads is co-incubated in a 96 well plate with AlF $_4^-$ activated G α o (Berman et al., 1996). Samples from each well are aspirated into the Luminex flow cytometer, the bead is detected and bead-associated fluorescence is measured, providing a quantitative reading of the amount of G α o bound to RGS4. Figure 1c demonstrates several strengths of this method for high throughput screening. Only the activated G α o incubated with microspheres precoupled to RGS4 resulted in high-affinity, saturable G α o binding. The K $_d$ for specific binding was 4.7 \pm 0.4 nM (n=4, in duplicate). The relatively high affinity of the RGS4-G α o interaction in the presence of AlF $_4^-$ as well as the very low background fluorescence provide an outstanding signal/noise ratio and permit use of very low concentrations of the protein reagents (2 nM RGS4 and 0.4-100 nM AF532-G α o), demonstrating the suitability of FCPIA for high-throughput screening.

High throughput screen for RGS4 inhibitors.

Our high throughput screen focused on identifying compounds which bound to RGS4 and disrupted its binding to G α o. The library consisted of 3028 small molecule compounds from the Chembridge collection. RGS4 and activated G α o were added to the plates and 100 beads/events per well were read on the Luminex flow cytometer. The extremely high S/N of the assay (Z' score 0.74) permitted the use of small numbers of bead events to reduce reagent use and to speed data acquisition. The screen produced a number of hit compounds as defined by 50% inhibition of the protein-protein interaction signal (Table 1). Five were confirmed in follow-up concentration-response studies and two compounds, CCG-2046 and CCG-4986, reduced the RGS4-G α o interaction signal with single-digit micromolar IC $_{50}$ values (Figure 2).

Inhibitory Effect of CCG-2046 and CCG-4986 on the catalytic GTPase accelerating activity of RGS4.

We tested our two most potent hits in a single-turnover GTPase assay (Lan et al., 1998). Figure 3 shows the ability of RGS4 to accelerate the hydrolysis of GTP by approximately 50-fold as compared to the intrinsic $G\alpha_o$ GTPase activity. Furthermore, 10 μ M CCG-4986, inhibited the catalytic activity of RGS4 by 68% as measured by this single-turnover experiment. This is consistent with the 71% inhibition predicted from the 4.2 μ M IC_{50} in the RGS4/ $G\alpha_o$ binding experiment. Surprisingly, CCG-2046 did not inhibit the GTPase activity of RGS4, identifying it as a false positive in our screen.

Effect of CCG-4986 on RGS4 intrinsic tryptophanyl fluorescence.

The inhibition of $G\alpha_o$ /RGS4 binding and GAP activity could be due to an interaction of compound with either the RGS or the $G\alpha$ subunit. CCG-4986 did not affect baseline GTPase activity or the binding of BODIPY-FL GTP γ S to $G\alpha_o$ (data not shown) suggesting that it was acting on the RGS. To determine whether CCG-4986 bound directly to RGS4, we examined the intrinsic fluorescence of RGS4 which has 2 tryptophan residues. An alteration of the environment surrounding the tryptophan(s) by direct interaction with the compound or due to a conformational change upon compound binding could produce a change in net RGS4 fluorescence. CCG-4986 caused a concentration-dependent quenching of RGS4 intrinsic fluorescence (Figure 4). The fluorescence was quenched 38% at 30 μ M CCG-4986. This magnitude of quenching of RGS4 intrinsic fluorescence by CCG-4986 provides a notably robust signal. The addition of DMSO (vehicle) to RGS4 caused less than 10% quenching and CCG-4986 did not alter the fluorescence of a solution of L-tryptophan showing that the effect of CCG-4986 was dependent on the RGS4 structure and was not a spectral artifact such as absorption of excitation or emission light.

Effect of CCG-4986 on Gai/o-mediated signaling

A key test for any inhibitor of a protein-protein interaction is its ability to inhibit a signaling cascade that involves those proteins. To assess this activity of CCG-4986, we utilized a permeabilized cell adenylyl cyclase (AC) assay which measures accumulated

cyclic adenosine monophosphate (cAMP) as a readout (Clark et al., 2004). In these experiments, C6 glioma cells stably expressing the $G\alpha_{o/i}$ -coupled μ -opioid receptor were permeabilized with digitonin and exogenous purified RGS4 protein was added to the system. Figure 5 shows the effect of forskolin, a direct AC activator, on the accumulation of cAMP. The addition of DAMGO, a μ -opioid agonist, decreases cAMP accumulated via $G\alpha_{o/i}$ -mediated inhibition of AC. The addition of 1 μ M RGS4 “rescues” the AC from inhibition by $G\alpha_{o/i}$ by accelerating the deactivation (GTP hydrolysis) of the α subunit, returning it to the inactive, GDP-bound form. The addition of CCG-4986 reverses the RGS4-mediated inhibition of the opioid response. This is an effect on RGS4 as CCG-4986 did not have any effect on the forskolin or DAMGO responses.

CCG-4986 selectively inhibits RGS4

The experiments described thus far demonstrate that CCG-4986 interacts directly with RGS4 and inhibits RGS4 activity, however, its specificity for RGS4 is not known. Based on shared sequence identity, RGS8 is the RGS most closely related to RGS4 (76% similar). Therefore, we tested the effect of CCG-4986 on $G\alpha_o$ /RGS8 binding by FCPIA (Figure 6A) and it did not significantly effect on the binding of RGS8 to $G\alpha_o$, in contrast to its complete inhibition of $G\alpha_o$ binding to RGS4. We then tested CCG-4986 on RGS8 functional activity in the permeabilized cell cyclase assay. Even at 30 μ M, CCG-4986 did not significantly inhibit the activity of RGS8 on μ -opioid suppression of cAMP accumulation (Figure 6B).

Purity and Activity of CCG-4986

In the course of this study, three batches of CCG-4986 were obtained from Chembridge. The batches of CCG-4986 showed identical activity for inhibiting RGS4. The purity of CCG-4986 was determined by analytical HPLC to be ~96%. To ensure that the RGS4 inhibitory activity was from the reported structure of CCG-4986, semi-preparative HPLC was used to separate the major compound component (~96%) from a minor contaminant. Subsequent FCPIA experiments showed that the RGS4 inhibitory activity was contained within the major component and the minor impurity showed no

activity. The resolved components were subject to direct infusion MS, and the major peak showed an m/z of 374.9, consistent with the structure of CCG-4986.

DISCUSSION

Most high affinity inhibitors of protein-protein interactions target protein pairs in which one protein displays a small peptide that is buried in a relatively well-defined binding pocket on its cognate binding partner. Examples include both peptide and small molecule inhibitors for a subset of protein interactions, such as Bcl-2 and Bak-BH3 domains (Degterev et al., 2001), Myc/Max dimerization (Berg et al., 2002), and p53/mdm2 interaction (Duncan et al., 2001; Stoll et al., 2001). These types of interactions are amenable to very high throughput assays utilizing fluorescence polarization measurements of a labeled peptide combined with its protein binding partner (Nikolovska-Coleska et al., 2004). However, many protein-protein interactions can't be easily studied by fluorescence-polarization, either because the exact interaction interface is unknown or the contact surface lacks a discrete, isolated peptide binding domain and is instead a rather flat, large surface. Such is the case for the $G\alpha$ /RGS interaction. FCPIA is ideally suited for examining these types of protein-protein interactions. The nanomolar concentrations of protein necessary and excellent signal-to-noise ratio translates into an assay that is suitable even for protein pairs which may be difficult to express or purify in large quantity. While the throughput of flow cytometry has in the past not been very high, the availability of commercial 96-well plate-reading flow cytometry systems (e.g. Luminex) has improved this situation with the capability of reading a plate in less than 30 min. In addition, the Hypercyt® system developed by Sklar and colleagues (Young et al., 2005) is reported to collect flow cytometry data as fast as 2.5 min per 96-well plate making this approach truly viable for high-throughput screening.

In our screen we identified two compounds (CCG-4986 and CCG-2046) as primary screening hits. It is surprising that our other high-potency compound (CCG-2046) did not inhibit activity of RGS4 at $G\alpha_o$ despite its potency in the FCPIA interaction assay. It is possible that CCG-2046 had spectral interference effects but it did not inhibit RGS8 binding to $G\alpha_o$ in FCPIA (not shown) which makes that unlikely. There

are differences in the conditions of the GAP assay and the FCPIA assay. The latter uses GDP/AMF instead of GTP so either an interaction with the AMF or an effect that is specific to the transition-state conformation of $G\alpha_o$ could contribute.

The major advance in this study is the identification of the first small molecule inhibitor of an RGS protein, CCG-4986. It has good potency ($<10 \mu\text{M IC}_{50}$) for a primary screening hit. It has a molecular weight of 374.8 and a log P of 3.14 which are well within the range of active drugs. Efforts to establish the site of contact of CCG-4986 with RGS4 and to define initial structure activity relations to enhance potency are underway. While it is active *in vitro*, preliminary results in intact RGS4-transfected cells did not show activity (data not shown) suggesting that it may not be cell-permeable. This may be due to the relatively high topological polar surface area (130, calculated with the Chemaxon Marvin plugin) which predicts difficulty crossing membranes. Thus optimization of the structure for intact cell-based studies may be needed. Regardless, our primary screening hit CCG-4986 should prove to be a useful molecular tool for understanding the role and specificity of RGS4 and will serve as a first example defining an “RGS pharmacology”.

It is intriguing to speculate on potential applications of RGS inhibitors. In Parkinson's disease, RGS9-2 has been identified as being overexpressed in the striatum of levodopa treated patients (Tekumalla et al., 2001). Inhibiting its GAP activity which terminates $G\alpha_o$ signaling in dopaminergic neurons (Rahman et al., 1999) could be useful to enhance dopamine signaling (Neubig and Siderovski, 2002). Interestingly, a recent study by Ding et al. (Ding et al., 2006) showed effects of RGS4 on M_4 muscarinic autoreceptor function leading to increased levels of striatal acetylcholine which could be detrimental in Parkinson's. This recent discovery presents a model system in which an RGS4 inhibitor could be useful for both the study of the signaling phenomena in the laboratory as well as providing an initial step toward therapeutics.

The search for RGS inhibitors, and protein-protein interaction inhibitors in general, provides exciting opportunities for targeting novel steps in signal transduction pathways. This can provide valuable information about pathway functions and provides a marked increase in the number of potential pharmacological targets. This discovery of first small molecule RGS4 inhibitor represents a first step to chemical targeting of this

novel aspect of GPCR signaling. Additionally, the FCPIA screening approach may be useful in identifying additional protein-protein interaction inhibitors to aid the study of the complex protein interaction signaling nexus found within cells.

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Footnotes

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1 The RGS4-short was found to be indistinguishable from a full length 6X-HIS tagged RGS4 with regard to inhibition of activity or $G\alpha_o$ binding by CCG-4986.

Figure Legends

Figure 1. FCPIA Method and Saturation binding of $G\alpha_o$ to RGS4. (A) Schematic of an “active” protein-protein interaction complex. Biotinylated RGS is coincubated with LumAvidin microspheres and washed. Subsequently, AMF-activated AF532-labeled $G\alpha_o$ is added and the RGS and G-protein interact. The measurement of this protein-protein interaction is accomplished as shown in (B). Beads are drawn into the flow cytometer from a 96-well plate. As beads pass the lasers, the bead is excited and detected by one laser, and the associated AF532- $G\alpha_o$ is excited by a second laser. The detector identifies the bead and the amount of associated AF532- $G\alpha_o$ fluorescence. No wash step is necessary to remove unbound $G\alpha_o$, as the flow cytometer only measures bead-associated fluorescence. Data obtained from a fluorescent binding assay are depicted in (C). Two nM biotinylated RGS4 on LumAvidin® beads and increasing concentrations of activated AlexaFluor532- $G\alpha_o$ (0.1-100nM) were added to wells of a 96-well plate and bead-bound fluorescence reflecting $G\alpha_o$ binding is measured by flow cytometry. The K_d for specific binding was 4.7 ± 0.4 nM (n=4, in duplicate).

Figure 2. Dose-response curves for two CCG compounds using FCPIA. Two nM biotinylated RGS4 was coupled to LumAvidin® microspheres and preincubated with varying concentrations of compound before the addition of 5 nM AMF-activated $G\alpha_o$. CCG-2046 and CCG-4986 inhibited with IC_{50} values of 4.3 ± 0.2 and 4.2 ± 0.1 μ M (n=3), respectively.

Figure 3. Inhibition of RGS4 GAP activity by CCG-4986. The two most potent compounds from our RGS4/ $G\alpha_o$ binding screen were tested in single-turnover GTPase assays. 200 nM $G\alpha_o$ was loaded with a 3-fold molar excess of [32 P]GTP in 20 mM HEPES, 20 mM EDTA pH 8.0 for 5 min at RT then cooled on ice. This was then added to equal volumes of ice-cold initiation buffer (20 mM HEPES, 40 mM $MgCl_2$, and unlabeled GTP, pH 8.0) containing RGS4 +/- inhibitor (50nM RGS4, 10 μ M inhibitor, final). The reaction was quenched with 5% activated charcoal (in 20 mM sodium phosphate, pH 2.0), incubated for 20 min, centrifuged, and the supernatant was counted

using Cerenkov counting. The amount of [32 P]Pi released at each time point was fit to an exponential function: $\text{counts}(t) = \text{counts}(t=0) + \Delta\text{counts}(t=30 \text{ min}) \cdot (1 - e^{-kt})$. While CCG-2046 did not inhibit this activity, CCG-4986 did significantly. The 68% inhibition of the RGS GAP activity rate seen at 10 μM CCG-4986 ($n=5$, in triplicate) is consistent with the 3-4 μM IC_{50} seen in FCPIA.

Figure 4. Quenching of RGS4 intrinsic fluorescence by CCG-4986. Intrinsic tryptophanyl fluorescence of RGS4 (2 μM) was measured using a spectrofluorimeter. The addition of CCG-4986 quenched the intrinsic fluorescence in a concentration-dependent manner, with a maximal inhibition of 38% and an IC_{50} of $10.7 \pm 0.7 \mu\text{M}$ ($n=3$). The addition of DMSO as a control had a minimal effect on the RGS4 intrinsic fluorescence. The addition of CCG-4986 to a solution of 1 μM L-tryptophan did not affect the fluorescence of the free L-tryptophan.

Figure 5. Inhibition of RGS4 activity by CCG-4986 in permeabilized cells. C6 glioma cells stably expressing μ -opioid receptors were assayed for cAMP accumulation under various conditions. Data are expressed as a % of the forskolin-stimulated cAMP production and are from 3-5 independent experiments. Forskolin (10 μM) stimulates the production of cAMP which is inhibited by the μ -opioid agonist DAMGO (100 nM) through $G\alpha_{i/o}$ subunits. The addition of 1 μM RGS4 reverses the DAMGO-mediated inhibition. The activity of CCG-4986 on RGS4 is reflected by the increase in accumulated cAMP as RGS4 is inhibited.

Figure 6. Specificity of CCG-4986 for RGS4. (A) Effect of CCG-4986 on RGS binding to $G\alpha_o$ in the FCPIA assay. CCG-4986 inhibits RGS4 binding to $G\alpha_o$ in a dose-dependent manner, but did not effect the ability of RGS8 to bind $G\alpha_o$ even at 100 μM ($n=3$). (B) Effect of CCG-4986 on RGS activity in the permeabilized C6 cell assay (see Fig. 5). The activity of 1 μM RGS4 or RGS8 was measured in the absence or presence of 30 μM CCG-4986. 1 μM RGS4 reduced opioid inhibition by $89 \pm 2\%$ and RGS8 by $83 \pm 5\%$. In contrast to the elimination of the effect of RGS4, CCG-4986 did not significantly inhibit the activity of RGS8 ($n=3$).

Table 1. HTS Results

Initial Chembridge Library	3028
Primary Hits (>50% Inhibition)	18
Confirmed Hits	5
IC50 <10uM	2
Inhibit RGS4 GAP Activity	1

Figure 1

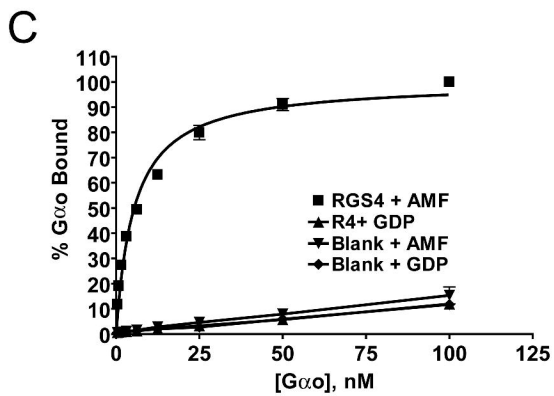
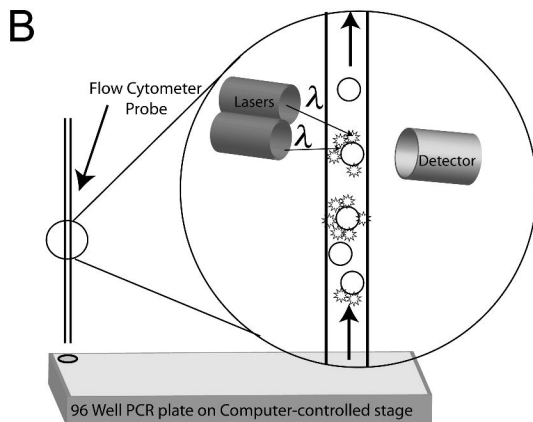
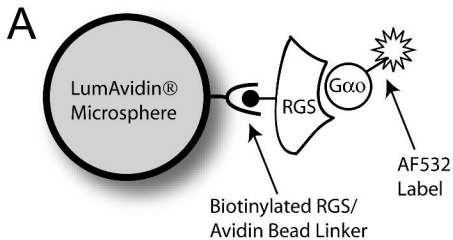


Figure 2

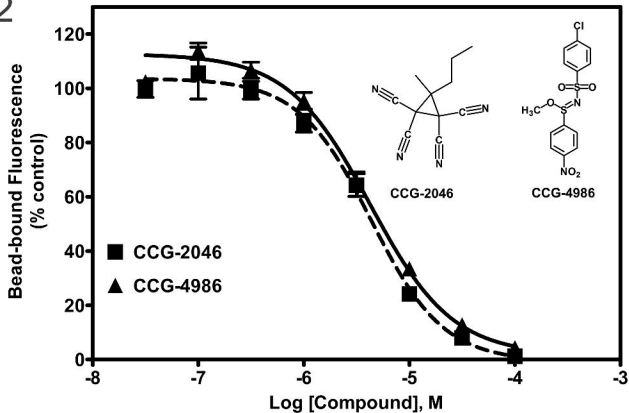


Figure 3

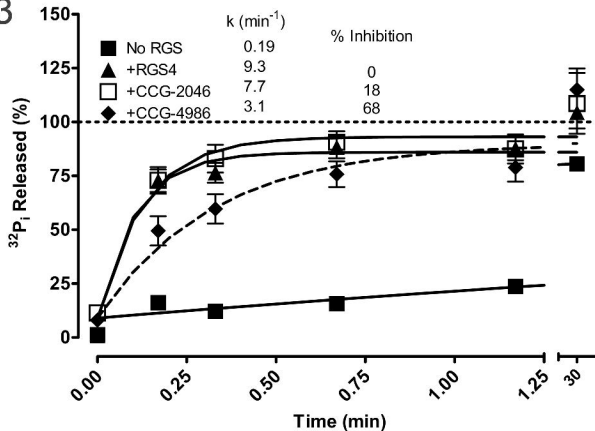


Figure 4

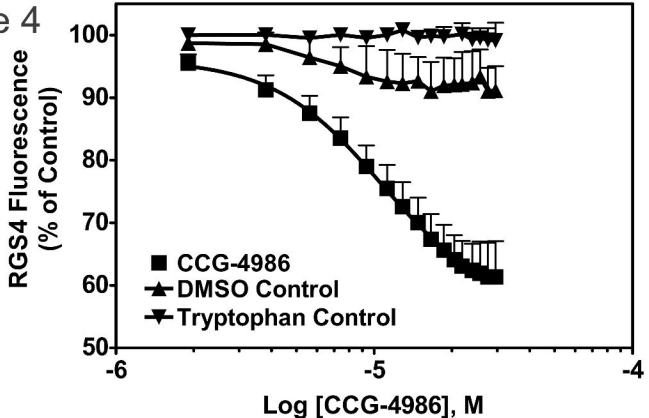
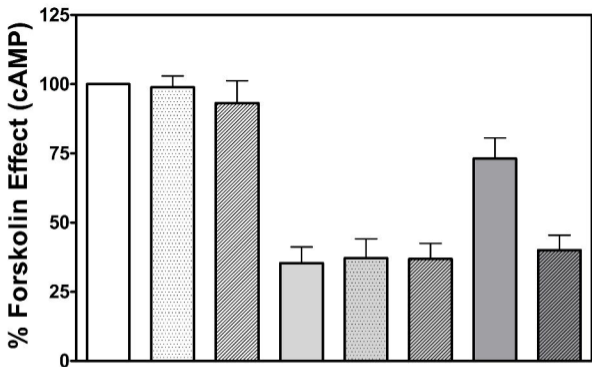


Figure 5



Forskolin	+	+	+	+	+	+	+	+
CCG-4986	-	-	+	-	-	+	-	+
DAMGO	-	-	-	+	+	+	+	+
RGS4	-	-	-	-	-	-	+	+
Vehicle	-	+	-	-	+	-	-	-

Figure 6

