Identification of Small-Molecule Inhibitors of RGS4 Using a High-Throughput Flow Cytometry Protein Interaction Assay


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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 (Gα) and thus shorten the time course and reduce the magnitude of G-protein α- and βγ-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 to assess Gα-RGS interactions in a high-throughput screen, we identified the first small-molecule inhibitor of an RGS protein. Of 3028 compounds screened, 1, methyl N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate (CCG-4986), inhibited RGS4/Gαi binding with 3 to 5 μM potency. It binds to RGS4, inhibits RGS4 stimulation of Gα2 GTase 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/Gα 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 the development of chemical tools and for possible therapeutic drug discovery (Arkin and Wells, 2004; Pagliaro et al., 2004). Whereas extracellular ligand binding domains, ion channels, and enzyme active sites are considered more “drugable,” 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 Gα subunits, which then undergo an activating conformational change releasing free α- and βγ-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 Gα subunit returns to its inactive, GDP-ligated form, and its signaling functions are terminated. The hydrolysis of GTP due to the Gα subunit’s intrinsic GTase activity is very slow but is accelerated up to 1000-fold by regulators of G protein signaling (RGS) proteins (Mukhopadhyay and Ross, 1999; Lan et al., 2000). The GTase accelerating protein (GAP) activity of RGS proteins (Mukhopadhyay and Ross, 1999; Lan et al., 2000). The GTase accelerating protein (GAP) activity of RGS proteins shortens the lifetime and, in general, reduces the magnitude of signaling by both the active Gα and the free Gβγ as Gαβγ reassociation occurs rapidly upon formation of the Gα-GDP complex (Neubig et al., 2003; Fu et al., 2006; Huang et al., 2006). This work was supported by National Institutes of Health grants GM039561 (to R.R.N.), F32-GM076821 (to D.L.R.), DA004087 (to J.R.T.), and T32-DA007268 (to J.R.T. and J.N.T.). Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org. doi:10.1124/mol.106.028670.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; FCPIA, flow cytometry protein interaction assay; RGS, regulator of G-protein signaling; GAP, GTase accelerating protein; CCG-4986, methyl N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate; CCG-2046, 3-methyl-3-propylcyclopropane-1,1,2,2-tetracarbonitrile; RT, room temperature; DMSO, dimethyl sulfoxide; cv, column volume; DAMGO, [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin; BCB, bead-coupling buffer; AC, adenylyl cyclase; buffer A, HEPES and dithiothreitol; [35S]GTPγS, guanosine 5′-O-[32P]triphosphate.
The RGS proteins have been proposed as novel drug targets (Zhong and Neubig, 2001; Neubig and Siderovski, 2002; Cho et al., 2004; Liebmann, 2004; Riddle et al., 2005) because 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 (Mirnics et al., 2001; Williams et al., 2004; Erdely et al., 2006), Parkinson’s disease (Tekumalla et al., 2001; Kovoor et al., 2005; Ding et al., 2006), hypertension (Heximer et al., 2003a; Riddle et al., 2006), and addiction (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 an action in tissues that express that particular RGS protein (Zhong and Neubig, 2001). RGS specificity for discrete $\alpha$ subunits could also permit a pathway-specific enhancement of agonist action (Zhong and Neubig, 2001; Neubig and Siderovski, 2002).

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 laboratory identified a cyclic octapeptide derived from the RGS interaction site of $\alpha_{2}B$ subunits that inhibits RGS4 and alters G protein-coupled inwardly rectifying potassium channel 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 two-hybrid readout (Young et al., 2004) that yielded some low-affinity inhibitors, but no structures or follow-up information was 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, because it involves a single-turnover assay of $[^{32}P]$GTP hydrolysis using a charcoal precipitation step. We chose to measure the high-affinity protein-protein interaction between purified $\alpha$ subunits and RGS proteins when the $\alpha$ subunit is in the transition-state conformation induced by GDP and $\text{AlF}_4^{-}$. We used a flow cytometry protein interaction assay (FCPIA) approach based on previous studies measuring $\alpha$ and $\beta$ subunits interactions (Sarvazyan et al., 1998) and ligand-receptor-G-protein interactions (Sklar et al., 2002; Simons et al., 2003).

Using FCPIA, we screened a 3028-compound library from the Chembridge screening collection to identify inhibitors of the RGS4/$\alpha_{2}$ protein-protein interaction. This screen resulted in five potential RGS4 inhibitors, two of which had IC_{50} values less than 10 $\mu$M. One compound, CCG-4986, inhibited RGS4 GAP activity and RGS4 activity on $\mu$-opioid receptor-mediated signaling. In addition, CCG-4986 is selective for RGS4 compared with 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). $[^{32}P]$GTP and $[^{35}S]GTP\gamma S$ were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). The screening compound library was from the Chembridge screening collection (http://www.hit2lead.com), and the identity and purity (>96%) of CCG-4986 was confirmed by liquid chromatography/mass spectrometry through the University of Michigan Department of Pharmacology Mass Spectrometry 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, G418 (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 Prism 4.0 (GraphPad Software, San Diego, CA).

**RGS Protein Expression and Purification.** N-terminally truncated (initiating at Met19) rat RGS4, designated pQE60RGS4short (Krumins et al., 2004), was used for the experiments in this study.1 The $\Delta N$-variant was selected because it expresses higher levels of soluble protein in Escherichia coli compared with 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 70-ml S Sepharose Fast Flow (GE Healthcare, Little Chalfont, Buckinghamshire, UK) fast-performance liquid chromatography column. The column was washed with 3 column volumes (cv) of buffer A (50 mM HEPES and 1 mM dithiothreitol, pH 7). A two-stage linear gradient was run, with 0 to 20% buffer B (buffer A + 1 M NaCl) in 3 cv and 20 to 55% in 13 cv. RGS4 eluted in a single broad peak between 290 and 380 mM NaCl. Purified RGS4 was isolated using a Superdex 75 75 gel-filtration column. A glutathione transference-tagged rat full-length RGS8 was purified according to兰 et al. (1998).

**Expression and Purification of $\alpha_{2}$, 6X-His-tagged $\alpha_{2}$, was expressed and purified from transformed BL21-DE3 E. coli as described previously (Lee et al., 1994). Activity of purified $\alpha_{2}$ was determined by $[^{35}S]GTP\gamma S$ binding (Sternweis and Robishaw, 1984).

**Chemical Biotinylation of Purified RGS Proteins.** RGS proteins were biotinylated with amine-reactive biotinamidohexanoic acid N-hydroxysuccinimide ester (mol. wt., 454.54; Sigma) in a 2:1 (biotin/RGS) stoichiometry. Biotin-N-hydroxysuccinimide ester (1 mg) was resuspended, with sonication, in 1 ml of double-distilled H_{2}O; 40 $\mu$M (100 nmol) was added to 1 mg of $\Delta N$-RGS4 (~50 nmol) in a total volume of 300 $\mu$L of HEN buffer (50 mM HEPES, 1 mM EDTA, and 100 mM NaCl, pH 8), and incubated, rotating, at 4°C. The reaction was terminated with 10 $\mu$L of 1 M glycine, and excess biotin was removed by applying the sample to a 1-ml Sephadex G25 spin column and eluting with HEN buffer.

**Fluorescent Labeling of $\alpha_{2}$, Purified $\alpha_{2}$ was chemically labeled with Alexa Fluor 532 carboxylic acid succinimidyl ester (Invitrogen) at a 3:1 fluorophore/protein ratio. Purified $\alpha_{2}$ (500 $\mu$g, 12.5 nmol) was diluted in 250 $\mu$L of H_{2}O/E_{100}N_{100} (50 mM HEPES, 100 mM EDTA, and 100 mM NaCl, pH 8) buffer supplemented with 10 $\mu$M GDP. Then, 2.8 $\mu$L (~38 nmol) of an Alexa Fluor 532 solution (1 mg/100 $\mu$L of DMSO) was added, and the solution was incubated at 4°C in the dark for 1.5 h. The reaction was quenched with 20 $\mu$L of 1 M glycine 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 $\alpha_{2}$ was determined after labeling using $[^{35}S]GTP\gamma S$ binding (Sternweis and Robishaw, 1984).

**FCPIA and High-Throughput Screen.** Each test compound was spotted in 1 $\mu$L of DMSO into individual wells of 96-well Axygen polypropylene plate using a Beckman BioMek FX robot.

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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 $\alpha_{2}$, binding by CCG-4986. 

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Downloaded from multipharm.aspetjournals.org on November 7, 2017.
the library was prepared based on compound mass. The RGS con-
absence of RGS) were used as controls in some experiments to
each compound-containing well of the 96-well screening plate and
Na2HPO4, and 10 mM glucose, pH 7.45) with 1 mM 3-isobutyl-1-
screening compounds in DMSO was between 10 and 30
After 10 min to ensure the activation of the G
( at 10 nM final concentration), which binds RGS with high affinity.
K
nM. Signal-to-background ratio was robust at approximately 50 to
2003). Inhibition of cAMP formation was determined as a percentage
incubated for 15 min at 37°C, and reactions were stopped by the
2006). Compounds were preincubated with the RGS protein for 10 to
1% bovine serum albumin, pH 8.0). Beads (50 µl) were added to
was repeated for three washes. The beads were then resuspended
in 500 µl of BCB, and biotinylated RGS protein was added to yield the
concentration of 40 nM. The beads were incubated for 30 min at RT.
were spun down, washed with 1 ml of BCB three times, and finally resuspended in 5
of flow buffer (50 mM HEPES, 100 mM NaCl, 0.1% Lubrol, and 1%
bovine serum albumin, pH 8.0). Beads (50 µl) 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 Gαo solution was prepared. GDP
(5 µl, 10 mM), MgCl2 (500 µl, 50 mM), AlCl3 (500 µl, 50 µM), and
NaF (500 µl, 50 mM) were added to 3.5 ml of flow buffer, and AF532-Gαo
was added to form the activated Gαo-GTP-AIF532 complex (at 10 nM final concentration), which binds RGS with high affinity.
After 10 min to ensure the activation of the Gαo, 50 µl 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. Me-
dian fluorescence intensity values for bead-bound AF532 Gαo were
computed and used for data analysis. The effective concentration of screening compounds in DMSO was between 10 and 30 µM, because
the library was prepared based on compound mass. The RGS con-
centration in the assay was 2 nM, and the Gαo concentration was 5
nM. Signal-to-background ratio was robust at approximately 50 to
100:1. Compounds were considered hits if they inhibited the RGS4-
Gαo interaction at least 50% compared with controls.
For FCPIA saturation experiments, the RGS4 beads and AF532
Gαo were prepared as above, except that AF532-Gαo was prepared at
200 nM. This solution was then diluted serially down the 96-well plate to yield final Gαo concentrations between 0.78 and 100 nM. The
incubation and plate reading were performed as in the high-through-
put screen. FCPIA compound dose-response experiments were done
similarly to the flow cytometry screening assay, except that the total
assay volume was 150 µl, with 50 µl of RGS4 beads (coupled at 3×
final concentration, 6 nM), 50 µl of Gαo-AF532 (15 nM), and varying concentrations of CCG compounds to give a final range of 100 to
0.01 µM.
**GAP Assay.** Single-turnover GTP hydrolysis measurements in
96-well plates were performed as described previously (Roof et al.,
2006). Compounds were preincubated with the RGS protein for 10 to
15 min on ice before initiating the GAP assay.
**cAMP Accumulation Assay.** Rat C6 glioma cells expressing the
µ-opioid receptor were grown to confluence, suspended, and perme-
abilized by treatment with digitonin, essentially as described previ-
ously (Alt et al., 2001). Cells were resuspended in assay buffer (128
mM NaCl, 2.4 mM KCl, 2 mM NaHCO3, 3 mM MgSO4, 10 mM
Na2HPO4, and 10 mM glucose, pH 7.45) with 1 mM 3-isobutyl-1-
methylxanthine and 100 µM ATP and then combined with drugs
(forskolin, DAMGO) and purified RGS proteins. Assay mixtures were
incubated for 15 min at 37°C, and reactions were stopped by the
addition of ice-cold perchloric acid. cAMP concentration was mea-
sured by radioimmunoassay as described previously (Clark et al.,
2003). Inhibition of cAMP formation was determined as a percentage
of the concentration of cAMP accumulated 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 experi-
ments were performed on a Photon Technology International Al-
phaScan Spectrofluorometer (Photon Technology International,
Lawrenceville, NJ) with 2.5-nm slits using wavelengths of 285 and
340 nm for excitation and emission, respectively. ΔN-RGS4 (2 µM) in
600 µl of buffer (20 mM HEPES, 100 mM NaCl, 500 µM 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-µl injections of a 1.2-M 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 nM. For
control experiments, the DMSO solvent for CCG-4986 was injected
with RGS4, or CCG-4986 was added to a solution of t-tryptophan,
following the same procedure and concentration steps.

**Results**

**FCPIA Measurements of the RGS4/Gαo Protein-Protein Inter-
action.** FCPIA consists of three key components (Fig. 1, A and B): 1) avidin-coated microspheres; 2) biotinyl-
ated RGS4; and 3) activated Gαo labeled with Alexa fluor 532. For the experiment, biotinylated RGS4 coupled to avi-
din-coated beads is incubated in a 96-well plate with AIF532-
activated Gαo (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, pro-
viding a quantitative reading of the amount of Gαo bound to
RGS4. Figure 1C demonstrates several strengths of this
method for high-throughput screening. Only the activated
Gαo incubated with microspheres precoupled to RGS4 re-
sulted in high-affinity, saturable Gαo binding. The Kd value
for specific binding was 4.7 ± 0.4 nM (n = 4, in duplicate).
The relatively high affinity of the RGS4-Gαo interaction in
the presence of AIF532 and the very low background fluores-
cence provide an outstanding signal-to-noise ratio and permit
the use of very low concentrations of the protein reagents (2
nM RGS4 and 0.4–100 nM AF532-Gαo), demonstrating the
suitability of FCPIA for high-throughput screening.

**High-Throughput Screen for RGS4 Inhibitors.** Our high-
throughput screen focused on identifying compounds
that bound to RGS4 and disrupted its binding to Gαo. The
library consisted of 3028 small-molecule compounds from the
Chembridge collection. RGS4 and activated Gαo were added to
the plates, and 100 beads/events per well were read on the
Luminex flow cytometer. The extremely high SN of the assay
(Z' score of 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-Gαo interaction signal with single-
digit micromolar IC50 values (Fig. 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
compared with the intrinsic Gαo GTPase activity. Further-
more, 10 µM CCG-4986 inhibited the catalytic activity of
RGS4 by 68%, as measured by this single-turnover experi-
ment. This is consistent with the 71% inhibition predicted
from the 4.2 μM IC₅₀ value in the RGS4/Gαₒ binding experi-
iment. It is surprising that 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 Gαₒ/RGS4 binding and
GAP activity could be due to an interaction of compound with

**TABLE 1**

<table>
<thead>
<tr>
<th>High-throughput screening results</th>
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<tr>
<td>Initial Chembridge library</td>
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<tr>
<td>Primary hits (&gt;50% inhibition)</td>
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<tr>
<td>Confirmed hits</td>
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<td>IC₅₀ &lt; 10 μM</td>
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<td>Inhibit RGS4 GAP activity</td>
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Fig. 1. FCPIA method and saturation binding of Gαₒ to RGS4. A, schematic of an “active” protein-protein interaction complex. Biotinylated RGS is coinuculated with LumAvidin microspheres and washed. Thereafter, AMF-activated AF532-labeled Gαₒ 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-Gαₒ is excited by a second laser. The detector identifies the bead and the amount of associated AF532-Gαₒ fluorescence. No wash step is necessary to remove unbound Gαₒ, because the flow cytometer only measures bead-associated fluorescence. Data obtained from a fluorescent binding assay are depicted in C. Two nanomolar concentration of biotinylated RGS4 was coupled to LumAvidin microspheres and preincubated with varying concentrations of compound before the addition of 5 nM AMF-activated Gαₒ. CCG-2046 and CCG-4986 inhibited with IC₅₀ values of 4.3 ± 0.2 and 4.2 ± 0.1 μM (n = 3), respectively.

Fig. 2. Dose-response curves for two CCG compounds using FCPIA. Two nanomolar concentration of biotinylated RGS4 was coupled to LumAvidin microspheres and preincubated with varying concentrations of compound before the addition of 5 nM AMF-activated Gαₒ. CCG-2046 and CCG-4986 inhibited with IC₅₀ values of 4.3 ± 0.2 and 4.2 ± 0.1 μM (n = 3), respectively.

Fig. 3. Inhibition of RGS4 GAP activity by CCG-4986. The two most potent compounds from our RGS4/Gαₒ binding screen were tested in single-turnover GTPase assays: 200 nM Gαₒ was loaded with a 3-fold molar excess of [³²P]GTP in 20 mM HEPES and 20 mM EDTA, pH 8.0, for 5 min at RT and then cooled on ice. This was then added to equal volumes of ice-cold initiation buffer (20 mM HEPES, 40 mM MgCl₂, and unlabeled GTP, pH 8.0) containing RGS4 (50 nM RGS4, 10 μM 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 [³²P]Pi released at each time point was fit to an exponential function: counts(t) = counts(t = 0) + Δcounts(t = 30 min) × (1−e⁻ᵏᵗ). Whereas 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 to 4 μM IC₅₀ value seen in FCPIA.
either the RGS or the Gα subunit. CCG-4986 did not affect baseline GTPase activity or the binding of BODIPY-FL GTPγS to Gαo (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 two tryptophan residues. An alteration of the environment surrounding the tryptophan(s) by direct interaction with the compound or conformational change upon compound binding could produce a change in net RGS4 fluorescence. CCG-4986 caused a concentration-dependent quenching of RGS4 intrinsic fluorescence (Fig. 4). The fluorescence was quenched 38% at 30 μM 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 Gaαi/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 used a permeabilized cell adenyl cyclase (AC) assay, which measures accumulated cAMP as a readout (Clark et al., 2004). In these experiments, C6 glioma cells stably expressing the Gaαi/o-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 accumulation via Gaαi/o-mediated inhibition of AC. The addition of 1 μM RGS4 “rescues” the AC from inhibition by Gaαo, 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, because 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 Gaαo/RGS8 binding by FCPIA (Fig. 6A), and it did not significantly affect the binding of RGS8 to Gaαo, in contrast to its complete inhibition of Gaαo binding to RGS4. We then tested CCG-4986 on RGS8 functional activity in the permeabilized cell cyclase assay. Even at 30 μM, CCG-4986 did not significantly inhibit the activity of RGS8 on μ-opioid suppression of cAMP accumulation (Fig. 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 high-performance liquid chromatography to be ~96%. To ensure that the RGS4 inhibitory activity was from the reported structure of CCG-4986, semipreparative high-performance liquid chromatography 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 mass spectrometry, 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...
These types of interactions are amenable to very high throughput assays using fluorescence polarization measurements of a labeled peptide combined with its protein binding partner (Nikolovska-Coleska et al., 2004). However, many protein-protein interactions cannot 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 Go/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 translate into an assay that is suitable even for protein pairs, which may be difficult to express or purify in large quantity. Whereas 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 Go, despite its potency in the FCPIA interaction assay. It is possible that CCG-2046 had spectral interference effects, but it did not inhibit GRS8 binding to Go in FCPIA (data not shown), which makes that unlikely. There are differences in the conditions of the GAP assay and the FCPIA assay. The latter uses GDP/aluminum-magnesium-fluoride instead of GTP so either an interaction with the AMF or an effect that is specific to the transition-state conformation of Go 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 (IC50 < 10 μM) 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. Although 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 plug-in), 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 Go, signaling in dopaminergic neurons (Rahman et al., 1999), could be useful to enhance dopamine signaling (Neubig and Siderovski, 2002). It is interesting that a recent study by Ding et al. (2006) showed effects of RGS4 on M4 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 studying the signaling phenomena in the laboratory and 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 the first small-molecule RGS4 inhibitor represents a first step to chemical targeting of this novel aspect of GPCR signaling. In addition, the FCPIA screening approach may be useful in identifying additional protein-protein interaction inhibitors to aid the study of the complex protein interaction inhibitors found within cells.

![Fig. 6. Specificity of CCG-4986 for RGS4. A, the effect of CCG-4986 on RGS binding to Go in the FCPIA assay. CCG-4986 inhibits RGS4 binding to Go in a dose-dependent manner but did not affect the ability of RGS8 to bind Go 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 ± 2% and RGS8 by 83 ± 5%. In contrast to the elimination of the effect of RGS4, CCG-4986 did not significantly inhibit the activity of RGS8 (n = 3).](Image)
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References


