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Reversible Inhibitors of Regulators of G-protein Signaling Identified in a High-throughput Cell-based Calcium Signaling Assay

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Abstract

Regulator of G-protein signaling (RGS) proteins potently suppress G-protein coupled receptor (GPCR) signal transduction by accelerating GTP hydrolysis on activated heterotrimeric G-protein α subunits. RGS4 is enriched in the CNS and is proposed as a therapeutic target for treatment of neuropathological states including epilepsy and Parkinson's disease. Therefore, identification of novel RGS4 inhibitors is of interest. An HEK293-FlpIn cell-line stably expressing M₃-muscarinic receptor with Doxycycline-regulated RGS4 expression was employed to identify compounds that inhibit RGS4-mediated suppression of M₃-muscarinic receptor signaling. Over 300,000

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Contributions

S.M.W. developed the cell-line and S.M.W., M.W., M.L., Z.L., J.J.B., O.M., B.S. contributed to screening implementation and analysis; C.R.H, C.W.L. provided compound analysis, chemistry support and discussion of screening results; J.P.M, A.J.S., L.L.B designed and conducted experiments; R.R.N. discussed results, and reviewed the article. All authors contributed to the writing of the article and A.J.S. wrote the article. All authors have approved the final article.

Conflict of Interest

The authors declare no conflict of interest.

compounds were screened for an ability to enhance $G\alpha_q$ -mediated calcium signaling in the presence of RGS4. Compounds that modulated the calcium response in a counter-screen in the absence of RGS4 were not pursued. Of the 1,365 RGS4-dependent primary screen hits, thirteen compounds directly target the RGS-G-protein interaction in purified systems. All thirteen compounds lose activity against an RGS4 mutant lacking cysteines, indicating that covalent modification of free thiol groups on RGS4 is a common mechanism. Four compounds produce >85% inhibition of RGS4-G-protein binding at 100 μ M, yet are >50% reversible within a ten-minute time frame. The four reversible compounds significantly alter the thermal melting temperature of RGS4, but not G-protein, indicating that inhibition is occurring through interaction with the RGS protein. The HEK cell-line employed for this study provides a powerful tool for efficiently identifying RGS-specific modulators within the context of a GPCR signaling pathway. As a result, several new reversible, cell-active RGS4 inhibitors have been identified for use in future biological studies.

Keywords

G-protein coupled receptors; M_3 muscarinic acetylcholine receptor; Regulator of G-protein signaling; Small molecule inhibitor; High-throughput screen

1. Introduction

Signaling through G-protein coupled receptors (GPCRs) controls many vital physiological functions. As a result, therapeutics that target GPCRs constitute a major portion of the prescription drug market [1]. Despite this success, achieving selectivity among closely related GPCR subtypes has limited therapeutic development in many areas, particularly for Alzheimer's disease [2, 3]. One strategy to attain greater selectivity is to modulate receptor activation by engaging less conserved allosteric surfaces on the receptors, either independently [4], or with the use of bitopic molecules that engage both the orthosteric and allosteric sites simultaneously [5]. Another approach is to target the downstream protein-protein interactions (PPI) that regulate GPCR signal transduction [6]. Targeting downstream regulatory PPIs provides an ability to more subtly modulate endogenous signaling, similar to a purely allosteric GPCR ligand, but uniquely, therapeutic action can also be compartmentalized in distinct tissues where both the receptor and target protein are co-expressed [7].

Regulators of G-protein Signaling (RGS) suppress GPCR signal transduction by selectively interacting with GTP-bound (activated) heterotrimeric $G\alpha$ subunits to enhance their intrinsic rate of nucleotide hydrolysis [8-10]. The expression profiles among the >20 human RGS proteins are relatively diverse, with some isoforms being highly expressed in specific tissues, such as RGS4 in the CNS [11, 12]. Within these tissues, RGS proteins can co-localize with specific receptors [13-16], which may in part drive the receptor-specific regulation by certain RGS isoforms [14, 17]. Pathway-specific regulation is also due to the binding specificity of certain RGS proteins for $G\alpha_i/o$ or $G\alpha_q$ family members observed *in vitro* [18-21]. As a result of the expression patterns and pathway-specific effects, modulating GPCR signaling up or down in a particular tissue could be achieved by inhibiting or activating a specific RGS isoform. Therefore, RGS proteins have been proposed as intriguing drug targets [22-24].

RGS4 is highly expressed in cortex, thalamus, and other brain regions [11], and potentially affects numerous centrally-acting GPCR signaling pathways. Within the dorsolateral striatum, RGS4 serves as a bridge between D_2 -dopamine and A_2 -adenosine receptors and the endocannabinoid mobilization driving the striatal plasticity associated with normal

motor behavior. As a result, RGS4 knockout mice are more resistant than WT animals to motor behavior deficits occurring from 6-OHDA depletion of dopamine [25]. This suggests that RGS4 may be a new target for treating Parkinson's disease. Additionally, formation of an RGS4-A₁-adenosine receptor complex via the neurabin scaffolding protein can negatively regulate the neuroprotective effects of adenosine signaling in a kainate-induced seizure model. Genetic knockout of neurabin or small molecule antagonism of RGS4 reduces seizure severity in this model [26]. In either case, inhibition of RGS4 provides a beneficial enhancement of a particular GPCR signaling pathway in the context of these models. Such studies support the use of RGS inhibitors in therapy. As a result there is a critical need for continued development of selective small molecule RGS modulators.

Since RGS4 inhibitors identified in biochemical screening assays have shown limited or no cellular activity [27-30], we employed a novel cell-based calcium assay with regulated RGS4 expression. This system mitigates a major challenge to screening in cellular systems, which is the multiple potential sites of action of the compound in the pathway. By screening compounds in an inducible RGS4 cell line (Doxycycline treated cells), followed by a counter-screen of the hits in the absence of RGS4 (untreated cells) we could enrich for those that are actual RGS4 inhibitors. Using this approach we screened >300,000 compounds from NIH small molecule repository (MLSMR) to identify new RGS4 inhibitors. Here we describe the identification process and biochemical characterization of several new RGS4 inhibitors with cellular activity. Like all previously reported RGS4 inhibitors, these compounds are dependent on covalent modification of cysteine residues for activity. Several RGS inhibitors are reversible and have selectivity for RGS4 over other RGS isoforms tested. They should provide new tools to dissect the role of RGS4 in biology and as a therapeutic target.

2. Materials and methods

2.1 Materials

Chemicals were purchased from Fisher Scientific (Hampton, NH) or Sigma-Aldrich (St. Louis, MO). All materials are at least reagent grade. Avidin-coated Luminex beads were purchased from Luminex (Austin, TX). Ni-NTA resin was purchased from Qiagen (Valencia, CA). Amylose resin was obtained from New England Biolabs (Ipswich, MA). Antisera were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 M3-R4 cell-line development and characterization

The Invitrogen Flp-In T-Rex HEK 293 cells stably expressing the Tet repressor (pCDNA6/TR) and lacZ-Zeocin fusion gene (pFRT/lac-Zeo), containing the Flp Recombination Target (FRT) site, were used as host cells. HA-tagged RGS4 (C2S) was ligated into a pCDNA5/FRT/TO vector. Flp-In cells were plated in 6-well plates at 400,000 cells/well and co-transfected with 0.4 µg of RGS4-pCDNA5/FRT/TO and 3.6 µg of pOG44 (expressing Flp recombinase) using 10 µL Lipofectamine 2000 reagent. Stable integration of the RGS4-containing vector occurs between the FRT sites orienting the SV40 promoter and initiation codons in frame with the Hygromycin resistance gene, while inactivating the lacZ-Zeocin fusion gene, making the stably transfected cells Hygromycin resistant and Zeocin sensitive. Two days after transfection, 200 µg/mL Hygromycin was added to the wells to select for stably transfected cells. Cell pools were tested for Zeocin sensitivity and Doxycycline induced RGS4 expression was verified via Western blot. RGS4 expressing cells were then transfected with human M₃-muscarinic receptor cloned into pCDNA3.1(+) using 4 µg of plasmid and 10 µL of Lipofectamine 2000, followed by selection of neomycin resistant clones using G418. Single M3-R4 cells were then flow sorted into two 96-well plates.

Isolated single clones were expanded and tested for carbachol response and an RGS effect using the Fluo4 NW calcium signaling assay according to the manufacturer's instructions.

2.3 Western blot for RGS4 expression

Cell transfections and preparation of lysates were performed as described previously [31]. In brief, HEK 293T cells maintained in DMEM plus 10% FBS were grown to confluence in 6-well plates and transiently transfected with 2.5 μ g of HA-RGS4 or empty vector (mock) with 4 μ L of Lipofectamine 2000 per microgram of DNA, followed by incubation for 48 hours. The HEK293-FlpIn-TREx/M₃R/RGS4 cells were similarly plated in the presence or absence of 1 μ g/mL Doxycycline for 45 hours to induce RGS4 expression. Cell lysates were prepared by removing DMEM/FBS medium and rinsing wells with PBS at room temperature followed by the addition of 350 μ L of lysis buffer plus protease inhibitors at 4°C. Cells were scraped and transferred to a microcentrifuge tube and allowed to tumble at 4°C for 1 hour. Lysates were pelleted at 13,000 rpm for 15 minutes and the supernatant protein was quantified using Bradford reagent. Cell lysates were then run on a 12% SDS-PAGE gel and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA) and probed with either rabbit anti-HA at 1:400 and rabbit anti-Actin at 1:500, followed by probing both with anti-rabbit HRP secondary at 1:8,000.

2.4 High-throughput cellular screen

High-throughput screening was performed at Johns Hopkins Ion Channel Center, Johns Hopkins University, School of Medicine. The Molecular Libraries Small Molecule Repository (MLSMR) collection of > 300,000 compounds was used to screen for inhibitors of RGS4 using the HEK293-FlpIn-TREx/M₃R/RGS4 cell line described above. Cell plating was achieved using high density cell freezes that were diluted to 200,000 cells/mL in DMEM (high glucose with glutamine) 10% FBS, 1% Pen/Strep, 15 μ g/mL Blasticidin, 400 μ g/mL G418, and 200 μ g/mL Hygromycin. RGS4 expression was induced with the addition of 10 ng/mL Doxycycline. The day before the assay, 50 μ L/well of the diluted cells was plated in black, clear bottom, poly-D-lysine coated 384-well plates and incubated overnight at 37°C with 5% CO₂. On the day of the experiment the cell medium was removed and 20 μ L/well of Fluo4-AM dye solution was added, followed by a 30 minute incubation at 37°C. The dye solution was removed followed by addition of 20 μ L of assay buffer (Hank's Balanced Salt Solution in HEPES, pH 7.4) with a second 30 minute incubation at room temperature. The cells were then treated with 4 μ L of 6 $\times$ compound in assay buffer (10 μ M final) for 20 minutes at room temperature. Plates were imaged using a Hamamatsu FDSS 6000 kinetic plate reader and a baseline was recorded for 10s at 1Hz. This was followed by injection of 4 μ L of 7 $\times$ carbachol (7 nM) and calcium-mediated fluorescence was recorded for 100 seconds. The fluorescence ratio (maximum minus the minimum intensity divided by the baseline over the 100 seconds) was integrated for each well, followed by B-score normalization [32]. Hit selection was based on two criteria, that a compound's initial-fluorescence B-score is within five standard deviations of the mean initial fluorescence ratio B-score of the library, and the B-score of the compound following carbachol injection is greater than three standard deviations above the mean. Compounds that lacked activity in the absence of Doxycycline were confirmed in concentration-response format using concentrations 1 nM – 30 μ M in a 1:3 serial dilution in duplicate.

2.5 Protein expression and purification

N-terminally truncated Δ 51 RGS4 (residues 52-205) was expressed as a single open reading frame with maltose binding protein (MBP) fusion protein N-terminally fused via a linker region containing a 10 $\times$ histidine sequence and a tobacco etch virus (TEV) cleavage site. Cysless Δ 51 RGS4 RGS4 (cys(-) RGS4), described previously [29], was prepared via mutation of the seven cysteines within the RGS-box to alanine: Cys 71, 95, 132, 148, 183,

197, 204. RGS19 C-terminally truncated (Δ C11) and full length RGS16 were both expressed as MBP fusions. All MBP fusion proteins were prepared as previously described using the pMALC2H10 vector [29]. RGS8 (residues 61-198) was expressed with an N-terminal 6 $\times$ his-tag in a pQE80 vector, as described previously [33]. G α_{i1} protein was expressed with an internal 6 $\times$ his-tag and G α_o using an N-terminal 6 $\times$ his-tag, both using a pQE80 vector, as described previously [34].

2.6 Chemical labeling of purified RGS and G α_o proteins

RGS proteins were biotinylated on free amines and G α_o was labeled with AlexaFluor-532 on free thiols exactly as described before [35].

2.7 Flow Cytometry Protein Interaction Assay (FCPIA)

FCPIA was performed as previously described with the use of AlexaFluor 532-tagged G α_o and biotinylated RGS proteins [35]. To assay the compounds in concentration-response format, RGS protein was immobilized on streptavidin-coated Luminex beads in assay buffer containing 50 mM HEPES buffer pH 7.4 with 100 mM NaCl, 0.1% lubrol and 1% BSA, and treated with compound or DMSO for 15 minutes at room temperature in 96-well plates (Genemate T-3082-1). G α_o -tagged AlexFluor 532 (30 nM final) was mixed with GDP $\cdot$ AlF $_4^-$ and 1 mM MgCl $_2$, then added to the RGS beads and allowed to incubate at room temperature for 30 minutes before measuring binding. Compounds were tested in concentration-response from 100 nM-100 μ M. To determine reversibility, RGS-coated beads were incubated with 100 μ M compound or DMSO for 30 minutes at room temperature. The beads were then washed three times with assay buffer. The beads solution was then split into two groups in which one group was retreated with 100 μ M compound (to determine maximum inhibition) and the other DMSO, then each added to a separate G α_o mixture as before. Binding between RGS and G-protein was observed using a Luminex 200 flow cytometer. GraphPad Prism software v5.01 was used for the non-linear regression analysis of inhibition curves.

2.8 Receptor-independent, Steady-state GTPase Acceleration Protein Assay

In order to measure changes to RGS4 stimulation of G-protein steady-state GTP hydrolysis, independent of a receptor, an R178M/A326S mutant of G α_{i1} was utilized [36]. GTP hydrolysis was monitored colorimetrically using a malachite green dye that increases absorbance at 630 nm in complex with free inorganic phosphate. Malachite green was prepared using the method described previously [37]. Compounds were serially diluted in 50 mM HEPES at pH 7.4, including 100 mM NaCl, 0.01% Lubrol, 5 mM MgCl $_2$, and 10 μ g/mL BSA using half-log steps between 100 nM-100 μ M in 384-well plates (Corning 3680 clear flat-bottom plates, Corning, NY). To each well, 200 nM Δ 51 RGS4-MBP and 6 μ M G α_{i1} (R178M/A326S) was added before initiating the reaction with 300 μ M GTP in 8 μ L final volume. The reaction was incubated at room temperature for 120 minutes followed by quench with 10 μ L of HCl/Malachite green dye, then immediately followed by 2 μ L of a 32% w/v stock solution of sodium citrate as a colorimetric stabilizer. Each plate was incubated at room temperature for 20 minutes before reading Malachite green absorbance at 630 nm using a Victor II plate reader (Perkin Elmer). GraphPad Prism software v5.01 was used for the non-linear regression analysis of inhibition curves.

2.9 Thermal stability measurements

Thermal stability changes of G α_{i1} or Δ 51 RGS4 (cleaved from MBP) in the presence of compound were determined using a ThermoFluor instrument (Johnson & Johnson, Langhorne, PA). Each protein (10 μ M final) was mixed with compound (100 μ M final) or DMSO, 1-anilinonaphthalene-8-sulfonic acid (1,8 ANS) at a final concentration of 200 μ M,

and overlaid with 5 μ L of silicon oil before incubating for 15 minutes at room temperature in a black 384-well PCR plate (Fisher Scientific, Cat# TF-0384K). Thermal stability was measured between 30-90 °C increasing the temperature by 1 °C with intervening fluorescence measurements at 25 °C for each point. The melting temperature (T_m) was determined by applying a sigmoidal fitting procedure to determine the midpoint transition from the folded to unfolded state, using GraphPad Prism v5.01.

3. Results

3.1 High-throughput cellular screen for RGS4 inhibitors

An M3-R4 cell-line was developed to efficiently identify compounds capable of inhibiting RGS4 in a cellular environment. These cells stably express human M_3 -muscarinic receptor with Doxycycline (Dox)-regulated RGS4 expression. The full length RGS4 expressed in these cells contains a cysteine to serine point mutation at position two in the peptide sequence (C2S), which abrogates proteosomal degradation to enhance cellular levels of RGS4 [31]. Stimulation of the cells with carbachol produces a $G\alpha_q$ -mediated calcium transient that is monitored using the calcium-sensitive Fluo4 fluorescent dye. Overnight Dox treatment induces RGS4 expression leading to a marked suppression of calcium release through GAP activity on $G\alpha_q$ (Figure 1a & b). In the screen cells were plated in 384-well plates and stimulated with an approximate EC_{20} concentration of carbachol (1 nM) in order to obtain an optimal RGS4-effect. This produced an observed Z-factor of 0.5-0.6 across three separate plates (approximately 80 -Dox and 80 +Dox wells on each plate).

In the primary screen (PubChem AID: 463165), 305,721 compounds were tested for their ability to increase carbachol-stimulated calcium transient in RGS4-expressing cells. Compounds that produce high background fluorescence during the 10 second baseline recording were removed from further analysis. Hit identification followed with selection of compounds producing a B-score [32] greater than three standard deviations above the mean for the library. This resulted in 1,365 compounds capable of enhancing carbachol-stimulated fluorescence above the threshold B-score value (Table 1). Results from representative hits are illustrated in figure 1c.

To exclude compounds that increase calcium flux through a mechanism unrelated to RGS4 (e.g. positive allosteric modulator of the receptor or through interactions with downstream targets in the calcium signaling pathway), the primary hits were tested in a counter-screen in the absence of Dox induction. Compounds that enhanced the carbachol-stimulated fluorescence more than five standard deviations above the mean for the negative controls were discarded at this stage (Table 1). Furthermore, in order to remove muscarinic receptor agonists, the cells were left untreated with Dox and compound alone was injected before recording data. Compounds were discarded based on the same activity criteria as before. Finally, the remaining hits were confirmed in a concentration-response format to evaluate the EC_{50} concentrations. As a result of the primary screen validation steps, a total of 58 compounds were selected on the basis of their ability to enhance carbachol-stimulated calcium signaling specifically in Dox treated cells and in a concentration-dependent manner (Table 1).

3.2 Inhibition of the RGS- $G\alpha$ interaction

Two assays were employed to determine whether the primary screen hits directly block the RGS- $G\alpha$ interaction. The first, a multiplexed Flow Cytometry Protein Interaction Assay (FCPIA) monitors the equilibrium association between RGS-coupled Luminex beads and GDP- AlF_4^- -activated $G\alpha_o$ [35]. Each of the 58 compounds was reordered and dissolved in DMSO prior to testing in multiplexed FCPIA, which initially tested activity against MBP-

tagged RGS4, RGS4 lacking cysteines (-Cys), and RGS8. The multiplexing is achieved by coupling each protein to individually identifiable bead sets and then adding the bead sets to the same well with fluorescently-labeled $G\alpha_o$. Thirteen compounds inhibited RGS4- $G\alpha_o$ binding with IC_{50} values less than 100 μ M, though most were less than 15 μ M (Figure 2a). Inhibition of $G\alpha$ binding to RGS4 (-Cys) or RGS8 occurred with only two of the 58 compounds tested (CID: 11957531 and 10069059, supplemental table 1), which are structurally similar trihydroxy apomorphine derivatives (Supplemental figure 1). The results of the multiplexed FCPIA suggest that, with the exception of the apomorphine compounds, all of the active inhibitors covalently modify cysteine residues on RGS4. This has been the common mechanism for all small molecule RGS4 inhibitors to date [27, 29, 38].

In order to test the compounds in a system that more closely resembles the function of RGS4 inside the cell, all 58 compounds were tested in an assay that monitors RGS4 stimulation of steady-state GTP hydrolysis by $G\alpha$ (SS-GAP). The reaction was performed in the absence of a receptor using a $G\alpha_{i1}$ mutant (R178M, A326S) capable of spontaneous nucleotide exchange [36]. Using this simplified system it is possible to rapidly evaluate large compound sets in 384-well concentration-response format using malachite green dye to quantify GTP hydrolysis [37]. In contrast to FCPIA, 27 of the 58 compounds produced IC_{50} values less than 100 μ M. All 13 compounds active in FCPIA were also active in SS-GAP (Figure 2b and supplemental figure 2).

Although there are more compounds active in SS-GAP, the majority are weak inhibitors with IC_{50} values greater than 30 μ M. The more transient association of RGS4 with $G\alpha$ during nucleotide turnover in SS-GAP may be more sensitive to inhibitors compared to the equilibrium association of RGS4 with a permanently activated $G\alpha_o$ protein in FCPIA. A second possibility is that the greater amount of bovine serum albumin required for FCPIA (1% w/v in FCPIA vs. 0.01% w/v in SS-GAP) may bind some of the compounds and reduce their effective concentrations. The 13 compounds that are active in both assays were relatively consistent in potency between the two assays. One compound in particular stood out with high potency in both assays (CID: 1472216). This higher potency could result from being significantly more reactive than most of the compounds tested, which is consistent with the level of promiscuity reported for this compound in PubChem (active in 187 bioassays, see supplemental table 1). Further characterization focused on the thirteen compounds active in both assays.

3.3 Reversibility of RGS inhibitors

Covalent drugs can provide advantages over nonreactive compounds including greater potency and prolonged therapeutic effect [39], though risk of toxicity associated with reactive compounds [40] has deterred widespread use. Reversible covalent molecules may provide the benefits associated with the covalent interaction, but with reduced physiological and toxicological problems resulting from a permanent association [41-43]. Since eleven out of thirteen compounds show a loss of activity against RGS4 (-Cys), it is likely the compounds work through a covalent mechanism. Reversibility of the compounds was tested using FCPIA since the compounds can be easily removed from the bead-coupled protein by pelleting and re-suspending the beads three times in fresh buffer. Less than half of the compounds showed significant reversibility, although four compounds produced greater than 85% inhibition at 100 μ M, yet were more than 50% reversible within the ten minute washing period (CID: 5428579, 1905297, 1389577, & 1777233 in figure 3). Interestingly, the two apomorphine compounds that showed activity on RGS4 (-Cys) were not reversible in this assay. The products formed through autoxidation of hydroxy apomorphine derivatives are highly reactive [44], thus inhibition of both RGS and $G\alpha$ by these two compounds would explain this observation, but that has not been tested.

3.4 Selectivity of reversible RGS inhibitors

Although the four reversible compounds do not appear to functionally modulate the G-protein since there is no effect on RGS8 or RGS4 (-Cys) binding in FCPIA, we assessed their binding to both RGS4 and Gα using a thermal stability shift assay. This assay provides a measure of direct protein-ligand interactions by monitoring changes in thermal stability in the presence of compound. The melting temperature of each protein was determined with and without 100 μM compound using DMSO as control. Consistent with a mechanism of covalent modification [45], all four compounds significantly destabilize RGS4 by as much as 13 °C. In contrast, none of the compounds tested significantly alter Gα thermal melting temperature (figure 4).

Selectivity was also determined using multiplexed FCPIA with a panel of RGS proteins (RGS7, RGS16, & RGS19). The four reversible RGS inhibitors showed variable activity, with 1905297 being relatively selective for RGS4 (figure 5). RGS19 appears to be highly sensitive to cysteine modification as it is consistently inhibited by most of the compounds tested (supplemental table 2). Even though RGS4 and RGS19 show nearly comparable sensitivity to most of the inhibitors, other closely related RGS4 isoforms (i.e. RGS8 and RGS16) remain relatively insensitive at these concentrations. It is also notable that several compounds (including 5428579 and 1389577) showed activity towards RGS7, which lacks cysteine residues. It is unclear from these data whether the compounds acting on RGS7 bind to a defined pocket, or are reactive towards other groups besides thiols.

3.5 Inhibition of RGS4 activity in M3-R4 cells

The challenge facing thiol-reactive compounds to permeate the cell membrane and overcome the reductive cytoplasmic environment has limited the number of cell-active RGS inhibitors. As a result, this system monitoring RGS4 activity within intact cells was employed to identify novel RGS inhibitors. The thirteen RGS inhibitors identified in this screen antagonize RGS4 cellular activity at multiple concentrations, and this is illustrated specifically for the four reversible RGS inhibitors (figure 6). The cellular EC₅₀ concentrations (table 2) are very close to the values observed using FCPIA and SS-GAP, which suggests the mechanism of disrupting the RGS-Gα interaction is the same inside the cells.

4. Discussion

Although therapeutics targeting GPCRs have been remarkably successful, there remains a critical need for improved therapeutic options to selectively modulate GPCR signaling. A promising new approach towards this goal is to selectively modulate mechanisms that regulate these receptors. Among these, RGS proteins are recognized as important targets for their role in negatively regulating the amplitude and duration of GPCR output. The varying tissues expression profiles and receptor coupling efficiencies suggests RGS modulation may provide therapeutic specificity towards individual receptors, or receptor populations. This effect has been shown using genetic models of RGS “insensitivity” with knock-in mice that carry a Gα_{i2} (G184S) mutant allele in place of the WT gene. This mutation renders Gα_{i2} incapable of binding to any RGS protein [46]. Animals carrying this knock-in mutation show potentiated responses that are specific to 5-HT_{1A}-mediated anti-depressant behavior [47], with no significant increase in the hypothermic response that is also known to be mediated through these receptors [48].

Targeting PPIs, like the RGS-Gα interaction, is a significant challenge due to the structural topology generally associated with signal transduction proteins. In particular, the RGS-Gα interface covers a surface area of 1100 Å² [49] without well-defined pockets into which

small molecules can be targeted to block the interaction. The absence of any non-covalent small molecules capable of acting on RGS proteins is one potential consequence of the relatively featureless structural surface. As a result, thiol-reactive compounds have predominated as RGS inhibitors since the formation of a covalent bond with cysteine residues often provides enough binding energy to surmount the high affinity interaction with G-proteins. The challenge of blocking RGS function within the reducing cellular environment has likely limited the number of RGS inhibitors thus far identified.

A cell-based high-throughput screen was implemented to directly identify cell-active RGS inhibitors. This assay takes advantage of the strong inhibitory effects of RGS4 on G_{α_q} signaling, which substantially suppresses the calcium fluorescence readout shown in figure 1. A critical component of the assay is the Dox-inducible RGS4 expression, which provides an efficient means of eliminating compounds targeting other parts of the calcium signaling pathway. Additional steps were taken to rule out muscarinic receptor agonists, positive allosteric modulators, or partial agonists in order to ensure calcium signaling effects were mediated through antagonism of RGS4. As a result of these triage steps, approximately half of the 58 compounds evaluated here were able to directly block the RGS- G_{α} interaction in a biochemical assay (SS-GAP). For reasons outlined in section 3.2, there were fewer active compounds in FCPIA, but there was direct overlap between the two assays.

It remains possible the compounds found inactive in FCPIA and SS-GAP could still inhibit RGS4 effects in the cell by modulating localization to receptor or G-protein pools at the membrane. The N-terminal 33 residues of RGS4 contains an amphipathic helical domain that plays an important role in trafficking [50] and localization to both the membrane and specific receptors [16, 51]. A compound that binds to this critical region could inhibit RGS4 localization to receptor complexes at the membrane, thereby preventing or reducing association of RGS4 with G_{α_q} . In FCPIA and SS-GAP the proteins are freely diffusible, so compounds that only affect localization would not have activity. Furthermore, the $\Delta 51$ RGS4 protein used in these assays lacks the N-terminal localization domain. In this report we have characterized the compounds that specifically block the RGS- G_{α} interaction, which provide the advantage of not being limited to a specific receptor or receptor-scaffolding complex.

Like all previously reported small molecule RGS inhibitors, most compounds in this study lack activity towards RGS4 (-Cys). This suggests that the mechanism of inhibition is, at least in part, due to a covalent interaction with cysteine sidechains on the RGS proteins. The thermal destabilization of RGS4 in the presence of the four reversible inhibitors (figure 5) is also consistent with a covalent mechanism of action, and has been observed with all previous RGS4 inhibitors [27, 29, 38]. Despite the reactive mechanism, the compounds are specific for RGS4 in the thermofluor assay as they do not significantly change the G_{α} melting temperature. A reaction with G_{α} cannot be completely ruled out as an interaction with a solvent exposed and functionally isolated cysteine sidechain is still possible without significantly altering melting temperature. The lack of an effect on the folding→unfolding transition energy of G_{α} strongly suggests there is no functional influence. Furthermore, the specificity for RGS is also corroborated by the selectivity for certain RGS isoforms. If the compounds were able to modulate G_{α} function, it is unlikely specificity would be observed (table 2 and supplemental table 2).

The biggest hurdle associated with the use of covalent inhibitors as therapeutics or even biological probes is the concern over the specificity and toxicity. As a therapeutic, irreversible compounds or metabolites sometimes form the basis of immune-related adverse drug reactions [40]. In a research setting toxicity can also obscure interpretation of biological data. These problems can often be mitigated if the covalent compounds are

reversible over a biologically relevant time scale [41, 43]. Reversibility of the RGS inhibitors was determined using FCPIA, which identified four compounds with both strong inhibition and significant reversibility over a 10 minute washing period. Additionally, these four compounds were some of the least promiscuous [52] based on biological activity reported in the PubChem project (pubchem.ncbi.nlm.nih.gov), which is compiled in supplemental table 1.

5. Conclusions

This report details the identification and mechanistic evaluation of several new reversible, cell active RGS inhibitors. Thirteen compounds were identified that consistently inhibit the RGS-G α interaction, although most lost activity towards an RGS4 mutant lacking cysteine residues. This is a common characteristic among all RGS4 small molecule inhibitors, and most cysteine-reactive compounds in general. Despite the apparent reactivity, many of the inhibitors show specificity for RGS4 over other RGS isoforms tested. We further determined that four compounds are significantly reversible, which is an important characteristic both as a therapeutic or pharmacological tool. The significance of this study is that now a greater number of pharmacological tools are available to disseminate the roles of RGS4 in biology and disease. Recent publications have already begun using RGS inhibitors previously described, but the lack of activity in intact cells has no doubt limited the use of most RGS inhibitors currently available. Finally, as a part of the identification process we have also described a novel cellular signaling assay that provides an efficient mechanism to rapidly evaluate large numbers of compounds for activity against RGS4 (or possibly other RGS proteins) within the context of the calcium signaling cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	Bovine Serum Albumin
DMSO	dimethyl sulfoxide
FCPIA	Flow Cytometry Protein Interaction Assay
GAP	GTPase Accelerating Protein
MBP	Maltose Binding Protein
PPI	Protein-protein interaction
RGS	Regulator of G-protein Signaling
T_m	melting temperature

Literature References

1. Williams C, Hill SJ. GPCR signaling: understanding the pathway to successful drug discovery. *Methods Mol Biol.* 2009; 552:39–50. [PubMed: 19513640]
2. Messer WS Jr. The utility of muscarinic agonists in the treatment of Alzheimer's disease. *J Mol Neurosci.* 2002; 19(1-2):187–93. [PubMed: 12212779]
3. Thathiah A, De Strooper B. The role of G protein-coupled receptors in the pathology of Alzheimer's disease. *Nat Rev Neurosci.* 2011; 12(2):73–87. [PubMed: 21248787]
4. Lane JR, Abdul-Ridha A, Canals M. Regulation of G protein-coupled receptors by allosteric ligands. *ACS Chem Neurosci.* 2013; 4(4):527–34. [PubMed: 23398684]
5. Lane JR, Sexton PM, Christopoulos A. Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends Pharmacol Sci.* 2013; 34(1):59–66. [PubMed: 23177916]
6. Magalhaes AC, Dunn H, Ferguson SS. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br J Pharmacol.* 2012; 165(6):1717–36. [PubMed: 21699508]
7. Blazer LL, Neubig RR. Small molecule protein-protein interaction inhibitors as CNS therapeutic agents: current progress and future hurdles. *Neuropsychopharmacology.* 2009; 34(1):126–41. [PubMed: 18800065]
8. Berman DM, Wilkie TM, Gilman AG. GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. *Cell.* 1996; 86(3):445–52. [PubMed: 8756726]
9. Hepler JR, et al. RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. *Proc Natl Acad Sci U S A.* 1997; 94(2):428–32. [PubMed: 9012799]
10. Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem.* 2000; 69:795–827. [PubMed: 10966476]
11. Larminie C, et al. Selective expression of regulators of G-protein signaling (RGS) in the human central nervous system. *Brain Res Mol Brain Res.* 2004; 122(1):24–34. [PubMed: 14992813]
12. Gold SJ, et al. Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. *J Neurosci.* 1997; 17(20):8024–37. [PubMed: 9315921]
13. Bernstein LS, et al. RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq/11alpha signaling. *J Biol Chem.* 2004; 279(20):21248–56. [PubMed: 14976183]
14. Wang Q, Liu-Chen LY, Traynor JR. Differential modulation of mu- and delta-opioid receptor agonists by endogenous RGS4 protein in SH-SY5Y cells. *J Biol Chem.* 2009; 284(27):18357–67. [PubMed: 19416973]
15. Kovoov A, et al. D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knock-out mice develop dyskinesias associated with dopamine pathways. *J Neurosci.* 2005; 25(8):2157–65. [PubMed: 15728856]
16. Zeng W, et al. The N-terminal domain of RGS4 confers receptor-selective inhibition of G protein signaling. *J Biol Chem.* 1998; 273(52):34687–90. [PubMed: 9856989]
17. Colver J, Sharma M, Kovoov A. RGS9-2 mediates specific inhibition of agonist-induced internalization of D2-dopamine receptors. *J Neurochem.* 2010; 114(3):739–49. [PubMed: 20477943]
18. Talbot JN, et al. Differential modulation of mu-opioid receptor signaling to adenylyl cyclase by regulators of G protein signaling proteins 4 or 8 and 7 in permeabilised C6 cells is Galpha subtype dependent. *J Neurochem.* 2010; 112(4):1026–34. [PubMed: 20002516]
19. Heximer SP, et al. RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc Natl Acad Sci U S A.* 1997; 94(26):14389–93. [PubMed: 9405622]
20. Hunt TW, et al. RGS10 is a selective activator of G alpha i GTPase activity. *Nature.* 1996; 383(6596):175–7. [PubMed: 8774883]
21. Hooks SB, et al. RGS6, RGS7, RGS9, and RGS11 stimulate GTPase activity of Gi family G-proteins with differential selectivity and maximal activity. *J Biol Chem.* 2003; 278(12):10087–93. [PubMed: 12531899]

22. Neubig RR, Siderovski DP. Regulators of G-protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov.* 2002; 1(3):187–97. [PubMed: 12120503]
23. Sjogren B, Blazer LL, Neubig RR. Regulators of G protein signaling proteins as targets for drug discovery. *Prog Mol Biol Transl Sci.* 2010; 91:81–119. [PubMed: 20691960]
24. Kimple AJ, et al. Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev.* 2011; 63(3):728–49. [PubMed: 21737532]
25. Lerner TN, Kreitzer AC. RGS4 is required for dopaminergic control of striatal LTD and susceptibility to parkinsonian motor deficits. *Neuron.* 2012; 73(2):347–59. [PubMed: 22284188]
26. Chen Y, et al. Neurabin scaffolding of adenosine receptor and RGS4 regulates anti-seizure effect of endogenous adenosine. *J Neurosci.* 2012; 32(8):2683–95. [PubMed: 22357852]
27. Blazer LL, et al. Reversible, allosteric, small-molecule inhibitors of RGS proteins. *Mol Pharmacol.* 2010
28. Kimple AJ, et al. The RGS protein inhibitor CCG-4986 is a covalent modifier of the RGS4 Galpha-interaction face. *Biochim Biophys Acta.* 2007; 1774(9):1213–20. [PubMed: 17660054]
29. Roman D, et al. Allosteric Inhibition of the RGS-G{alpha} Protein-Protein Interaction by CCG-4986. *Mol Pharmacol.* 2010
30. Roman DL, et al. Identification of small-molecule inhibitors of RGS4 using a high-throughput flow cytometry protein interaction assay. *Mol Pharmacol.* 2007; 71(1):169–75. [PubMed: 17012620]
31. Bodenstein J, Sunahara RK, Neubig RR. N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol.* 2007; 71(4):1040–50. [PubMed: 17220356]
32. Brideau C, et al. Improved statistical methods for hit selection in high-throughput screening. *J Biomol Screen.* 2003; 8(6):634–47. [PubMed: 14711389]
33. Soundararajan M, et al. Structural diversity in the RGS domain and its interaction with heterotrimeric G protein alpha-subunits. *Proc Natl Acad Sci U S A.* 2008; 105(17):6457–62. [PubMed: 18434541]
34. Lee E, Linder ME, Gilman AG. Expression of G-protein alpha subunits in Escherichia coli. *Methods Enzymol.* 1994; 237:146–64. [PubMed: 7934993]
35. Blazer LL, et al. Use of flow cytometric methods to quantify protein-protein interactions. *Curr Protoc Cytom.* 2010 **Chapter 13**: p. Unit 13 11 1-15.
36. Zielinski T, et al. Two Galpha(i1) rate-modifying mutations act in concert to allow receptor-independent, steady-state measurements of RGS protein activity. *J Biomol Screen.* 2009; 14(10):1195–206. [PubMed: 19820068]
37. Chang L, et al. High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. *Anal Biochem.* 2008; 372(2):167–76. [PubMed: 17904512]
38. Blazer LL, et al. A nanomolar-potency small molecule inhibitor of Regulator of G protein Signaling (RGS) proteins. *Biochemistry.* 2011
39. Singh J, et al. The resurgence of covalent drugs. *Nat Rev Drug Discov.* 2011; 10(4):307–17. [PubMed: 21455239]
40. Park BK, et al. Managing the challenge of chemically reactive metabolites in drug development. *Nat Rev Drug Discov.* 2011; 10(4):292–306. [PubMed: 21455238]
41. Lee CU, Grossmann TN. Reversible covalent inhibition of a protein target. *Angew Chem Int Ed Engl.* 2012; 51(35):8699–700. [PubMed: 22806944]
42. Smith AJ, et al. Beyond picomolar affinities: quantitative aspects of noncovalent and covalent binding of drugs to proteins. *J Med Chem.* 2009; 52(2):225–33. [PubMed: 19053779]
43. Tummino PJ, Copeland RA. Residence time of receptor-ligand complexes and its effect on biological function. *Biochemistry.* 2008; 47(20):5481–92. [PubMed: 18412369]
44. El-Bacha RS, Netter P, Minn A. Mechanisms of apomorphine cytotoxicity towards rat glioma C6 cells: protection by bovine serum albumin and formation of apomorphine-protein conjugates. *Neurosci Lett.* 1999; 263(1):25–8. [PubMed: 10218902]

45. Cimperman P, et al. A quantitative model of thermal stabilization and destabilization of proteins by ligands. *Biophys J*. 2008; 95(7):3222–31. [PubMed: 18599640]
46. Fu Y, et al. RGS-insensitive G-protein mutations to study the role of endogenous RGS proteins. *Methods Enzymol*. 2004; 389:229–43. [PubMed: 15313569]
47. Talbot JN, et al. RGS inhibition at G(alpha)i2 selectively potentiates 5-HT1A-mediated antidepressant effects. *Proc Natl Acad Sci U S A*. 2010; 107(24):11086–91. [PubMed: 20534514]
48. Richardson-Jones JW, et al. 5-HT1A autoreceptor levels determine vulnerability to stress and response to antidepressants. *Neuron*. 2010; 65(1):40–52. [PubMed: 20152112]
49. Tesmer JJ, et al. Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. *Cell*. 1997; 89(2):251–61. [PubMed: 9108480]
50. Bastin G, et al. Amino-terminal cysteine residues differentially influence RGS4 protein plasma membrane targeting, intracellular trafficking, and function. *J Biol Chem*. 2012; 287(34):28966–74. [PubMed: 22753418]
51. Srinivasa SP, et al. Plasma membrane localization is required for RGS4 function in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*. 1998; 95(10):5584–9. [PubMed: 9576926]
52. McGovern SL, et al. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J Med Chem*. 2002; 45(8):1712–22. [PubMed: 11931626]

Highlights

- A novel cell-based calcium signaling assay for chemical RGS modulators is described
- A high-throughput screen identified thirteen cell-active RGS inhibitors
- Several of the RGS inhibitors show specificity for RGS4
- Four compounds have a reversible mechanism of inhibition

Figure 1.

Inhibitors targeting RGS4 enhance carbachol-stimulated calcium signaling in cells treated with Doxycycline. (A) HEK-FlpIn cells stably transfected with M₃-muscarinic receptor produce a calcium transient in response to carbachol. Overnight treatment with Doxycycline (Dox) markedly suppresses the G_α_q-mediated calcium response through induction of RGS4 protein. (B) HA-tagged RGS4 is minimally detectable in HEK-FlpIn cells prior to treatment with Dox. Probing with an anti-HA antibody after a 45 hour treatment with Dox shows a marked increase in RGS4 protein, producing levels similar to transiently transfected cells (HEK Trans). (C) Representative primary screening hits that increase the carbachol-stimulated calcium transient towards the -Dox control (open squares) by antagonizing

RGS4-mediated suppression of G_{α_q} signaling. The primary screening hits were tested in duplicate wells at 10 μ M (final). Carb, carbachol; Dox, Doxycycline; HEK Trans, transiently transfected cells; HEK Mock, mock transfection with empty pCDNA vector.

Figure 2.

Activity summary of the compounds tested on RGS4 in FCPIA and SS-GAP. All 58 primary screen hits were assayed for an ability to block RGS4- $G\alpha_o$ equilibrium binding in FCPIA and inhibit RGS4-stimulation of $G\alpha_{i1}$ nucleotide hydrolysis in SS-GAP. Compounds with an IC_{50} value less than 100 μM were selected for further analysis, resulting in 27 compounds meeting this criterion in SS-GAP and 13 in FCPIA. All 13 compounds active in (A) FCPIA were also active in (B) SS-GAP. The IC_{50} values range from approximately 2-55 μM in both assays. CID 1472216 is the most potent RGS4 inhibitor tested, $IC_{50} = 1.6 \pm 0.4$ μM in SS-GAP and 1.7 ± 0.5 μM in FCPIA. The data are the mean $\pm$ S.E.M. of three independent experiments in duplicate wells. SS-GAP, steady-state GTPase acceleration protein assay; FCPIA, flow cytometry protein interaction assay; pIC_{50} , $\log IC_{50}$.

Figure 3.

Determination of RGS4 inhibitor reversibility using FCPIA. RGS4-labeled Luminex beads were treated with 100 μ M compound or DMSO for 30 minutes at room temperature before washing the beads three times. The beads were then split in two sets with DMSO or 100 μ M compound added back to the no wash set (open bar). Both treated and washed RGS4-coupled beads were tested for inhibition of G_{α_o} binding using FCPIA. Statistics: One-Way ANOVA with Bonferroni's multiple comparisons *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are the result of three independent experiments $\pm$ S.E.M.

Figure 4.

The reversible RGS4 inhibitors alter the thermal melting temperature of RGS4, but not Gα. RGS4 and Gα_{i1} were treated with 100μM compound or DMSO for 30 minutes prior to measuring thermal stability changes of each protein across a temperature gradient of 30-90°C. Melting was monitored as an increase in 1,8 ANS fluorescence using a ThermoFluor instrument. Data are plotted as temperature change relative to the DMSO control for each protein. None of the compounds significantly changed the thermal stability of Gα_{i1} compared to DMSO control. Statistics: One-Way ANOVA with Bonferroni's multiple comparisons *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are the result of three independent experiments ± S.E.M.

Figure 5.

Compounds 1905297 and 1777233 show selectivity for RGS4 over four other RGS isoforms tested in FCPIA. (A) Compound 1905297 is at least 3.6-fold selective for RGS4 over all other RGS proteins tested. (B) Compound 1777233 is slightly more potent towards RGS4, although there is only a 2-fold potency difference from RGS19 and 5-fold from RGS16. (C) & (D) Both 5428579 and 1389577 have approximately equal potency towards RGS4 and RGS19. In addition, activity was observed against RGS7 and RGS16 for 5428579 and RGS7 for 1389577. Compounds were tested from 100 nM to 100 μ M and data are the mean of three independent experiments.

Figure 6.

Cellular activity of the four reversible RGS4 inhibitors. (A) 1905297 increased the calcium response over Dox-treated control cells at 30 and 10 μM , although effects at lower concentrations may be mitigated as a result of lower overall carbachol response. (B)–(D) The carbachol response is increased over Dox-treated control cells at 1 μM compound and above. Data shown are representative responses performed in duplicate wells.

Table 1
Screening and Biochemical Confirmation Summary

	<i>Compounds Tested</i>	<i>Active/ Confirmed</i>	<i>Percent of Tested</i>
Primary Screen	305,721	1,365	0.45
Counter Screen: (-)Dox, (+)Carb.	1,365	1,078	79
Counter Screen: (-)Dox, (-)Carb.	1,078	467	43
CRC ^a Confirmation	467	58	12
Biochemical Confirmation	58	13	22

^a Concentration-response curves.

Table 2
Activity Summary of Reversible Compounds

<i>CID</i>	<i>FCPIA</i> <i>IC</i> ₅₀ (<i>μM</i>)	<i>SS-GAP</i> ^{<i>a</i>} <i>IC</i> ₅₀ (<i>μM</i>)	<i>Cellular Activity</i> <i>IC</i> ₅₀ (<i>μM</i>)	<i>Specificity (FCPIA)</i> ^{<i>b</i>}
5428579	8.2	55.8	35.5	R19 ≈ R4 > R7 > R16 > R8
1905297	27.9	35.6	23.8	R4 > (R7, R8, R16, R19)
1389577	18.3	10.9	15.0	R4 ≈ R19 > R7 > (R8, R16)
1777233	15.3	9.4	20.0	R4 > R19 > R16 > (R7, R8)

^{*a*}Steady-state GTPase Activating Protein Assay;

^{*b*}Flow cytometry protein interaction assay.