Discovery of Small Molecule Inhibitors for RGS Proteins using the RGScreen™ Platform

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Introduction and Summary

Members of the "regulator of G-protein signaling" (RGS)-protein superfamily act as "GTPase-accelerating proteins" (GAPs) for heterotrimeric G-protein alpha subunits, thereby attenuating GPCR signaling. Their selectivity for specific GPCRs and downstream signaling components makes them attractive as potential therapeutic targets, with the potential to modulate the effects of existing GPCR agonists. The most direct way to detect RGS protein function is by measuring the increased GTPase activity of the associated Gα protein. However, the GTPase activity of isolated Gα proteins is limited by GDP dissociation, so RGS protein GAP activity cannot be measured using simple biochemical assays. To overcome this kinetic constraint, we developed the RGScreen™ platform which uses proprietary Gα protein variants with altered GTP hydrolysis and GDP dissociation rates that enable detection of RGS protein GAP activity using the Transcreener® GDP Assay. Importantly, the selectivity of the Gα/RGS domain interactions tested was not altered by the mutations. We performed a 50k compound screen with RGS17 and RGS4 in combination with $G\alpha_{i1}$ and obtained a hit rate of 0.7% (>50% inhibition at 10μM) with an average Z' of 0.8. Follow up assays confirmed that more than 95% of the hits specifically inhibited the target RGS protein, without a significant effect on $G\alpha_{i1}$ alone. In addition, selectivity profiling with panels of RGS proteins and G alpha subunits revealed selectivity for both enzyme (RGS protein) and substrate (G alpha protein) for some inhibitors. This study lays the foundation for identification of small molecule probes to elucidate the function of RGS proteins and to explore their potential as drug targets.

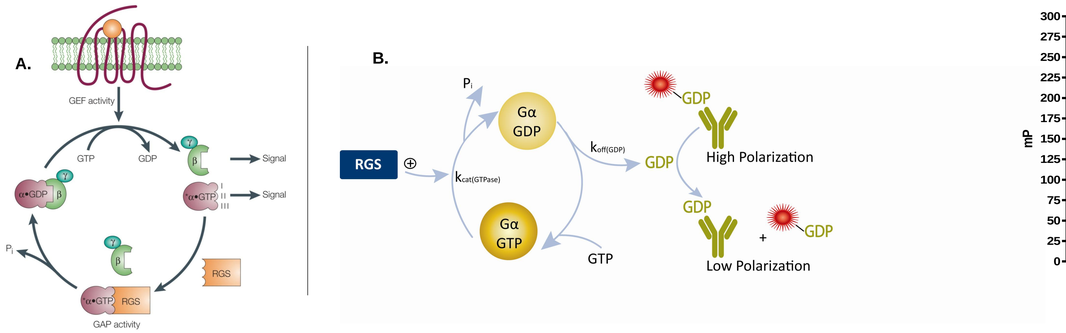
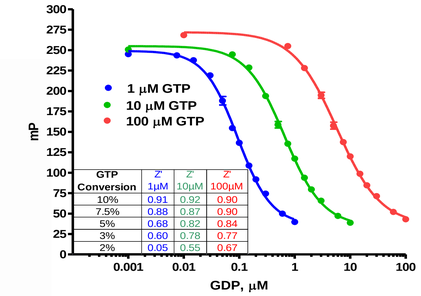


Figure 1. Coupling Kinetically Altered Gα Proteins With the Transcreener® GDP Assay Enables Direct Detection of RGS Protein GAP Activity. A) RGS proteins act catalytically on Gα-GTP complexes to stimulate GTP hydrolysis rates as much as 100-fold, thereby attenuating GPCR agonist signals. B) Detection of GDP using the Transcreener GDP Assay, a competitive fluorescence polarization immunoassay, offers an HTS compatible approach for directly measuring RGS GAP effects. However GDP dissociates from isolated Gα proteins very slowly, so RGS GAP effects are not detectable using steady state GTPase assays. To overcome this kinetic limitation, we combined Gα mutations that increased GDP dissociation and decreased GTPase activity to measure RGS GAP activity.



GTP Conversion	Z' 1μM	Z' 10μM	Z' 100μM
10%	0.91	0.92	0.90
7.5%	0.88	0.87	0.90
5%	0.68	0.82	0.84
3%	0.60	0.78	0.77
2%	0.05	0.55	0.67

Figure 2. The Transcreener® GDP Assay Allows Direct Detection of GDP Formation in a Homogenous, HTS Compatible Format. Standard curves mimicking enzyme reactions were constructed for conversion of GTP to GDP using initial GTP concentrations of 1 μM, 10 μM, and 100 μM GTP; as GDP was added, GTP was decreased proportionately. Fluorescence polarization assays were performed in black Corning® 384 Well Black Flat Bottom Microplates (Part #3654). Assays were read in a Tecan Safire$^{2™}$ multiwell reader. All points were done in 16 replicates so that Z' values could be determined (inset table).

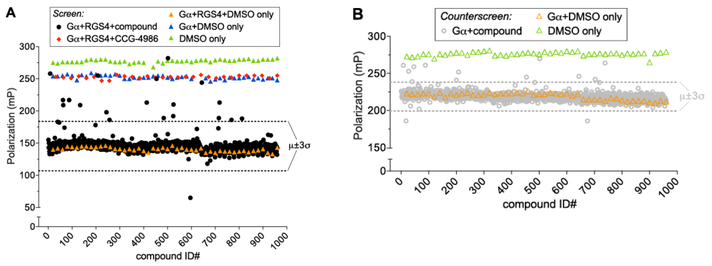


Figure 3. Pilot GenPlus Library Screen with Doubly-Mutated (DM) $G\alpha_{i1}$ in the Presence (A) and Absence (B) of RGS 4. GDP assay components were added to wells containing 50 nM $G\alpha_{i1}$ DM with (A) or without (B) 250 nM RGS4 protein and 10 μM compound (in 0.5% [v/v] final concentration of DMSO), 150 μM of reactive RGS4 inhibitor CCG-4986, or 0.5% DMSO only, as indicated in the legends. The range of signal observed (3 standard deviations [σ] about the mean [μ]) is denoted by the dashed lines for the 960-compound library screen using RGS4 and $G\alpha_{i1}$ DM. The Z' in **A** at 120 minutes was 0.83, which reflects only the RGS4-dependent increase in GTPase activity of the controls. The Z factor for the GenPlus library screen was 0.73, which was obtained by excluding values from wells containing hit compounds. Of the 17 hits in **A** that were outside 3 StdDevs, 10 were excluded as GTPase inhibitors by **B**, thus the RGS4-specific hits rate was 0.7%.

50,000 Compound Library Screen Strategy

1) Screened $G\alpha_{i1}$DM vs RGS4, RGS17 & No RGS (N=2)

↓

2) Determined Number of Compounds with 50% Inhibition or Specificity for RGS4 or RGS17 (excluded GTPase inhibitors or compounds causing interference)

↓

3) Performed ~10pt Dose Response Curves with a Subset of 160 Compounds with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_o$ vs. RGS4, RGS17 and no RGS

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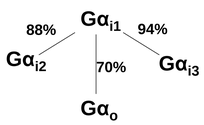

4) Plotted and Analyzed Inhibition Patterns of Dose Response Curves

Figure 4. Results of the 50,000 Compound Screen. 50,000 compounds drawn from the Chembridge DiverSet were screened as described for the Pilot Screen in Figure 3, with the exception that RGS17 was also screened with $G\alpha_{i1}$ DM. This diverse library represents a broad pharmacophore sample pre-filtered of undesirable compounds. The screen had an average Z' of 0.8 for both RGS4 and RGS17 dependent GTPase activity and an average Z factor of 0.7. Standard curves run on each plate allowed product formation determination, followed by % inhibition results (0% = + RGS, 100% = - RGS). 1.3% of the compounds were determined to be outside of 3 StdDevs of the mean. 0.7% of the compounds exhibited at least 50% inhibition. Only 0.1% of the compounds showed RGS specificity (8 RGS4 vs. 42 RGS17).

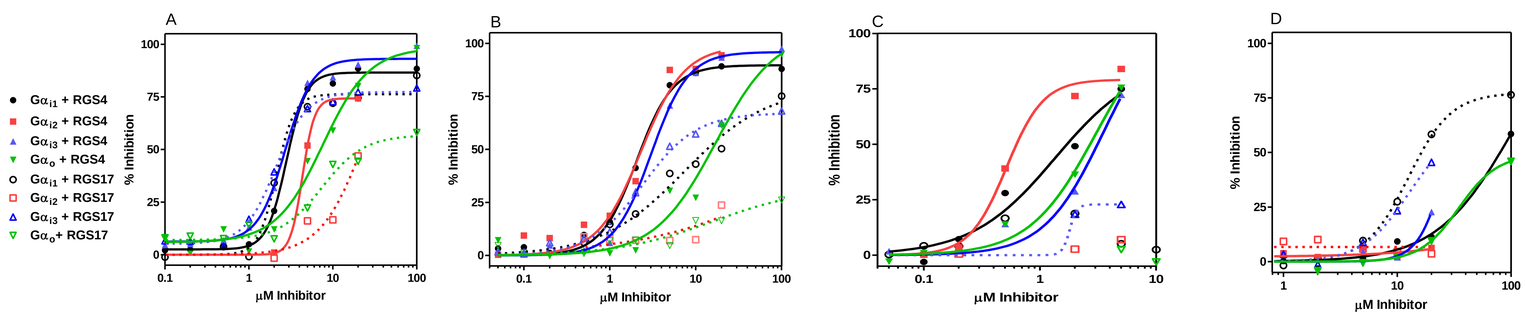

Figure 5. Profiling of Hits. Follow-up dose response curves were performed with 160 compounds from the 50K compounds screen (Figure 4) and tested for their relative effects on RGS4 and RGS17 GAP activity with doubly-mutated $G\alpha_{i1}$ $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$. Representative inhibition patterns are shown. **A)** 85 compounds exhibited greater potency with $G\alpha_{i1,2,\&3}$ than with $G\alpha_o$, with approximately equal potency for RGS4 and RGS17 with $G\alpha_{i1\&3}$, and higher potency/efficacy for RGS4 vs. RGS17 with $G\alpha_{i2\&O}$ **B)** 10 compounds showed potency with $G\alpha_{i1,2,3} > G\alpha_o$, potency/efficacy with RGS4 > RGS17. **C)** One compound showed high potency/efficacy for RGS4 vs. RGS17. **D)** 15 compounds had potency for RGS17 > RGS4 for $G\alpha_{i1\&3}$, and no inhibition with $G\alpha_{i2}$ or $G\alpha_O$. Other inhibition patterns were also observed.

Conclusions

- Using kinetically altered Gα protein variants in combination with the Transcreener ® GDP Assay provides a robust HTS method for identifying inhibitors of RGS protein GAP activity.
- The double mutant Gα strategy has been applied to $G\alpha_{i1}$, $G\alpha_{i2}$,$G\alpha_{i3}$ and $G\alpha_o$ with similar results.
- A 50K compound screen of RGS4 and RGS17 proteins with $G\alpha_{i1}$ yielded a 0.7% hit rate (>50% inhibition). Counter-screening without RGS allowed exclusion of non-specific GTPase inhibitors.
- Profiling of hits vs the two target RGS proteins and a panel of four Gα proteins revealed inhibition patterns that included selectivity for one RGS protein and one or more Gα proteins.

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