

FRET-based screening for potential modulators of the $G\alpha_{i1}$ protein/GoLoco interaction

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- CFP-YFP FRET based assay utilized to screen for potential inhibitors of $G\alpha_{i1}$ /GoLoco interaction
- Inhibitors of the G protein/GoLoco protein interaction are putative pharmacological tools
- The POLARstar Omega allows for fast screening and easy data evaluation

Introduction

Many extracellular signals, including hormones, neurotransmitters, and growth factors, are relayed intracellularly by binding the extracellular portion of a seven-transmembrane domain (7TM) receptor that is coupled to an intracellular heterotrimeric G protein. The stimulated G-protein coupled receptor (GPCR) propagates the extracellular signal through activation of the alpha subunit of heterotrimeric G proteins ($G\alpha$), which occurs when $G\alpha$ exchanges GDP for GTP. The $G\alpha$ subunit is also modulated by a family of proteins containing the GoLoco motif, which serves to inhibit the exchange of GDP for GTP by the $G\alpha$ subunit. The G protein/GoLoco interaction is therefore an attractive pharmacological target because it serves as a point of regulation for downstream GPCR signaling.¹

Presently, no small molecule inhibitors of the $G\alpha$ /GoLoco interaction are available for study. Although there is currently a lack of inhibitors for this recently discovered class of proteins, the potential applications of a GoLoco inhibitor are significant.² Combining existing GPCR agonists with specific GoLoco motif inhibitors could synergistically increase the specificity of existing drugs and serve as useful chemical probes.

To identify and assess potential modulators of the $G\alpha$ /GoLoco interaction, putative inhibitors were screened in a FRET (Fluorescence/Förster Resonance Energy Transfer) assay using BMG LABTECH's POLARstar Omega.

Assay Principle

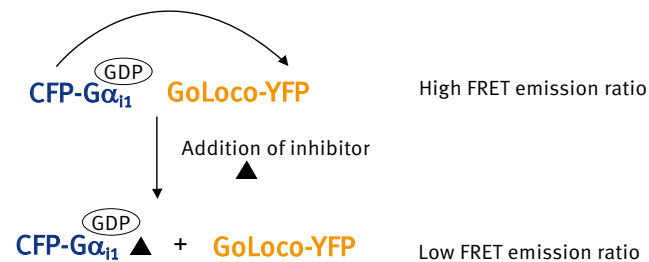


Fig. 1: Assay Principle for the FRET-based inhibitor screening

The ground state $G\alpha_{i1}$ binds GDP. The GoLoco motif selectively recognizes $G\alpha_{i1}$ (GDP) and binds with nanomolar affinity.³ In a FRET-based assay which measures the $G\alpha$ /GoLoco interaction, the FRET partners CFP- $G\alpha_{i1}$ -GDP and YFP-GoLoco yield a significantly increased (530/485 nm) FRET ratio when bound to each other than when

compared with the non-interacting state of CFP- $G\alpha_{i1}$ (as formed using aluminum tetrafluoride which mimics the transition state for GTP hydrolysis).

An inhibitor of this interaction could possibly perturb or displace the nucleotide from the $G\alpha$ binding pocket or it could occlude the GoLoco motif binding surface, thus preventing the protein/protein interaction.⁴ When $G\alpha$ and GoLoco are no longer interacting, the (530/485 nm) FRET ratio decreases (Figure 1).

Materials and Methods

- Black polystyrene 96-well plates, Corning (#3991)
- YFP: amino acids 1-237 of pEYFP-C1 open reading frame (ORF), BD Biosciences Clontech, Palo Alto, CA
- CFP: amino acids 1-239 of pECFP-C1 ORF, BD Biosciences Clontech
- POLARstar Omega, BMG LABTECH, Durham, USA



Fig. 2: BMG LABTECH's multidetection microplate reader POLARstar Omega

The RGS12 $G\alpha$ -GDP binding region (GoLoco motif, aa 1187-1221) was produced as a fluorescent chimera with the yellow fluorescent protein (YFP). Compounds were titrated into an optimized concentration of YFP-RGS12GoLoco and cyan fluorescent protein labeled $G\alpha_{i1}$ (CFP $G\alpha_{i1}$) to confirm inhibitor activity. Ground state $G\alpha_{i1}$ is GDP bound and the GoLoco motif binds readily, thus producing a higher emission ratio in the FRET assay from the non-directly excited fluorophore. In the mimicked GTP hydrolysis transition state, created by the binding of aluminum tetrafluoride (denoted AMF), the $G\alpha_{i1}$ /GoLoco interaction does not occur. This is evident by the decrease in the observed FRET ratio of non-directly excited fluorophore emission versus directly excited fluorophore emission.

For FRET measurements, the POLARstar Omega (Figure 2) was used with filters optimized for CFP/YFP FRET. Excitation filter with wavelength at 422 nm and dual emission wavelength filters at 530 nm and 485 nm were used. Instrument settings can be found below.

Instrument settings

Positioning Delay	0.2 sec
Measurement start time	0.0 sec
No. of flashes per well	10
No. of multichromatics	1
Simultaneous dual emission activated	
Excitation filter	422 nm
Emission A filter	530 nm
Emission B filter	485 nm

The FRET pair (100 nM CFP- $G\alpha_{i1}$ and 100 nM YFP-RGS12GoLoco) in either buffer (GDP or AMF buffer) was aliquotted in duplicate 60 μ L volumes with increasing concentrations of non-labeled competitor. The plate was read immediately and the FRET ratio (530/485) determined using the MARS Data Analysis Software.

GDP Buffer: 10 mM Hepes pH 7.4, 150 mM NaCl, 0.0005 % NP40 alternative, 100 μ M GDP, 50 μ M EDTA

AMF Buffer: 10 mM Hepes pH 7.4, 150 mM NaCl, 0.0005 % NP40 alternative, 100 μ M GDP, 50 μ M EDTA, 10 mM $MgCl_2$, 10 mM NaF, 30 μ M $AlCl_3$

Results and Discussion

The G-protein/GoLoco interaction is dependent on the nucleotide bound state of the G-protein. The interaction occurs with the GDP bound state of the G-protein, but not in the GTP hydrolysis-mimicking state (AMF). Figure 3 shows the FRET ratio obtained in both buffers for different concentrations of unlabeled competitor $G\alpha_{i1}$.

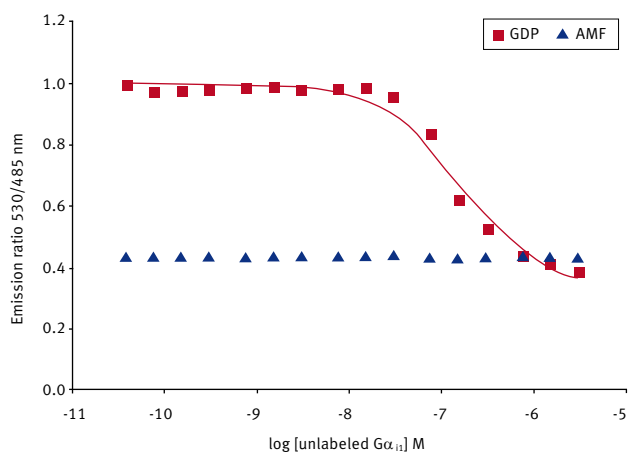


Fig. 3: 530/485 nm emission ratio of CFP- $G\alpha_{i1}$ and YFP-RGS12GoLoco measurements using different buffers (GDP-buffer and AMF buffer) and varied concentrations of unlabeled competitor G-protein.

A Z' value of 0.853 was obtained when the reaction volume was at 60 μ L. The assay system also demonstrated a robust tolerance to DMSO (up to 5%), a commonly used solvent for small molecule screening (Figure 4).

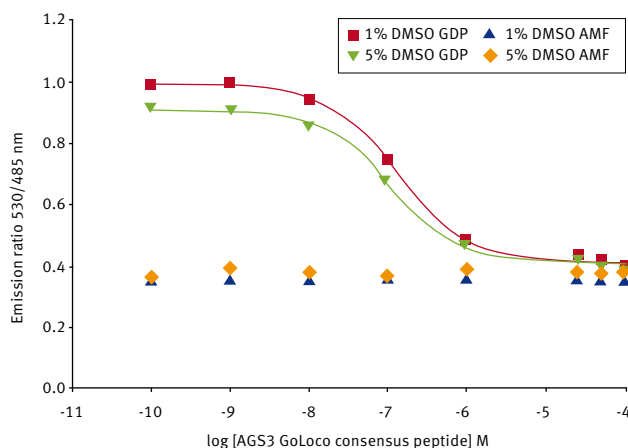


Fig. 4: DMSO tolerance of CFP- $G\alpha_{i1}$ and YFP-RGS12GoLoco measurements in GDP and AMF buffer with competitive inhibition of the interaction using varied concentrations of unlabeled GoLoco motif peptide.

The CFP/YFP FRET based assay is a robust assay and was used in a medium-throughput small molecule screen of 57 compounds (Figure 5).

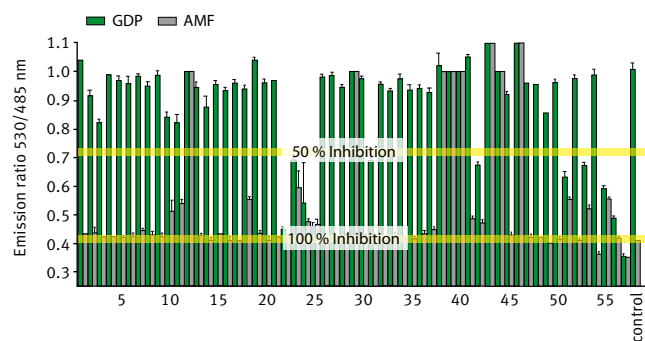


Fig. 5: GoLoco Screen of 57 compounds in GDP and AMF buffer.

Conclusion

The results show that the CFP-YFP FRET based assay is a robust assay that tolerates DMSO in concentrations often used to dissolve small molecules. A very good Z' value of 0.853 in a low volume of only 60 μ L shows the potential to perform the assay in 384-well format.

The POLARstar Omega proved to be a reliable and robust instrument to perform these FRET based measurements. With the MARS Data Analysis Software, the FRET ratio and Z' value are automatically calculated providing fast and efficient evaluation.

References

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