

Development of a Non-Radioactive Homogeneous HTS Platform to Measure the Activity of Guanylate Cyclase.

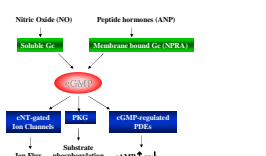


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1 Introduction

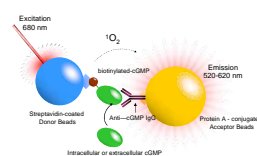
Using AlphaScreen, we have developed a very sensitive assay platform capable of detecting fmol levels of non-acetylated cGMP. A biotinylated derivative of cGMP is used as a tracer in competitive immunoassay format involving rabbit anti cGMP antibodies. The AlphaScreen signal is generated when Streptavidin coated Donor beads and protein A coated Acceptor beads are brought into proximity by the formation of the biotin-cGMP / anti-cGMP IgG complex. Production of cGMP by either particulate or soluble forms of guanylate cyclase leads to a decrease of the AlphaScreen signal by inhibiting the formation of the biotin-cGMP / anti-cGMP IgG complex. Using this assay, we have characterized the activity of the atrial natriuretic peptide receptor (NPR-A, particulate guanylate cyclase) overexpressed in CHO cells. We have also tested the activity of a soluble guanylate cyclase present in LLC-PK1 cells. Pharmacological parameters and Z' values obtained indicate that the assay platform is amenable to HTS.

2 Guanylate Cyclases as Drug Targets



cGMP is a second messenger produced from GTP by enzymes called guanylate cyclases (GC). GC's are either soluble (cytosolic) or associated to cell membrane (particulate). GC's activated by various modulators including NO and hormones such as ANP, produce cGMP which in turn affect different targets including ion channels, Protein Kinase G (PKG) and cGMP phosphodiesterase (PDE).

3 Assay Format

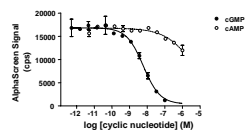


A biotinylated derivative of cGMP is used as a tracer in competitive immunoassay format involving rabbit anti cGMP antibodies (Calbiochem). The AlphaScreen signal is generated when Streptavidin coated Donor beads and protein A coated Acceptor beads are brought into proximity by the formation of the biotin-cGMP / anti-cGMP IgG complex. Presence of cGMP leads to a decrease of the AlphaScreen signal by inhibiting the formation of the biotin-cGMP / anti-cGMP IgG complex.

4 General Protocols

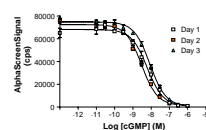
- | | |
|---|--|
| <p>Extracellular cGMP</p> <ul style="list-style-type: none"> • 5 ul cell suspension (3-10,000 cells) with Protein A Acceptor beads (20 ug/ml) and anti-cGMP IgGs (1/3000) • 5 ul antagonist (or assay buffer) (incubate 30 minutes at RT) • 5 ul agonist (incubate 30 minutes at RT) • 10 ul Streptavidin Donor beads (20 ug/ml) containing 0.3 nM biotin-cGMP in assay buffer • incubate 1 hour at RT and read on AlphaQuest | <p>Total cGMP</p> <ul style="list-style-type: none"> • 5 ul cell suspension (3-10,000 cells) with Protein A Acceptor beads (20 ug/ml) and anti-cGMP IgGs (1/3000) • 5 ul antagonist (or assay buffer) (incubate 30 minutes at RT) • 5 ul agonist (incubate 30 minutes at RT) • 10 ul Streptavidin Donor beads (20 ug/ml) containing 0.3 nM biotin-cGMP in lysis buffer* • incubate 1 hour at RT and read on AlphaQuest |
|---|--|
- All reagents are diluted in an assay buffer composed of PBS supplemented with 5 mM HEPES pH 7.4 and 0.5mM BSA.*
* Lysis buffer = assay buffer containing 0.3% Tween-20

5 cGMP detection: antibody specificity over cAMP



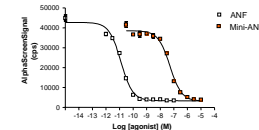
Competition isotherms performed with increasing concentrations of either cGMP and cAMP. The IC_{50} values derived from these experiments were 6 nM for cGMP and > 5 uM for cAMP. This result indicates that the anti-cGMP antibody obtained from Calbiochem has an excellent selectivity for cGMP (1000x) over cAMP, a second messenger present in high concentrations in the cells.

6 cGMP detection: reproducibility and sensitivity



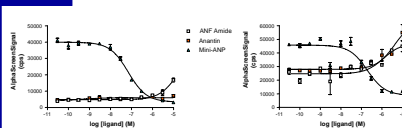
cGMP standard curves performed in absence of cells. Reagents are added as described in panel 4 except that both agonist and antagonist additions are substituted by the addition of 10 ul cGMP dilutions. Average IC_{50} value was (6 ± 3) nM and the limit of sensitivity estimated to be 25 fmol/well (1 nM).

7 Stimulation of cGMP production



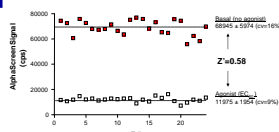
Agonist dose response curves performed in the presence of 10,000 cells. Reagents were added as described in panel 4 except that antagonist addition was substituted by the addition of 5 ul assay buffer. Cells were stimulated for 30 minutes at RT. EC_{50} values for ANF and mini-ANP were 30 pM and 100 nM respectively. The protocol used was for measuring total cGMP.

8 Inhibition of cGMP Production



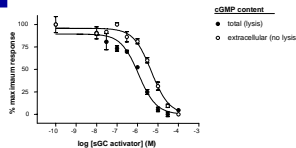
Antagonist dose response curves performed with 10,000 CHO cells in the presence of mini-ANP used at either its EC_{50} (left panel) or EC_{10} concentration. Cells were exposed for 30 minutes with the antagonists and then stimulated for 30 minutes with ANF at RT. Both antagonists were poorly active when ANF was used at its EC_{10} . However, the same compounds inhibited the ANF-induced cGMP production by CHO cells expressing NPR-A with an apparent IC_{50} value of 3 uM in the presence of a lower concentration (EC_{10}) of ANF.

9 Assay Reproducibility



Preliminary stage of assay validation for HTS: determination of intra-plate variability. 384 well microplates were filled with 10,000 cells and AlphaScreen reagents as described in panel 4. Cells were incubated in the presence (EC_{10} concentration) or the absence (basal) of mini-ANP for 30 minutes at RT. Z' value obtained was above 0.5 indicating the suitability of the platform for subsequent HTS validation phases. The example shown is representative of 3 independent experiments.

10 Detection of cGMP total vs intracellular contents



Comparison of total and extracellular cGMP production by endogenous soluble guanylate cyclase (sGC) present in LLC-PK1. Proprietary sGC activator used in this experiment lead to the production of cGMP with EC_{50} values of 1 and 5 uM when total or extracellular cGMP concentrations were analyzed respectively. Lower cGMP concentrations and kinetics of cGMP efflux may account for the higher EC_{50} value measured using the "no lysis" protocol.

11 Conclusion & Perspectives

- Using AlphaScreen, a reproducible and sensitive assay was developed to measure the activity of both particulate and soluble forms of guanylate cyclases.
- Anti-cGMP IgG's used for this assay are highly selective for cGMP.
- NPR-A receptors overexpressed in CHO cells were used to demonstrate the performance of the assay with the particulate form of guanylate cyclase.
- Endogenous source of soluble guanylate cyclase present in LLC-PK1 cells was also used to assess the potential of the platform.
- Limit of assay sensitivity was established at 25 fmol non-acetylated cGMP/well.
- Assay can detect either extracellular or total cGMP.
- Preliminary intra-plate variability studies indicate that the assay is well suited for HTS.
- Performance of the platform is currently assessed using various sources of cGMP: specific or not to cGMP.