Targeting the cGAS-STING Pathway Using a Homogenous, HTS Compatible Transcreener® cGAS Assay

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Overview

Cyclic GMP-AMP synthase (cGAS) is a recently discovered enzyme that acts as a foreign DNA sensor to elicit an immune response to pathogens via activation of the STING (stimulator of interferon genes) receptor. Shortly after its discovery in 2013, aberrant activation of cGAS by self-DNA was shown to underlie debilitating and sometimes fatal autoimmune diseases, such as systemic lupus erythematosus (SLE) and Aicardi–Goutieres Syndrome (AGS). Knockout studies in animal models have clearly indicated that inhibiting cGAS is a promising approach for therapeutic intervention. However, there are no HTS-compatible assay methods for measuring cGAS enzyme activity or for monitoring cGAMP in cell and tissue samples. To enable HTS efforts targeting cGAS, we developed Transcreener-based assays for cGAMP detection; i.e., homogenous, competitive immunoassays with fluorescence polarization (FP) and time resolved Förster resonance energy transfer (TR-FRET) signals. The key assay reagents are antibodies that selectively bind cGAMP in the presence of excess ATP and GTP and tracers that are displaced by cGAMP. We validated both assays with full length human cGAS and performed pilot screens for cGAS inhibitors with the FP assay. Similar to other Transcreener assays, the cGAMP assays have the performance characteristics needed for small molecule screening including sensitivity, robustness (good dynamic range, minimal signal variation), and low levels of compound interference. The availability of robust, HTS compatible assays for measuring cGAS enzyme activity will accelerate efforts to target the cGAS-STING pathway for autoimmune diseases.

The cGAS-cGAMP-STING Pathway Activates the Immune System in Response to Cytosolic DNA

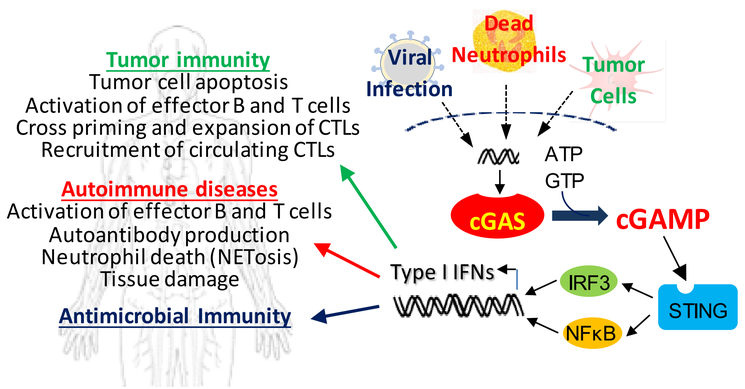


Figure 1. IFN-driven immune responses triggered by cGAS/STING are critical for protection against microbial pathogens and for tumor cell-specific T cell responses in cancer, but activation by self-nucleic acids can contribute to autoimmune diseases.

Mix and Read HTS Enzymatic Assays for cGAS Based on Immunodetection of cGAMP

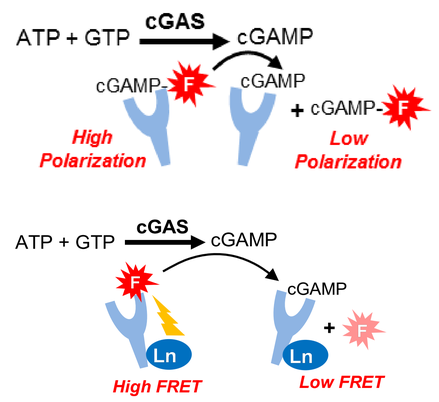


Figure 2. Transcreener cGAS Assay principle: Enzymatically generated cGAMP displaces a fluorescent tracer from mAb causing a decrease in its polarization or TR-FRET.

Assay Validation

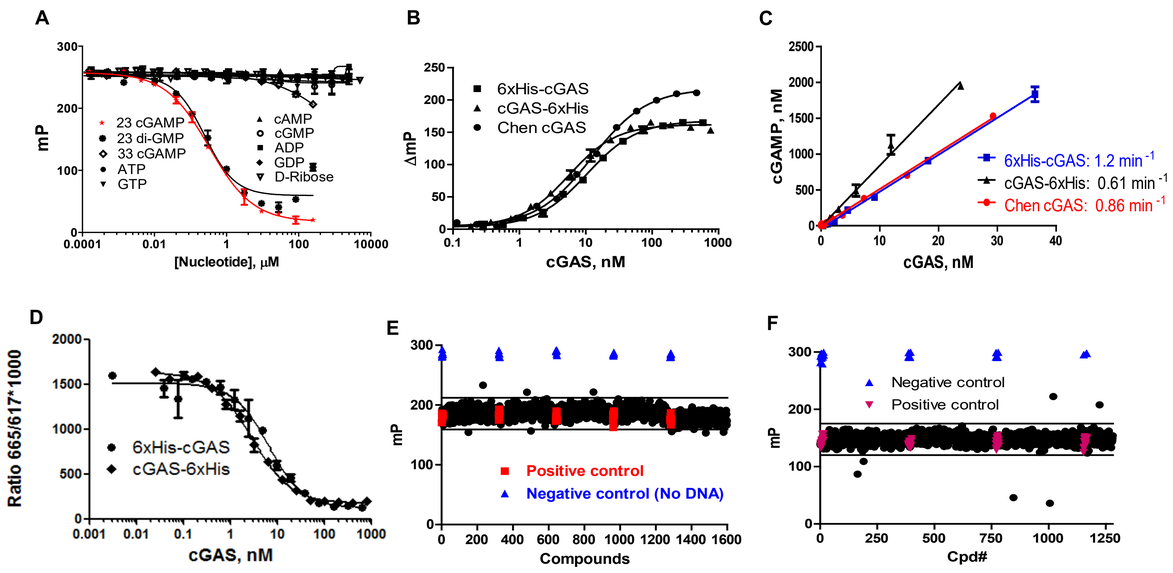
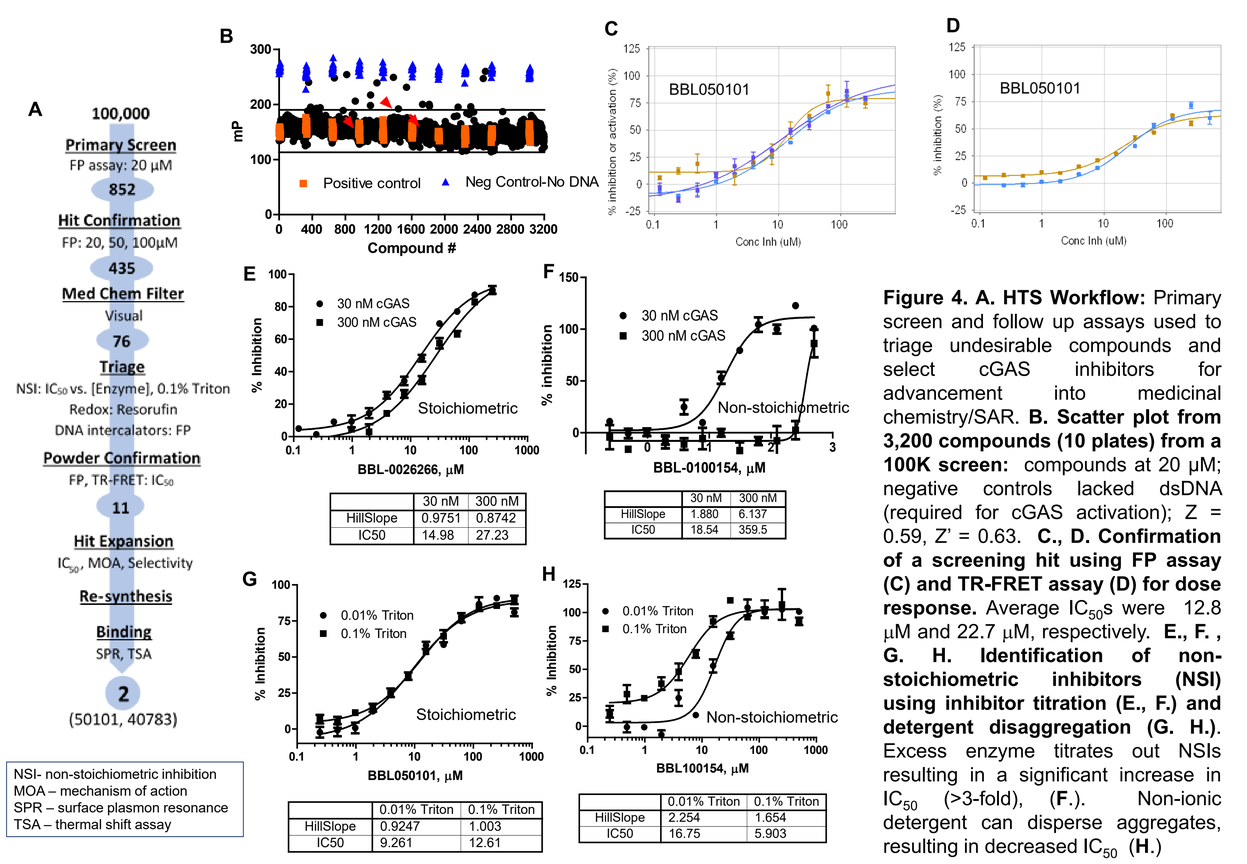


Figure 3. A. Specificity of mAb: Measuring cGAS initial activity requires detection of cGAMP in an excess of ATP and GTP. Competition binding curves show outstanding selectivity for cGAMP vs. substrates, ATP and GTP, as well as related molecules. **B. Detection of purified, full-length human cGAS with FP Assay.** cGAS enzyme reactions contained 100 µM ATP and GTP, 62.5 nM 45 bp ISD DNA, 60 min reactions. N- and C-terminal His-tagged cGAS was produced at BBL; 6xHis-cGAS was also generously supplied by Z. Chen (UTSW Medical Center). **C. Linear response**: Polarization data from A. was converted to cGAMP using a standard curve. **D. Detection of purified, full-length human cGAS with TR-FRET Assay.** TR-FRET was used as an orthogonal assay method for hit confirmation. As with the FP assay, conversion of FRET signal to cGAMP formation yielded a linear response (data not shown). **E. Interference screen**: The LOPAC library (1280 compounds) was used at 10 µM final concentration. Wells contained all cGAS enzyme reaction components except the cGAS enzyme; positive controls contained 1 µM cGAMP, negative controls contained no cGAMP. **F. Pilot screen with 1600 diversity compounds (Life Chemicals):** cGAS was used at 10 nM, compounds were at 10 µM; 60 min reaction; negative controls lacked dsDNA (required for cGAS activation); Z = 0.62, Z' = 0.7.

Screening and Triaging of Non-Stoichiometric Inhibitors



	30 nM	300 nM
HillSlope	0.9751	0.8742
IC50	14.98	27.23

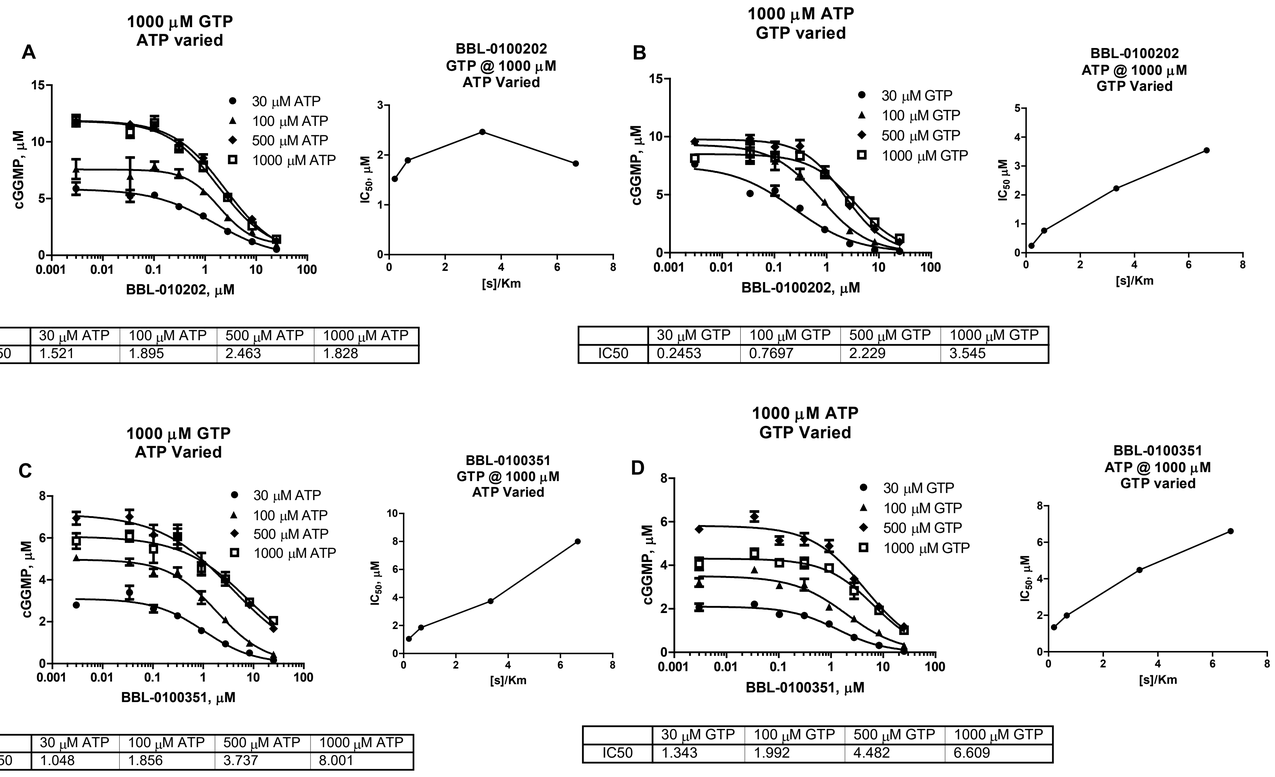
	30 nM	300 nM
HillSlope	1.880	6.137
IC50	18.54	359.5

	0.01% Triton	0.1% Triton
HillSlope	0.9247	1.003
IC50	9.261	12.61

	0.01% Triton	0.1% Triton
HillSlope	2.254	1.654
IC50	16.75	5.903

Figure 4. A. HTS Workflow: Primary screen and follow up assays used to triage undesirable compounds and select cGAS inhibitors for advancement into medicinal chemistry/SAR. **B. Scatter plot from 3,200 compounds (10 plates) from a 100K screen:** compounds at 20 µM; negative controls lacked dsDNA (required for cGAS activation); Z = 0.59, Z' = 0.63. **C., D. Confirmation of a screening hit using FP assay (C) and TR-FRET assay (D) for dose response.** Average IC_{50}s were 12.8 µM and 22.7 µM, respectively. **E., F. , G. H. Identification of non-stoichiometric inhibitors (NSI) using inhibitor titration (E., F.) and detergent disaggregation (G. H.).** Excess enzyme titrates out NSIs resulting in a significant increase in IC_{50} (>3-fold), (**F.**). Non-ionic detergent can disperse aggregates, resulting in decreased IC_{50} (**H.**)

Characterizing Mode of Inhibition



	30 µM ATP	100 µM ATP	500 µM ATP	1000 µM ATP
IC50	1.521	1.895	2.463	1.828

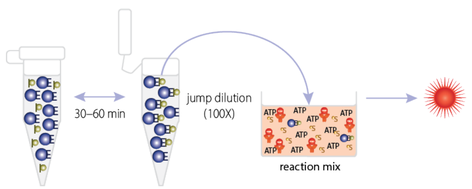
	30 µM GTP	100 µM GTP	500 µM GTP	1000 µM GTP
IC50	0.2453	0.7697	2.229	3.545

	30 µM ATP	100 µM ATP	500 µM ATP	1000 µM ATP
IC50	1.048	1.856	3.737	8.001

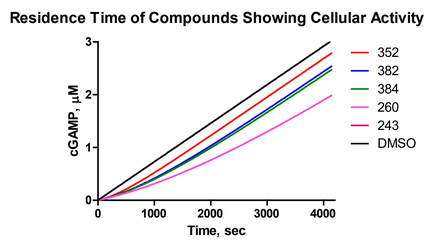
	30 µM GTP	100 µM GTP	500 µM GTP	1000 µM GTP
IC50	1.343	1.992	4.482	6.609

Figure 5. Characterization of Inhibitor MOA During Hit-to-Lead Using Substrate Competition Assays. A., B. Identification of a GTP-competitive inhibitor. C., D. Identification of an inhibitor that competes with both ATP and GTP. The Transcreener cGAS FP assay was used for all assays.

Measuring Inhibitor Residence Time



Jump Dilution Method: Inhibitors are preincubated with enzyme at saturating concentration to allow formation of E-I complexes, then diluted 100-fold into reaction mix. Recovery of enzyme activity correlates with inhibitor dissociation.



	352	382	384	260	243	DMSO
K	0.002126	0.001019	0.0008883	0.0003839	1.672E+13	1.65E+15
T1/2 (min)	7.84	16.36	18.76	43.41	<1	<1

Figure 6. A. Schematic of jump dilution method used to measure inhibitor residence time. B. Monitoring recovery of activity for a series of cell-permeable inhibitors following jump dilution. Activity recovers as the E-I complex dissociates, allowing calculation of off-rates.

Conclusions

- ***Competitive immunoassays for cGAMP with FP and TR-FRET readouts were validated for detection of cGAS enzyme activity and small molecule screening.***
- ***The assays served as a critical tool in a cGAS HTS campaign, including screening, hit confirmation and triaging of non-stochiometric inhibitors.***
- ***The Transcreeener cGAS FP assay was used to sort analogs based on mechanism of inhibition during hit-to-lead, which provided valuable information for driving SAR.***
- ***The ability to run the assay in continuous mode allowed measurement of inhibitor residence time in an automated format, allowing incorporation of binding kinetics into SAR.***

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