Development and Validation of a Novel cGAS Inhibitor Screening Assay

Julianna Blaylock, Yogan Khatri, and Daniel Tew

Cayman Chemical

— Developed in collaboration with BioLog - LIFE SCIENCE INSTITUTE -

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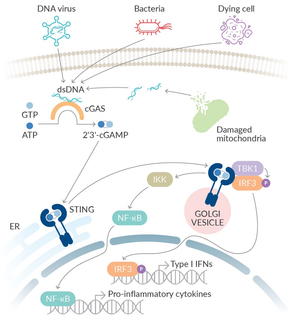
Abstract

Cyclic GMP-AMP synthase (cGAS) is a mammalian dsDNA sensor found in cytosol and involved in an innate immune response.[1] Dysregulation of this cGAS pathway can lead to chronic inflammation, autoimmune disorders, and even tumorigenesis. Activation of cGAS by pathogen-associated dsDNA and the production of 2′3′-cyclic GMP-AMP (2′3′-cGAMP) are important in host defense but may also play a role in the development of autoimmune diseases, such as systemic lupus erythematosus (SLE), which are characterized by increased expression of IFN-stimulated genes. Additionally, cGAS is activated in response to mitochondrial DNA leakage, which is associated with metastatic phenotypes and age-associated inflammation in cancer, and the accumulation of extrachromosomal telomere repeat DNA that results in IFN expression and inhibition of cell proliferation.[1,2] Late stage tumors with a high level of chromosomal instability exhibit decreased protein expression of cGAS as a mechanism to evade cGAS-mediated IFN-signaling and inhibition of tumor growth.[3] In contrast, carcinogen-induced cGAS activation and transfer of tumor cell cGAS to astrocytes through gap junctions promotes tumorigenesis and brain metastasis, respectively, in mouse models. Inhibition of cGAS activity suppresses IFN-stimulated gene expression and decreases type I IFN production in patient-derived samples and mouse models of autoimmune diseases, indicating therapeutic utility of cGAS inhibition. Proper regulation of the cGAS-STING pathway and finding modulators of cGAS is critical in maintaining immune homeostasis.

To facilitate the study of cGAS modulators, we have developed a novel cGAS Inhibitor Screening Assay that directly measures the product of the cGAS reaction, 2′3′-cGAMP, formed in the presence of DNA, ATP and GTP. The cyclic dinucleotide product is quantified via enzyme-linked immunoassay (ELISA) with a 2′3′-cGAMP specific polyclonal antiserum. This report will focus on the production of an active recombinant human cGAS protein and the optimization and validation of an inhibitor screening assay using the protein.

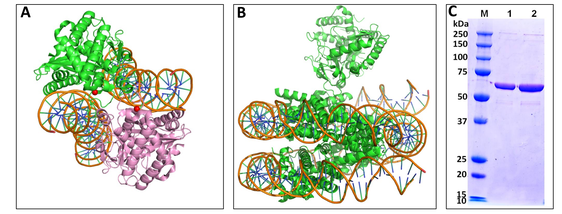
cGAS Pathway



The cGAS-STING pathway is activated by an exogenous (DNA/RNA virus or bacteria/dead cells/tumor cell) or an endogenous (mtDNA released by stressed mitochondria) agonistic DNA. cGAS, consisting of a two-lobed catalytic domain and an extended N-terminal domain, synthesizes the noncanonically linked dinucleotide 2′3′-cGAMP in which DNA stands sandwiched between the two cGAS promoters recognizing their own DNA thread during its active and stable confirmation. The second messenger 2′3′-cGAMP binds to stimulator of interferon genes (STING) dimers residing at the endoplasmic reticulum (ER) and translocates to ER-Golgi intermediate compartments, leading to STING activation.

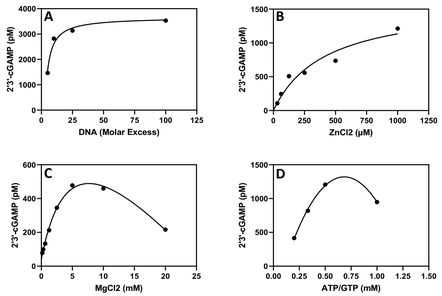
Upon activation, TANK-binding kinase-1 (TBK1) phosphorylates the CTT of STING and recruits interferon regulatory factors (IRFs) for phosphorylation, IRF dimerization, and nuclear translocation. This leads to the activation of downstream signaling and the transcription of target genes, including type I IFNs and ISGs (IFN-stimulated genes), in addition to NF-κB transcription factors.

Structure of cGAS enzyme and recombinant human cGAS



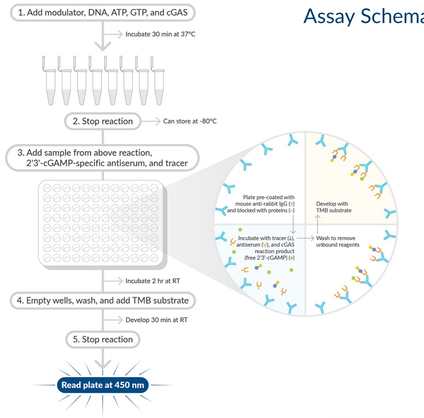
Structure of cGAS enzyme and recombinant human cGAS (Item No. 22810). **(A)** Typical cGAS dimeric crystal structure bound with DNA (orange coils) (PDB 5N6I). The red sphere represents zinc. **(B)** Cryo-EM structures of cGAS-nucleosome complexes (PDB 7CCQ). The structure shows that cGAS interacts with the nucleosome as a monomer, forming 1:1 (or 2:2 PDB 7CCR). The cGAS enzyme accumulates at chromosomes during mitosis or spontaneously in the nucleus, and binding of cGAS to the nucleosome competitively attenuates the dsDNA-mediated cGAS activation. **(C)** Recombinant N-terminal His-tagged cGAS (full length, 2-522) expressed in *E. coli*. Lane M- Marker (All Blue, BioRad), 1 and 2 – 2 and 4 µg of purified cGAS.

Optimization of cGAS Reaction Conditions

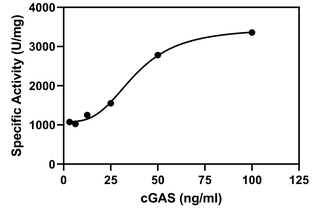


For optimization, cGAS reactions were performed as described in the kit booklet (Item No. 701930). The substrates and cofactors were individually titrated. Briefly, recombinant cGAS was incubated with the cofactors and substrates at the concentrations indicated above for 30 minutes at 37°C before stopping the reaction. All the reaction products were measured using the 2′3′-cGAMP ELISA. The DNA titration **(A)** peaked at a concentration of 25 times molar excess compared to the cGAS enzyme. The increasing zinc chloride titration **(B)** corroborated with increasing 2′3′-cGAMP output, confirming that cGAS is a zinc metalloenzyme. The titration of magnesium chloride **(C)** and substrates, ATP and GTP **(D)**, show inhibition of 2′3′-cGAMP production at higher concentrations. Together these experiments led to the optimized cGAS reaction conditions.

Assay Schematic

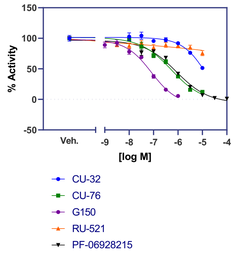


Concentration Dependent Specific Activity of cGAS



cGAS specific activity is dependent on cGAS concentration. cGAS reactions were performed with Cayman's cGAS Inhibitor Screening Assay kit (Item No. 701930) at the indicated concentrations of the enzyme. Specific activity was calculated using the definition that one unit of cGAS produces 1 pmol of 2′3′-cGAMP per minute at 37°C.

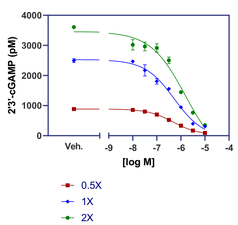
Validation of cGAS Inhibitors



Inhibitor	IC_{50} Value [µM]
CU-32 (Item No. 31029)	18.2
CU-76 (Item No. 31030)	0.530
G150 (Item No. 28318)	0.082
RU-521 (Item No. 31765)	--
PF-06928215 (Item No. 32515)	0.857

cGAS reactions were performed with Cayman's cGAS Inhibitor Screening Assay kit (Item No. 701930) with five inhibitors. Briefly, each inhibitor was tested in the assay at eight different concentrations and a 100% initial activity control. The reactions were incubated at 37°C for 30 minutes before being stopped and quantified by the ELISA. Data was normalized to 100% initial activity and plotted *versus* inhibitor concentration. IC_{50} values for each inhibitor are listed in the table.

Inhibitor Efficacy Depends on Substrate Concentration



Substrate Concentration	IC_{50} Value [µM]
0.5X	0.471
1X	0.529
2X	1.53

Inhibitor IC_{50} value is dependent on substrate concentration. cGAS reactions were performed with CU-76 (Item No. 31030) using Cayman's cGAS Inhibitor Screening Assay Kit (Item No. 701930). Briefly, CU-76 was assayed at seven different concentrations and a 100% initial activity control with indicated substrate concentrations. The reactions were incubated at 37°C for 30 minutes before being stopped and quantified by the ELISA. Data was back-calculated from the 2′3′-cGAMP standard curve and graphed *versus* inhibitor concentration. IC_{50} values for each substrate are listed in the table.

Conclusions

Cayman's novel cGAS Inhibitor Screening Assay Kit (Item No. 701930) provides an easy-to-use tool that can provide results within three hours. Customers need only to supply unknown modulators as all other optimized components have been provided. The sensitivity of the assay kit allows for detection of both potent and weak test compounds.

Acknowledgements

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References

1. Zhou, R., Xie, X., Li, X., *et al.* The triggers of the cGAS-STING pathway and the connection with inflammatory and autoimmune diseases. *Infect. Genet. Evol.* **77**, 104094 (2020).
2. Kwon, J. and Bakhoum, S.F. The cytosolic DNA-sensing cGAS-STING pathway in cancer. *Cancer Discov.* **10(1)**, 26-39 (2020).
3. Yum, S., Li, M., and Chen, Z.J. Old dogs, new trick: Classic cancer therapies activate cGAS. *Cell Res.* **30(8)**, 639-648 (2020).