Targeting ENPP1 for Cancer Immunotherapy: Development of an HTS Method Using the Transcreener® AMP²/GMP² Assay

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Overview

The innate immune response to cancer is initiated by DNA leaking into the cytosol of tumor cells where it binds to the cytoplasmic DNA sensor cyclic GAMP synthase (cGAS) and triggers type I IFN expression via the STING receptor (Stimulator of INterferon-sensitive Genes), leading to activation of tumor-specific T cells. Ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) has been identified as the primary enzyme responsible for degrading cGAMP and therefore is under intense investigation as a therapeutic target for cancer immunotherapy. ENPP1 hydrolyzes cGAMP to produce AMP, GMP and pyrophosphate products. We developed a robust, HTS-compatible enzymatic assay method for ENPP1 using the Transcreener AMP²/GMP² Assay, a competitive fluorescence polarization immunoassay that enables direct detection of AMP and GMP in a homogenous format. The monoclonal antibody used in the Transcreener AMP²/GMP² assay showed more than 10^4-fold selectivity for AMP and GMP versus cGAMP, indicating that detection of cGAS reaction products would not be compromised by cross-reactivity with the substrate, even if present in vast excess. A working concentration of 1 pM ENPP1 was determined as optimal for initial velocity detection with a 60 min reaction period, enabling screening with very low quantities of enzyme (e.g., 0.4 ng/well). ENPP1 showed little discrimination between various cyclic dinucleotide substrates tested, with EC_{50} values of 40, 83 and 168 nM for cdiGMP, cGAMP and cdiAMP, respectively. A Z' value of 0.72 was determined using ATP as substrate, indicating a high-quality assay. In summary, the Transcreener AMP²/GMP² Assay was validated as a robust assay for measuring ENPP1 activity under typical screening conditions. Direct detection of the nucleotide products of ENPP1 is advantageous over methods that use coupling enzymes to convert nucleotide or pyrophosphate products to other detectable analytes, as these methods are prone to interference with the coupling enzymes. The Transcreener AMP²/GMP² should prove to be a valuable tool for discovery of ENPP1 lead molecules.

Function of ENPP1 in the STING Pathway

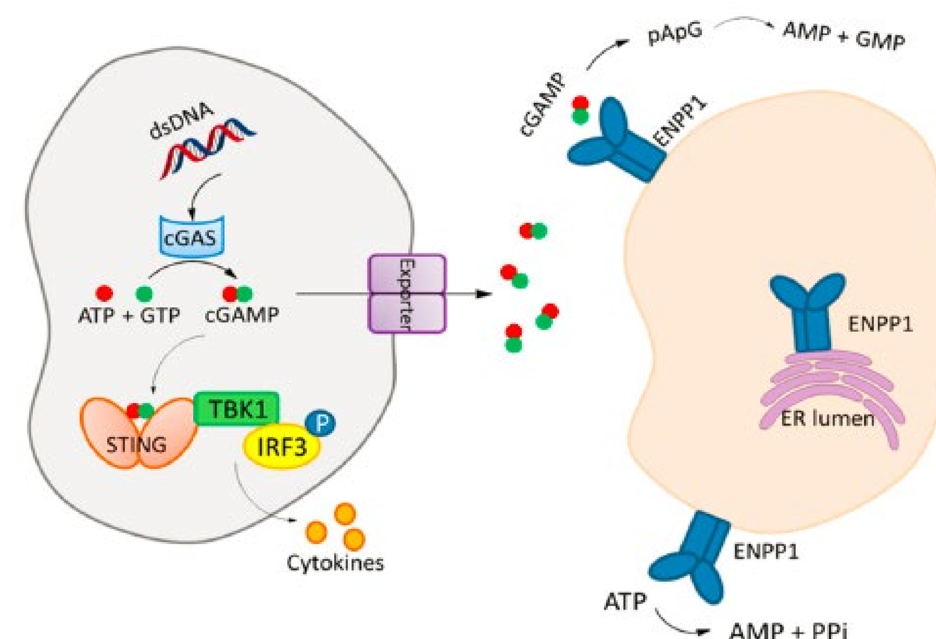


Figure 1. ENPP1 is a nucleotide pyrophosphatase and phosphodiesterase that regulates purinergic signaling and can degrade different nucleotides including ATP and cGAMP (photo credit. Onyedibe et al. Molecules 2019).

A Homogenous Enzymatic Assay for ENPP1 to Enable HTS

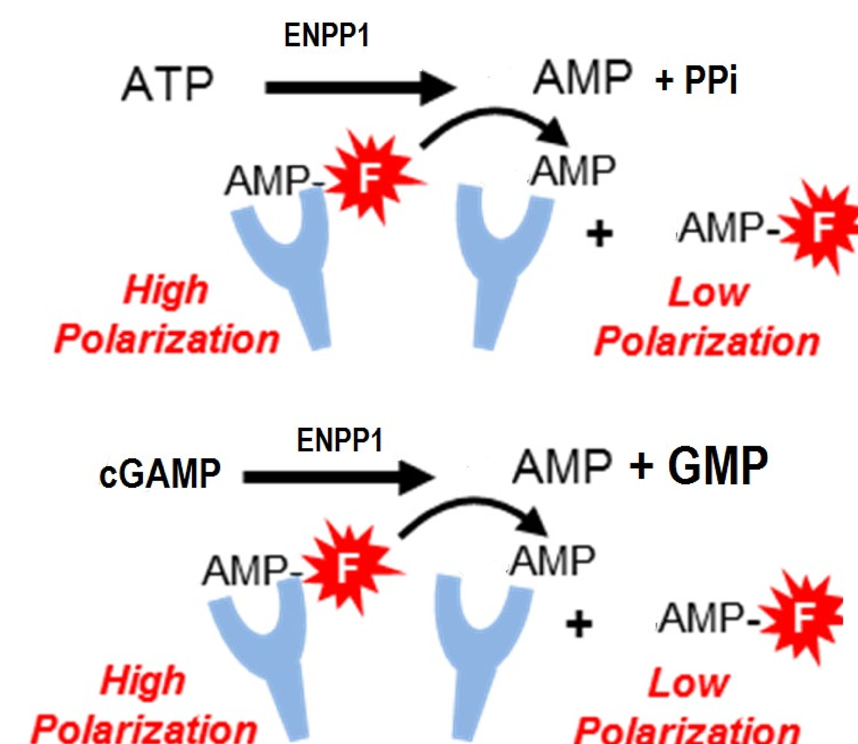
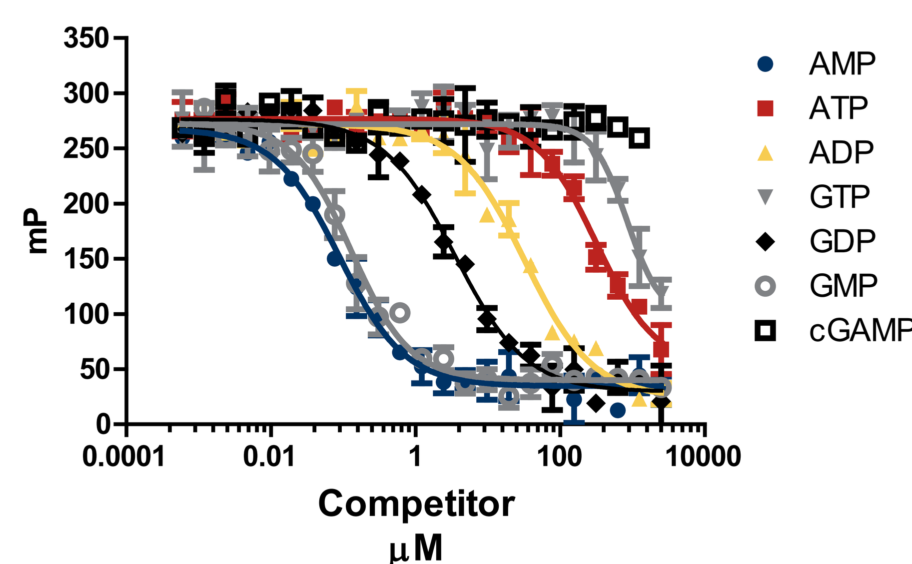


Figure 2. Transcreener ENPP1 Assay Principle: In the competitive fluorescence polarization (FP) immunoassay for ENPP1, enzymatically generated AMP/GMP displaces a fluorescent tracer from mAb causing a decrease in its polarization.

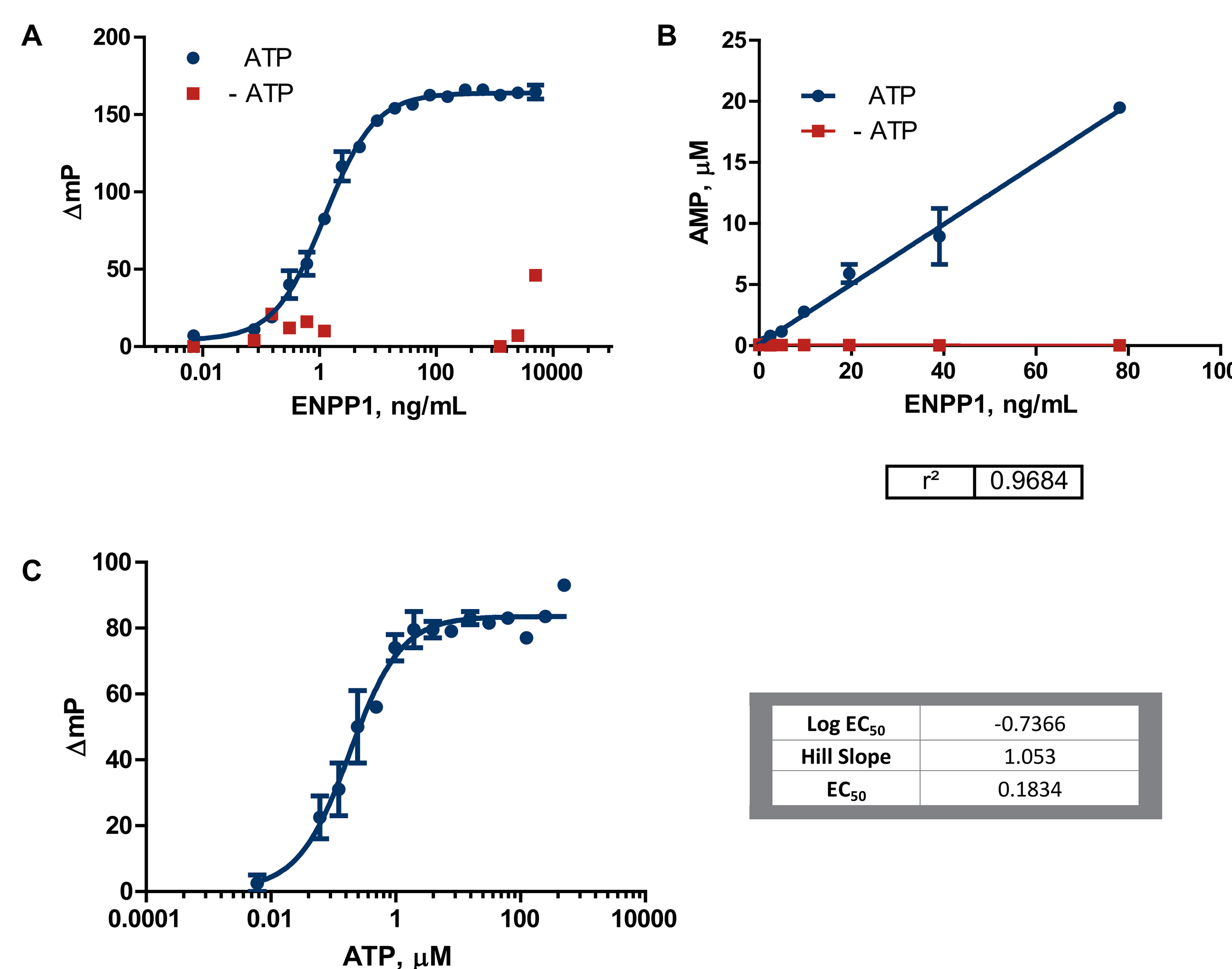
Competition Curves with AMP² Antibody



	Selectivity vs ATP	Selectivity vs cGAMP
AMP	3531	27927
ATP	1	8
ADP	10	77
GTP	<1	3
GDP	90	710
GMP	2424	19172
cGAMP	<1	1

Figure 3. Specificity of mAb: Equilibrium binding curves show outstanding selectivity for AMP vs. ENPP1 substrates, ATP and GTP, as well as related molecules.

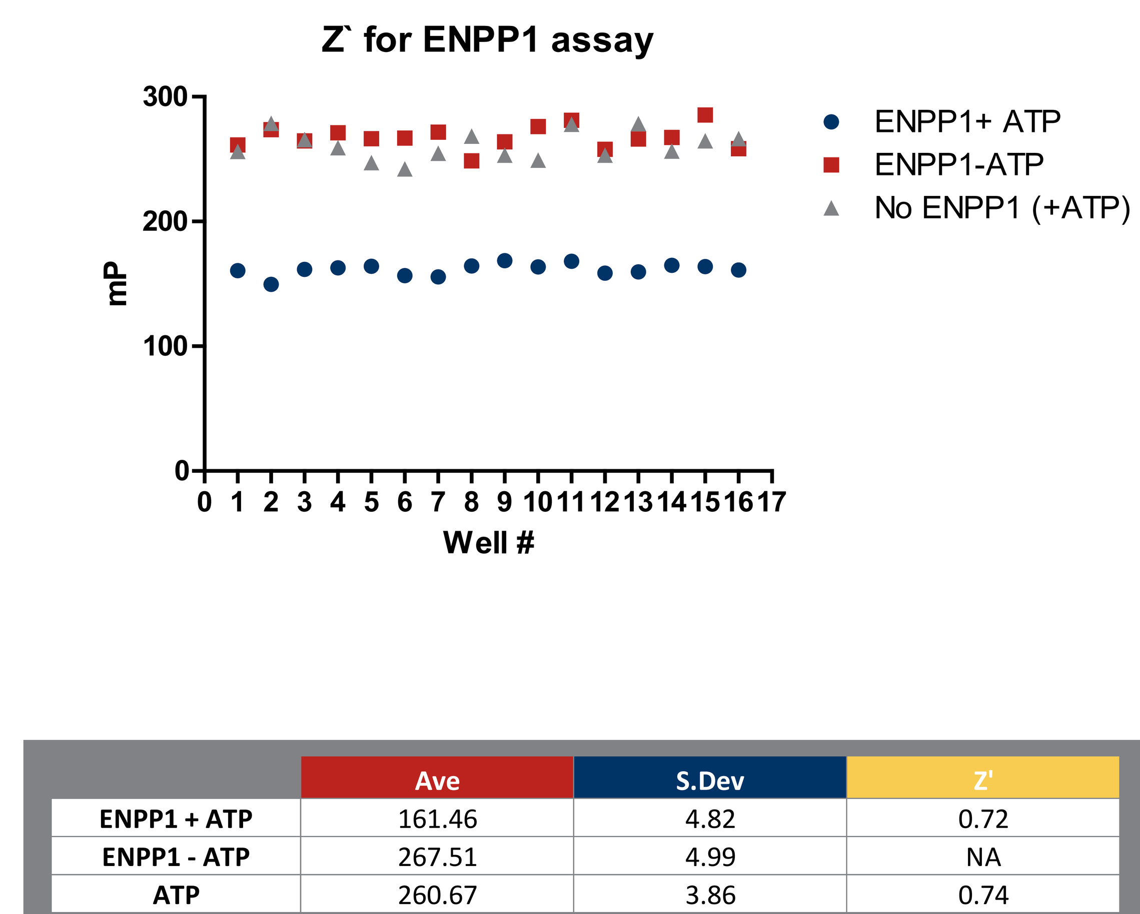
Hydrolysis of ATP by ENPP1



Log EC_{50}	-0.7366
Hill Slope	1.053
EC_{50}	0.1834

Figure 4. A. Hydrolysis of ATP: A. ENPP1 was titrated to determine optimal enzyme concentration. **B.** Raw data was converted to product formation (AMP) to show a linear correlation of the enzyme with the product formed. **C.** ATP was titrated using 20 ng/mL ENPP1 to determine substrate affinity.

HTS Conditions for ATP as Substrate



	Ave	S.Dev	Z'
ENPP1 + ATP	161.46	4.82	0.72
ENPP1 - ATP	267.51	4.99	NA
ATP	260.67	3.86	0.74

Figure 5. HTS Screening Conditions: Z' measurements using optimized ENPP1 reaction conditions indicate a robust assay.

Hydrolysis of cGAMP by ENPP1

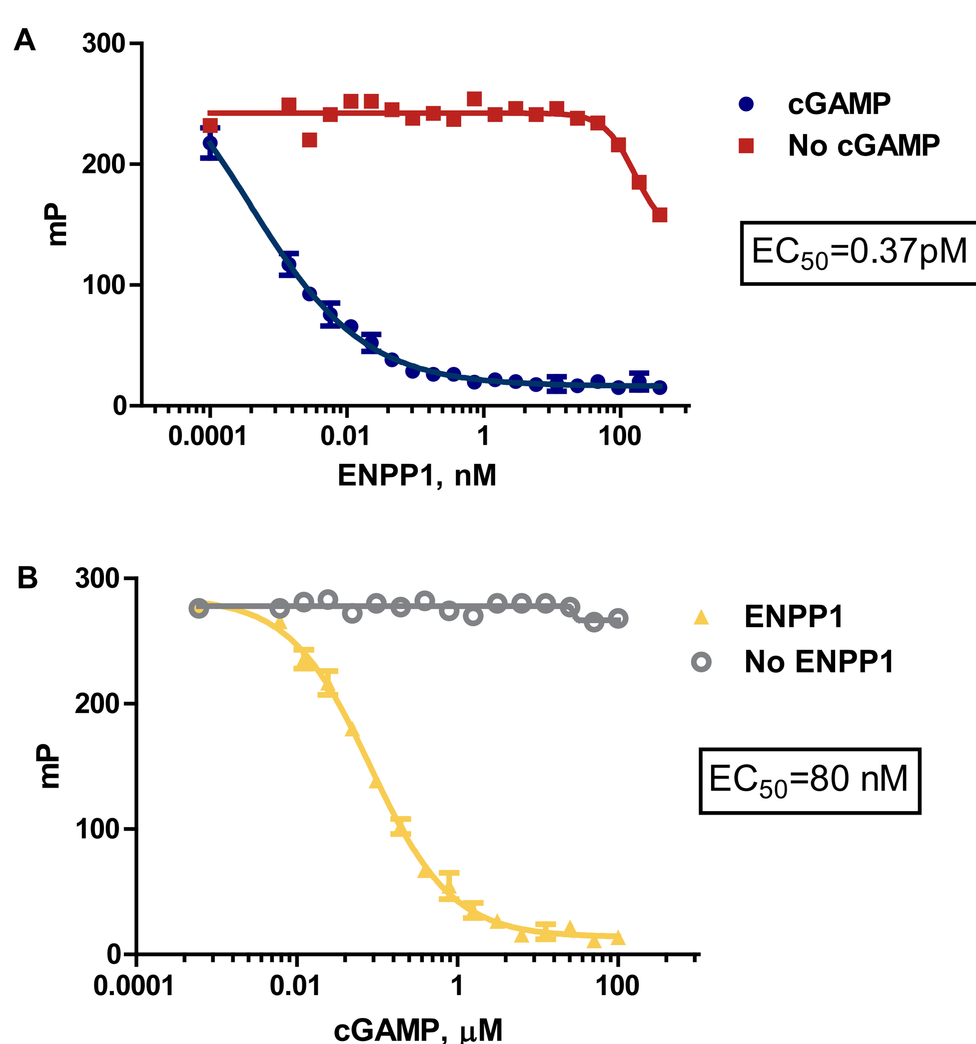
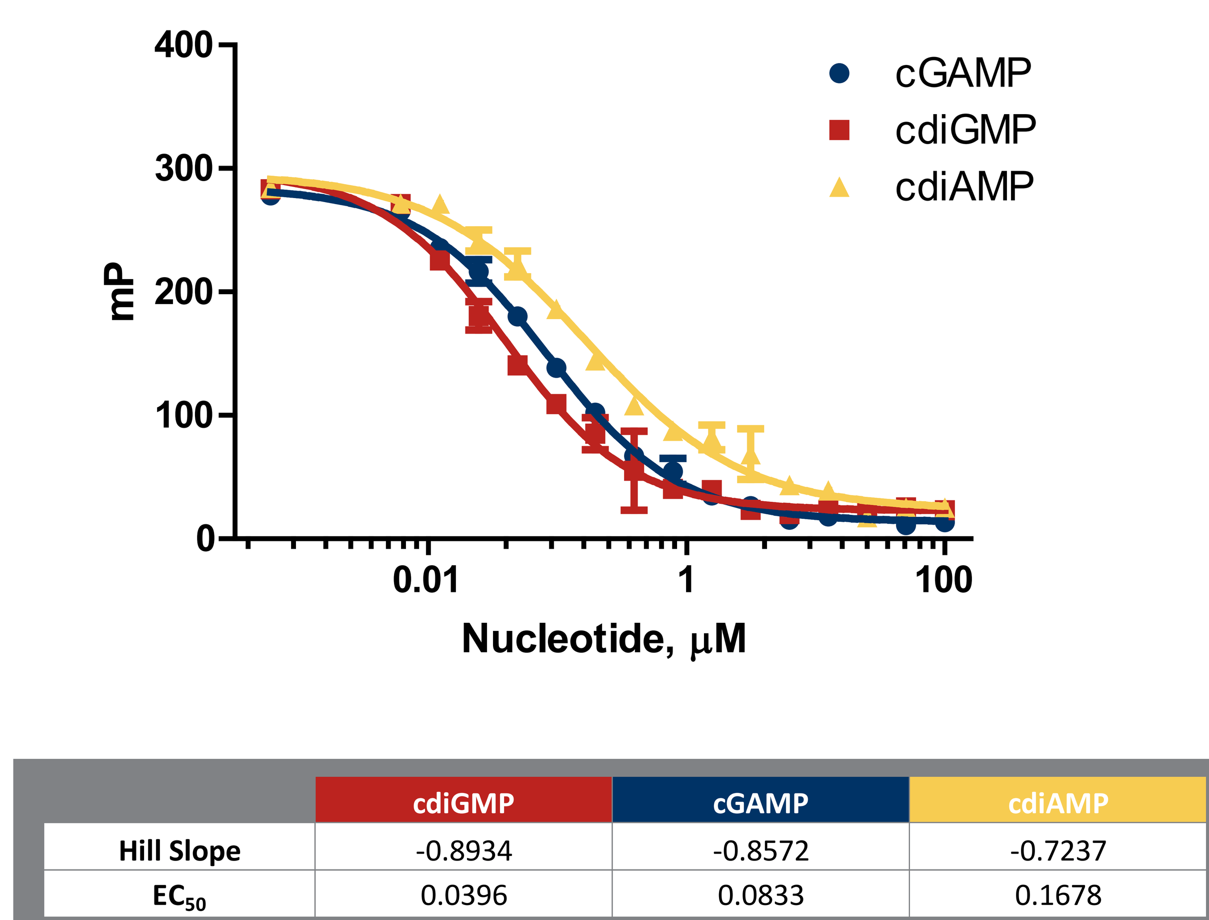


Figure 6. Hydrolysis of cGAMP A. Titration of ENPP1 in the presence of 10 uM cGAMP was done to determine 1 pM ENPP1 to be used for assay with cGAMP. **B. Titration of cGAMP with ENPP1** shows the EC_{50} to be around 80 nM.

Specificity of Substrates in STING Pathway



	cdiGMP	cGAMP	cdiAMP
Hill Slope	-0.8934	-0.8572	-0.7237
EC_{50}	0.0396	0.0833	0.1678

Figure 7. Selectivity of Cyclic Dinucleotides for ENPP1. ENPP1 is agnostic with respect to the type of cyclic dinucleotide required for its activity.

Conclusions

- ***The Transcreener AMP²/GMP² FP Assays are the only HTS assays that enable direct detection of AMP in a homogenous format.***
- ***The exquisite selectivity for AMP vs. ATP or cGAMP allows robust detection of ENPP1 under initial velocity conditions.***
- ***The assays provide a biochemical platform for lead discovery and optimization with ENPP1 .***

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