SUPPORTING INFORMATION

(-)-Epigallocatechin Gallate is a Noncompetitive Inhibitor of NAD Kinase

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General

Experimental details; parameters of high-throughput screening assay and supplementary data for several biophysical methods.

MATERIALS AND METHODS

Protein expression and purification

The NADK full-length gene fragment was cloned from the cDNA library of 293T cells. The gene was cloned into the pET-28a vector by a NovoRec® plus One step PCR Cloning Kit (NR005, novoprotein), and the N-terminus was linked with a TEV protease cleavage site. Single colonies were selected for sequence verification. The recombinant plasmid was introduced into Escherichia coli BL21 (DE3) cells for expression, and the single clone was cultured in LB medium at 37 °C until the OD600 was 0.4-0.6, and then the cells were induced at 16 °C with 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight. After the cells were centrifuged, the supernatant was discarded, washed again with PBS, and stored at -80 °C until further use. The pellet was thawed on ice, lysed by sonication in NiA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol) and centrifuged at 16,000 g for 1 h at 4 °C. A Histrap HP column (GE Healthcare) was used to specifically bind the recombinant protein, and the recombinant protein was then eluted using NiB buffer (50 mM Tris-HCl Ph 8.0, 150 mM NaCl, 500 mM imidazole, 10 mM β-mercaptoethanol) gradient. The purity of the eluted protein was confirmed by SDS-PAGE. Fractions with a purity greater than 90% were pooled and then cleaved by adding TEV protease overnight at 4 °C, and loaded onto a Histrap HP column again, and the effluent was collected. The flow-through is the detagged protein. For further purification using size exclusion chromatography, the protein was stored in gel filtration buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl).

The truncated Δ95-NADK DNA fragment was recombined into the pET-28a vector using a one-step PCR kit, and the purification method was the same as used full length NADK.

In-house compound library

The database of 3000 compounds screened was purchased from MedChemExpress (Cat. No.: HY-L021). The compound library includes 3333 natural products, including sugars and glycosides, phenylpropanoids, quinones, flavonoids, terpenes, steroids, alkaloids, phenols, acids and aldehydes, etc.

High-throughput screening (HTS) assay

By coupling the glucose-6-phosphate dehydrogenase (G6PD) enzyme, the NADP generated by NADK is converted into NADPH, and the absorbance value can be detected at A340nm using the Multiskan FC (Thermo Fisher). The optimized reaction system was 100 μl, conducted in a 96-well plate: 100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM ATP, 0.5-1 μg His-NADK protein, 10 mM 6-phosphate glucose (Sigma), 0.1 U glucose-6-phosphate dehydrogenase (G6378, Sigma), 1 mM NAD+, 0.5% DMSO. To obtain the enzyme kinetic curve, the reaction was measured every 30 s for 20 min at 37 ℃. Different concentrations of NADPH were used to generate a standard curve. The slope of the linear range of the enzyme activity reaction graph is the initial velocity V_0. The inhibition rate of the test compound could be obtained by the following formula：

$$\text{Inhibition (\%)} = 100\% \times (V_{0(\text{control})} - V_{0(\text{compound})}) / V_{0(\text{control})}$$

The concentration of the compound used in the preliminary screening was 50 μM (dissolved in DMSO), and 0.5% DMSO was used as a negative control to exclude the influence of DMSO on enzyme activity. The compound with an initial screening inhibition rate greater than 70% were used to generate. At the same time, G6PD enzyme assay was performed. The same buffer (100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$), 10 mM 6-phosphate glucose, 0.005U G6PD, 10 mM NADP+, 50 μM compound was used, and was set up DMSO negative control, calculate the corresponding inhibition rate.

NMR spectroscopy verification of the EGCG-NADK interaction

CPMG and saturation transfer difference (STD) NMR experiments were conducted to investigate the EGCG-NADK interaction. On a Bruker Avance III 600 MHz NMR spectrometer equipped with a cryoprobe, all NMR spectra were collected at 25 °C (Bruker Bio-Spin, Germany). Phosphate buffer (20 mM NaH_2PO4, 20 mM Na_2HPO4, 100 mM NaCl, pH 7.4, 5% DMSO, and 95% D_2O) was used to dissolve samples containing 200 μM of EGCG and 200 μM of EGCG in the presence of 10 μM NADK.

Surface plasmon resonance (SPR) assay

The SPR assay was applied to investigate the binding affinity of the NADK protein and the compounds on a Biacore T200 instrument (GE Healthcare) at 25 ℃ according to manufacturer's instructions. Recombinant NADK protein was coupled to a CM5 sensor chip (GE Healthcare) and immobilized to attain an optical density of 6000 resonance units at a flow rate of 30 μL/min. The path without the coupled protein served as a reference. The compounds were dissolved in SPR buffer (50 mM Tris-HCl, pH 8.0,150 mM NaCl, 0.5% v/v DMSO) and diluted in different concentrations. The last concentration was a 0 μM compound control. The compound was injected at a flow rate of 30 μL/min to bind with immobilized protein for 60 s and dissociated for 120 s. Finally, the K_d values of the compounds with NADK protein were analysed by Biacore T200 software Version 1.0.

Cell culture

All the cell lines in this study were obtained from ATCC. A549, H23, and H358 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% PS (penicillin/streptomycin) at 37 °C in a 5% CO_2 atmosphere. PC-9 cell was cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin(PS) at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay

Cells were grown in the presence or absence of EGCG for various times. Viable cells were determined using the ATP glow assay. The luminescence was measured by EnVision (Perkin Elmer).

Structural modeling

The crystal structure of NADK (PDB code: 3PFN) was chosen to form a molecular docking model, which contains amino acid

68-421. Waters were removed and a tetramer was used for docking. The protein was prepared with Protein Preparation Wizard Workflow, as provided in Maestro,98 with a pH of 7.0 ± 2.0. Other parameters were default. Ligand preparation was employed to generate various conformations of EGCG. Induced Fit Docking was performed to dock prepared ligands to prepared protein. The residues 411 and 415 were selected as box center of the receptor and the box size was set to auto. Other parameters were default. PyMOL was used to generate vacuum electrostatic images of NADK and modeled EGCG.

HDX-MS analysis

Δ95-NADK (8 μM) was incubated with and without 80 μM of EGCG for 30 min before the HDX reaction at 4 ℃. Four microlitres of Δ95/Δ95-EGCG complex was diluted into 16 μl of D_2O in exchange buffer (50 mM HEPES, 50 mM NaCl, 2 mM DTT, pH 7.5) and incubated for several time points (0 s,10 s,60 s,300 s,900 s) at 4 ℃ later quenched by mixing with 20 μl of ice-cold 3 M HCl 1% trifluoroacetic acid. Each quenched sample was then immediately injected into the LEAP Pal 3.0 HDX platform. The digested peptides were captured on a C18 PepMap300 trap column (Thermo Fisher Scientific) and desalted after passing through an immobilized pepsin column (2 mm×2 cm) at 120 μl/min after injection. The peptides were then separated over 6 minutes on a 2.1 mm×5 cm C18 separating column (1.9 μm Hypersil Gold, Thermo Fisher Scientific) with a linear gradient of 4% - 40% CH_3CN and 0.3% formic acid. A Fusion Orbitrap mass spectrometer (Thermo Fisher Scientific) with a determined resolving power of 65,000 at m/z 400 was used to obtain mass spectrometric data. Single preparations of each protein-ligand complex were used in HDX analyses, which were performed in triplicate. Each peptide envelope's intensity weighted mean m/z centroid value was calculated and then transformed into a percentage of deuterium incorporation. An unpaired t test for each time point determined the statistical significance for the differential HDX data, a method that is built into the HDX Workbench program. Back exchange corrections were performed based on a 70% deuterium recovery estimate and the deuterium exchange buffer's known deuterium level of 80%.

Data rendering

As previously described[1], using a residue averaging method, the HDX data from all overlapping peptides were condensed to individual amino acid values. The deuterium incorporation values and peptide lengths from all overlapping peptides were constructed for each residue. Shorter peptides were weighed more heavily, whereas longer peptides were weighed less heavily, using a weighting formula. The weighted deuterium incorporation readings for each amino acid were then averaged to obtain a single value. Proline residues and the first two residues of each peptide were not included in the computations.

data analysis

The data analysis and graphing software was GraphPad Prism 8.0. Significance analysis using two-tailed unpaired Student's t-test, *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

safety statement

There is no unexpected or unusually high safety hazards were encountered in all experiments involved in this work.

SUPPLEMENT FIGURES

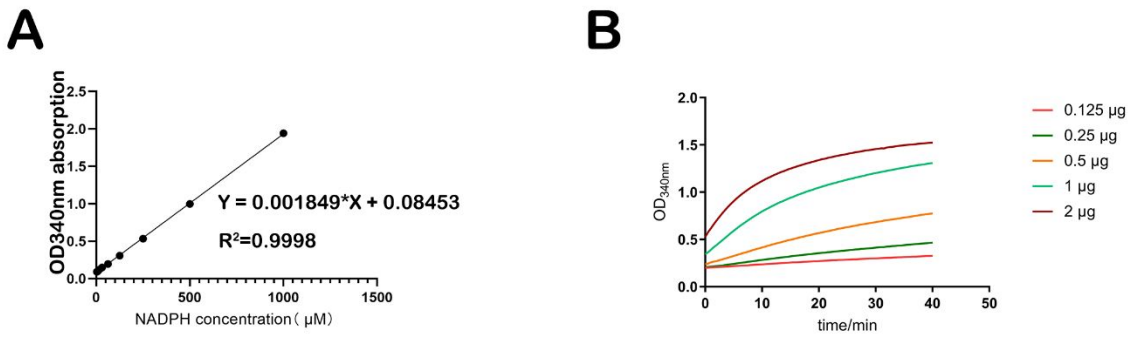

Figure S1. Development and optimization of the high-throughput screening assay of NADK inhibitors. A, Standard curve of NADPH. The absorption at 340nm is positively correlated with the concentration of NADPH, so the rate of change of OD340 nm represents the rate of change of NADPH, that is, the catalytic activity of NADK.B, Enzyme kinetic curves at different protein concentrations.

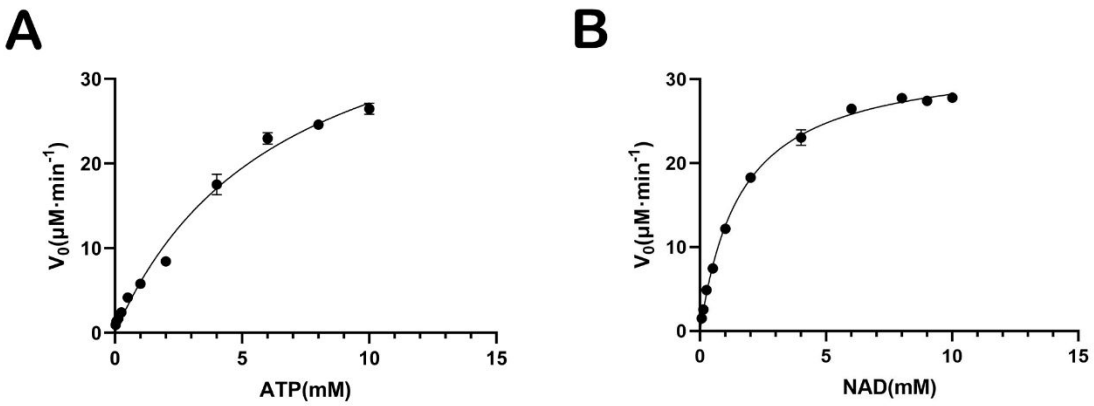

Figure S2. K_m of the substrate of NADK. A, The K_m of ATP was determined using various ATP concentrations at 10 mM NAD. B, The K_m of NAD was determined using various NAD concentrations at 10 mM ATP. The K_m was calculated by Michaelis–Menten nonlinear regression using GraphPad Prism software.

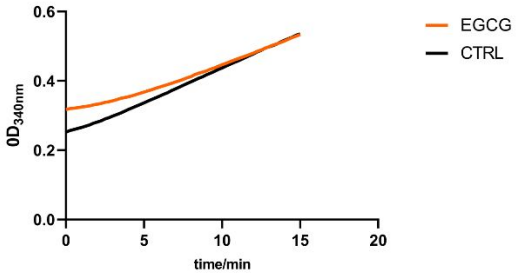

Figure S3. The enzyme activity curve of EGCG against G6PD. The control group used the same concentration of DMSO in the same reaction system. The same buffer (100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$), 10 mM 6-phosphate glucose, 0.005U G6PD, 10 mM NADP+, 50 μM compound was used, and was set up DMSO negative control. EGCG, (-)-epigallocatechin-3-gallate. CTRL, control.

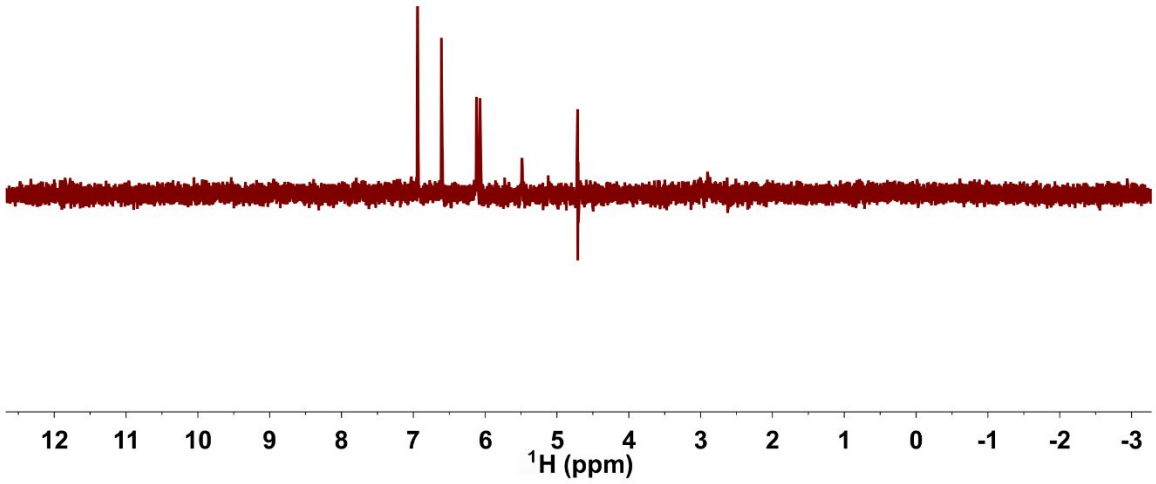

Figure S4. Saturation Transferred Difference (STD) data of 200 μM EGCG incubation with 10 μM NADK. NMR spectra were collected at 25 °C (Bruker Bio-Spin, Germany) on a Bruker Avance III 600 MHz NMR spectrometer equipped with a cryoprobe. 200 μM of EGCG was dissolved in phosphate buffer (20 mM NaH_2PO4, 20 mM Na_2HPO4, 100 mM NaCl, pH 7.4, 5% DMSO, and 95% D_2O) as control group. And 200 μM of EGCG in the presence of 10 μM NADK was dissolved in same buffer.

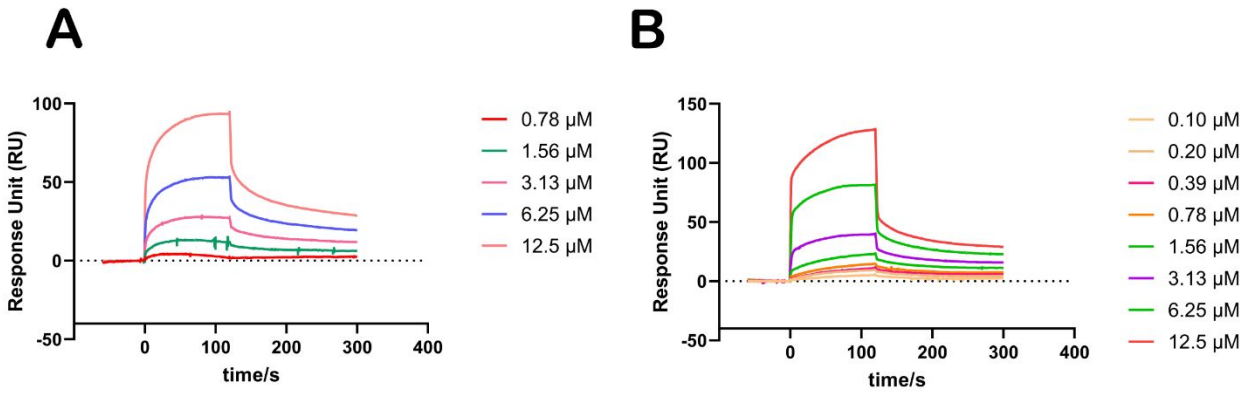

Figure S5. Two independent SPR experiments of EGCG. Two independent experiments were performed on Biacore T200. The results were analysed by Biacore T200 evaluation software. The K_d values were showed as mean ± SD.

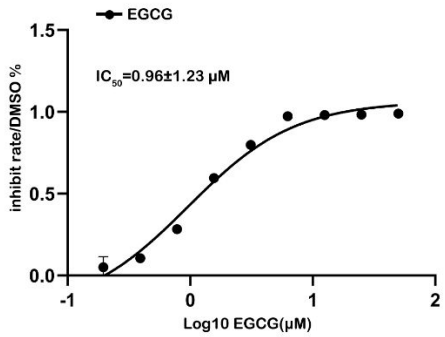

Figure S6. Determination of the IC_{50} of EGCG against truncated NADK-Δ95 using the enzymatic activity assay. 0.5 μg NADK-Δ95 was dissolved in gel filtration buffer and incubated with different concentrations of EGCG for 15 minutes. Then add other substrates to measure the absorbance. Graphpad was used for data analysis.

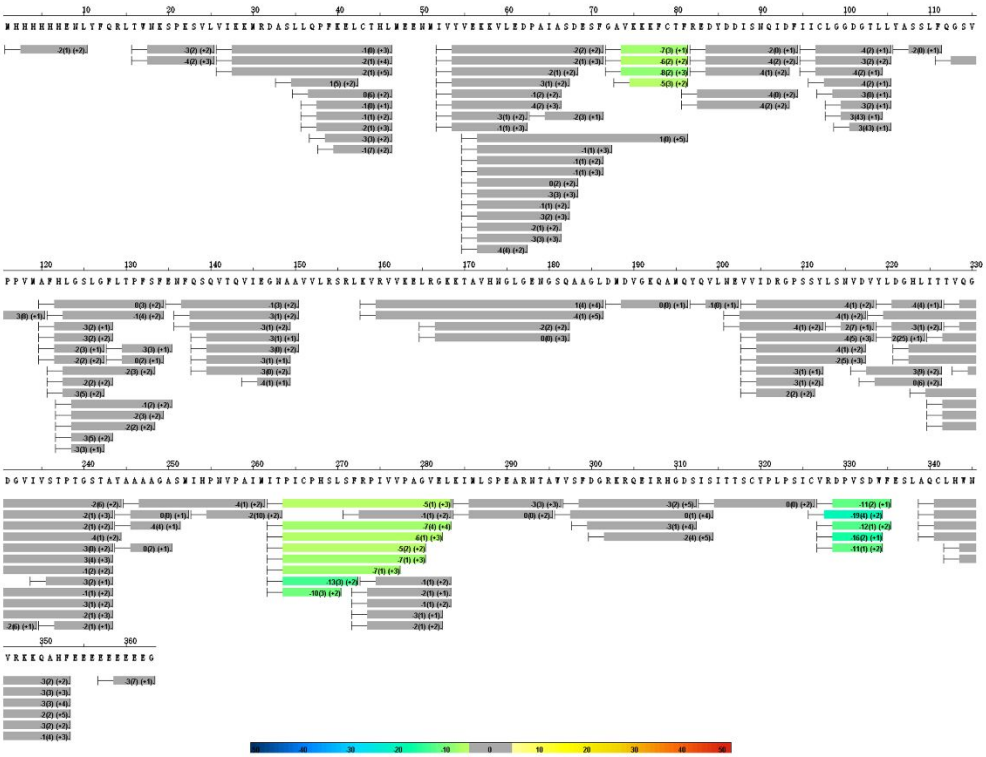

Figure S7. Schematic diagram of HDX rate changes of EGCG binding to Δ95. Numbers represent amino acid positions of Δ95, amino acids are represented by single letters. The values listed under each HDX experiments demonstrate the averaged difference in percentage of deuterium incorporation of that corresponding peptide derived from two different states across all exchange time points (i.e., 0s, 10s, 60s, 300s, 900s). When comparing the two samples, the perturbation %D is determined by calculating the difference between the two samples. HDX Workbench colors each peptide according to the smooth color gradient HDX perturbation key (D%) shown in each indicated figure. Differences in %D between -5% to 5% are considered nonsignificant and are colored gray according to the HDX perturbation key. A negative value represents decreased deuterium incorporation or stabilization while a positive value represents increased deuterium incorporation or destabilization in the corresponding region of the receptor when a binding event takes place. Blank region represents undetected peptide for corresponding experiment.

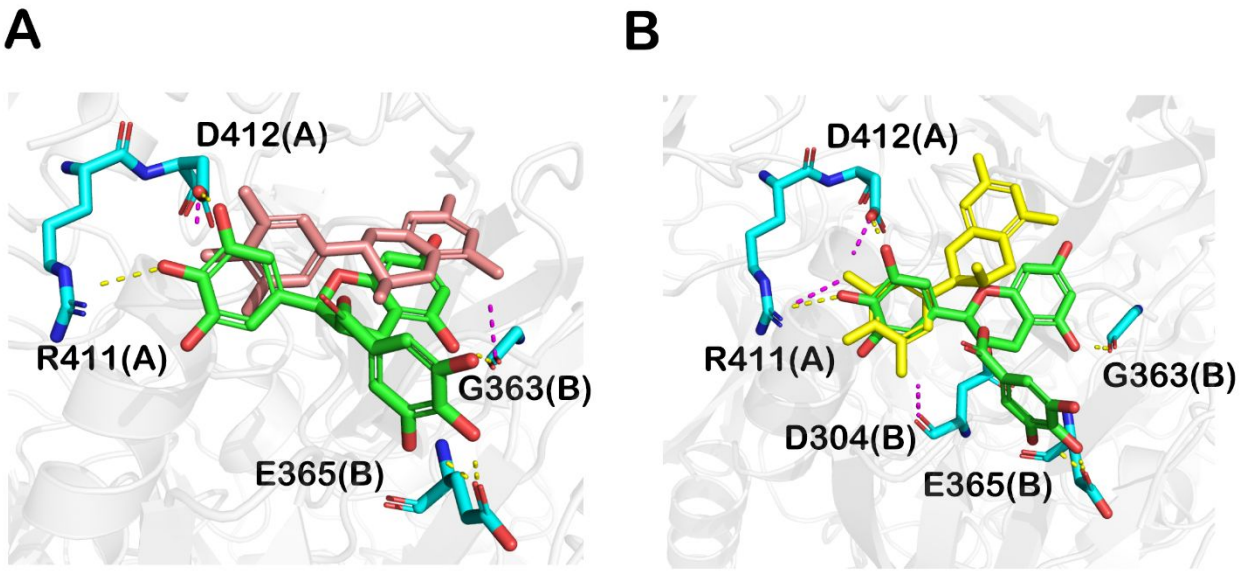

Figure S8. Docking models of (-)-gallocatechin and (-)-epigallocatechin onto the NADK. A, The (-)-gallocatechin (salon) was docked in the pocket to accommodate EGCG. B, The (-)-epigallocatechin (yellow) was docked in the pocket to accommodate EGCG. The yellow dotted lines represent hydrogen bond interactions formed by EGCG with NADK. The magenta dotted lines represent hydrogen bond interactions formed by (-)-gallocatechin and (-)-epigallocatechin with NADK. The critical residues are shown as sticks. Single letters represent amino acids.

REFERENCES

(1) Li, W.; Liao, L.-P.; Song, N.; Liu, Y.-J.; Ding, Y.-L.; Zhang, Y.-Y.; Zhou, X.-R.; Sun, Z.-Y.; Xiao, S.-H.; Wang, H.-B.; Lu, J.; Zhang, N.-X.; Jiang, H.-L.; Chen, K.-X.; Liu, C.-P.; Zheng, J.; Zhao, K.-H.; Luo, C. Natural Product 1,2,3,4,6-Penta-O-Galloyl-β-D-Glucopyranose Is a Reversible Inhibitor of Glyceraldehyde 3-Phosphate Dehydrogenase. *Acta Pharmacol Sin* **2021**.