

Electronic Supplementary Material (ESI) for

**Global Profiling of Cellular Targets of Gambogic Acid by
Quantitative Chemical Proteomics**

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Table of Contents

1. Synthesis of GA-yne	Pages 2-3
Scheme S1. Synthesis of GA-yne .	Page 2
Figure S1. ¹ H-NMR spectrum of GA-yne .	Page 2
Figure S2. ¹³ C-NMR spectrum of GA-yne .	Page 3
Figure S3. HRLC-MS analysis of GA-yne .	Page 3
2. Procedures and Methods for Biological Experiments	Pages 4-8
Figure S4. Full imaging of coomassie blue staining gels in Fig. 2A	Page 5
Figure S5. Labeling profiles of GA-yne and IA-yne in both living cells.	Page 5
3. Bioinformatics analysis of GA targets	Pages 9-12
Figure S6. Venn diagram of GA targets in both cells lines.	Pages 9
Figure S7. Ingenuity Pathway Analysis of 116 GA targets in K562 cells.	Pages 10
Figure S8. GA -engaged network in K562 cells.	Pages 11
Figure S9. Ingenuity Pathway Analysis of 80 GA targets in HeLa cells.	Pages 12
Figure S10. GA -engaged network in HeLa cells.	Pages 13
4. Protein lists of identified targets of GA	Page 13
Table S1. Full lists of protein targets of GA in K562 and HeLa cells	Page 13

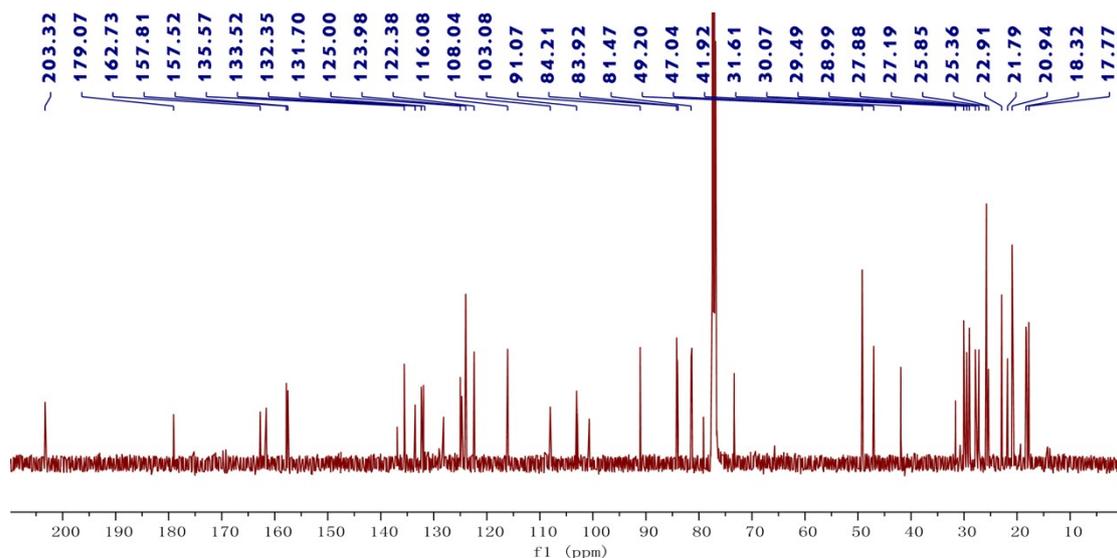


Figure S2. ¹³C-NMR spectrum of GA-yne.

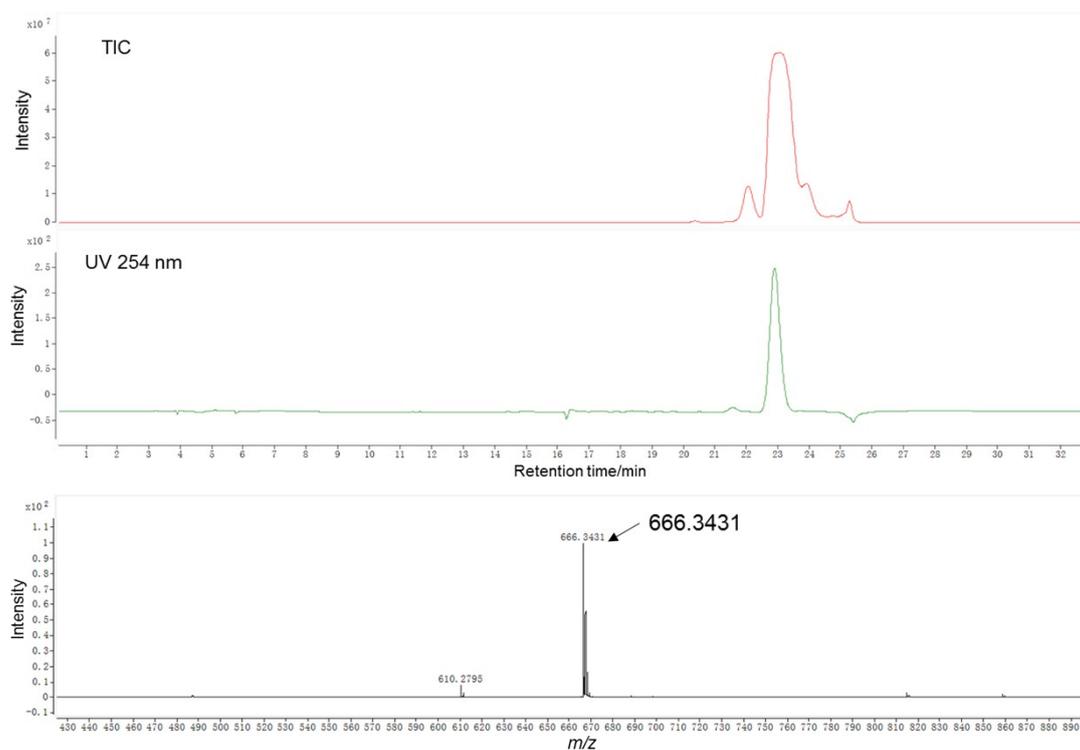


Figure S3. High resolution LC-MS analysis of GA-yne. Upper: total ion chromatography (TIC); Middle: UV (254 nm) detection; Lower: mass spectrometry result.

2. Procedures and Methods for Biological Experiments

2.1 Cell culture

Human cervical carcinoma HeLa and chronic myelogenous leukemia K562 cell lines were ordered from ATCC. HeLa cell line was grown in DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS, Gibco). K562 cell line was grown in RPMI medium 1640 (Roswell Park Memorial Institute 1640, Gibco) containing 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% FBS. The culture incubator set is 37 °C with 5% CO₂.

2.2 Cell proliferation assay

HeLa and K562 cells cultured for three passages were diluted in respective culture medium to 8000 cells/mL. 100 µL of cell suspension were seeded to each well of 96-well plate and incubated at 37 °C overnight. Various concentrations of compounds were dissolved in culture medium containing 0.5% DMSO. Cells in 96-well plate were treated with 100 µL of various concentrations of compounds and DMSO (negative control) for 36 hours in a 37 °C incubator. Cell viability was assessed by CellTiter-Glo® Luminescent Kit (Promega).

2.3 Gel-based ABPP of HeLa and K562 cell lines *in situ* and *in vitro*

HeLa and K562 cells were grown in culture medium until 90% confluence. For *in situ* study, the medium was removed and cells were incubated respectively with DMSO, **IA-yne** (1.0 µM), **GA-yne** (1.0 µM), and **GA-yne** (1.0 µM) with excess **GA** (10 µM) for 3 hours. After harvesting, cell pellets were sonicated and homogenized in cold lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100 and complete protease inhibitors) for 30 minutes. The lysed cells were centrifuged at 14000 g for 5 minutes and the soluble fractions were collected as whole cellular proteomes. For *in vitro* study, the cell lysates were incubated with DMSO, **IA-yne** (1.0 µM), **GA-yne** (1.0 µM), and **GA-yne** (1.0 µM) with excess **GA** (10 µM) for 3 hours. Both the *in situ* and *in vitro* samples were diluted to 2.0 mg/mL with lysis buffer and 1% SDS (w/v) was added to each sample and click reaction was performed as below: for each reaction, 20 µL of protein samples were added to freshly prepared 0.25 µL each of TAMRA-N₃ (10 mM stock in DMSO, Lumiprobe), CuSO₄ (100 mM stock in H₂O), THPTA (Tris(3-hydroxypropyl)triazolylmethyl)amine, 10 mM stock in H₂O, Sigma) and sodium ascorbate (NaVc, 100 mM stock in H₂O). The samples were incubated at room temperature for 1.0 hour, added with sample loading buffer, applied to SDS-PAGE and imaged by FUJIFILM FLA 9000 plus DAGE fluorescence scanner.

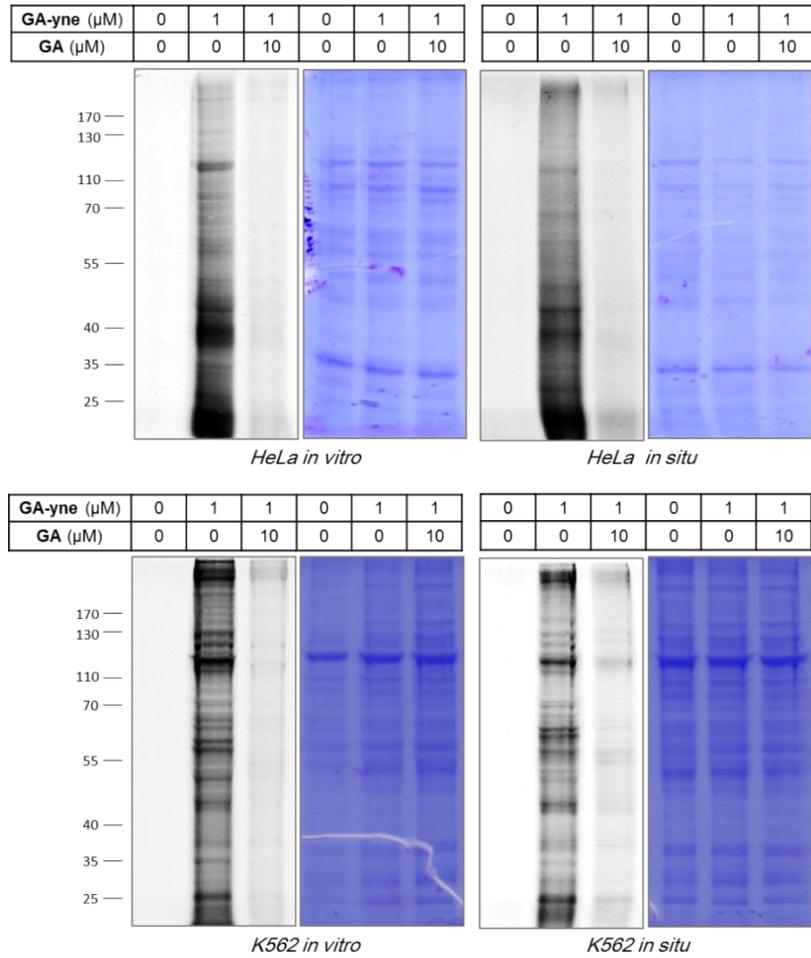


Figure S4. Full imaging of coomassie blue staining gels in Fig. 2A.

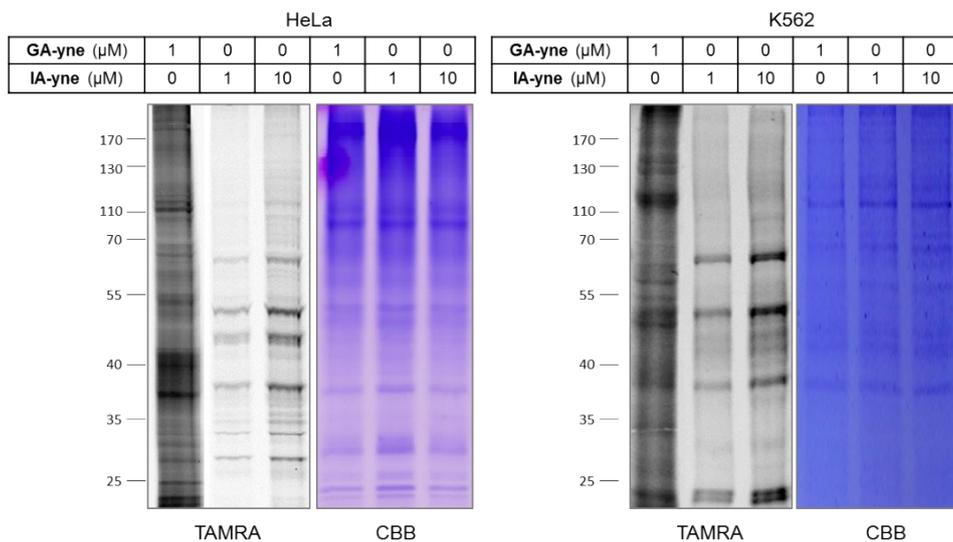


Figure S5. Labeling profiles of GA-yne and IA-yne in both living cells. (CBB: Coomassie blue staining)

2.4 Microscopy

HeLa cells were seeded on coverslips in a 6-well plate (Corning) and grown until 70% confluence. Cells were incubated with 1.0 μM of **GA-yne** in 2.0 mL of fresh DMEM growth medium for different time periods (1 hour and 3 hours) with medium containing same volume of DMSO used as a negative control. The cells were then washed with DMEM growth medium and ice-cold PBS twice each, followed by fixed with 3.7% paraformaldehyde in PBE for 30 min at room temperature. After washing with PBS twice, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed twice with PBS again and blocked with 3% BSA in PBS (with 0.05% Tween-20) for 30 min at room temperature, and washed twice with PBS. The cells were then treated with a freshly prepared click chemistry reaction cocktail containing of TAMRA-N₃ (100 μM final concentration), THPTA (100 μM final concentration), NaVc (1.0 mM final concentration), and CuSO₄ (1.0 mM final concentration), in 1.0 mL PBS for 2 h at room temperature. The cells were then washed with PBS (0.05% Tween-20 and 0.5 mM of EDTA) for 3 times, and with PBS twice with gentle agitation. Cells were washed twice with PBS and treated with 10 $\mu\text{g}/\text{mL}$ of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) dissolved in PBS for 15 min at room temperature, followed by imaging with Leica TSC SP8 STED 3X fluorescence microscope.

2.5 Mass spectrometry-based ABPP in living HeLa/K562 cells and TMT labeling

The probe incubation and proteome preparation procedures were same as that of labeling studies mentioned above in section 2.3. Each of 500 μL of proteome sample was subjected to click reaction with Biotin-N₃ (500 μM , Biomatrix Inc.), CuSO₄ (1.0 mM), THPTA (100 μM) and NaVc (1.0 mM). The samples were precipitated with CH₃OH (600 μL) / CHCl₃ (150 μL) / H₂O (300 μL) sequentially and vortexed for a while. After centrifuge at 14,000 g for 3 minutes, the protein disk was washed twice with CH₃OH (500 μL), air-dried and re-dissolved in 200 μL of click buffer (50 mM HEPES pH 8.0, 1% SDS) by sonication. 50 μL of streptavidin-sepharose (GE Healthcare) beads were added to each sample and incubated at room temperature with continuous rotation for 1 hour. The beads were washed with PBS with 1% SDS (w/v) three times, PBS with 0.5 M NaCl three times, 4.0 M Urea in 100 mM triethylammonium bicarbonate (TEAB) twice, and 100 mM TEAB five times. Each wash was performed on a rotator for 15 minutes. The bounded proteins were subjected to on-beads reductive alkylation with 200 μL of 10 mM of tris(2-carboxyethyl)phosphine (TCEP) at 50 °C for 30 minutes and 200 μL of 55 mM iodoacetamide at 37 °C in dark for another 30 minutes, followed by wash with 100 mM TEAB three times. Bounded proteins on beads were digested with 1.0 μg of sequencing grade modified trypsin (Promega) reconstituted in 50 μL of 100 mM TEAB for 16 hours at 37 °C. The digests were labeled with respective TMT-sixplex™ Isobaric Label Reagent Set (Thermo Scientific) according to the manufacturer's procedures. The labeled peptides were desalted by Pierce C18 spin columns and evaporated to dryness on a SpeedVac. The dried peptides were resuspended in 30 μL of formic acid/acetonitrile/H₂O (v:v:v = 0.5%/2%/97.5%) with sonication. The proteomics

experiment was carried out in biological duplicates.

2.6 LC-MS analysis

After filtration through 22 μm membrane the clear solution was subjected to nano LC-MS/MS separation. A volume of 3.0 μL of each sample was desalted by loading on a Thermo C18 PepMap100 precolumn (300 μm \times 5 mm) and eluted on a Thermo Acclaim PepMap RSLC analytical column (75 μm \times 15 cm). Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish the 120-minute gradient comprised of 85 min of 4–30% B, 15 min of 30–50% B, and 5 min of 90% B, followed by re-equilibrating at 4% B for 15 min. The flow rate was 0.3 $\mu\text{L}/\text{min}$. Peptides were then analyzed on a Q-Exactive proteomic mass spectrometer (Thermo Scientific) in a data-dependent manner, with automatic switching between MS and MS/MS scans using a top-20 method. MS spectra were acquired at a resolution of 70000 with a target value of 3×10^6 ions or a maximum integration time of 50 ms. The scan range was limited from 375 to 1400 m/z. Peptide fragmentation was performed via higher-energy collision dissociation (HCD) with the energy set at 32 NCE. The MS/MS spectra were acquired at a resolution of 35000 with a target value of 1×10^5 ions or a maximum integration time of 100 ms. The fixed first m/z was 100, and the isolation window was 1.2 m/z.

2.7 Proteomics data processing

Protein identification and TMT quantification were performed using Proteome Discoverer 2.1 software (Thermo Scientific). Peptide sequences (and hence protein identity) were determined by matching protein databases (Uniprot) with the acquired fragmentation pattern by SEQUEST HT algorithm. The precursor mass tolerance was set to 10 ppm and fragment ion mass tolerance to 0.02 Da. One missed cleavage site of trypsin was allowed. Carbamidomethyl (C) and TMT-sixplex (K and N-terminal) were used as a fixed modification. Oxidation (M) and deamination (N, Q) were used as variable modifications. All spectra were searched against protein database using a target false discovery rate (FDR) of 1%. The proteins identified in positive group (**GA-yne**-treated samples) were additionally filtered by at least two spectral counts and one unique peptides in each experimental replicate. Protein ratios were calculated as the median of all peptide hits belonging to a protein. The *p*-values of each category of TMT ratios were subjected to the Benjamini–Hochberg statistical correction (FDR = 0.05), and only proteins identified have *p*-values of competition ratio less than 0.05 were considered statistical significant targets. The raw data and database search results were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005243.

2.8 siRNA transfection

HeLa cells were transfected in OPTI-MEM with lipofectamin RNAiMAX (Invitrogen) with 25 nM of siRNA of RPS27A (5'-ACA UUC AUC AGA AGG GCA CTT-3') or scrambled RNA (5'-UUC UCC GAA CGU GUC ACG UTT-3', negative control) for 24

hours according to the manufacturer's protocols. The medium was removed and replaced for DMEM containing 10% FBS. Cells were treated with various concentrations of compounds and DMSO for 36 hours in a 37 °C incubator. Cell viability was assessed by CellTiter-Glo® Luminescent Kit (Promega).

2.9 Western blot

Cell lysates were boiled in SDS-PAGE sample loading buffer for 15 minutes and separated by SDS-PAGE. Then the proteins were transferred onto nitrocellulose membrane (Millipore) and the membrane was blocked with 3% (w/v) BSA in tris-buffered saline (TBS) for 2 hours at room temperature. After blocking, membranes were incubated with the primary antibodies of RPS27A and β -actin (Sangon Biotech) for one hour, respectively. After washing with TBST (TBS containing 0.1% Tween-20) for three times, blots were further incubated with the HRP-conjugated anti-rabbit (Sangon Biotech) secondary antibody for 1 hour at room temperature. After incubation, the blot was washed again with TBST three times and developed by enhanced ECL chemiluminescent substrate kit (Pierce).

3. Bioinformatics analysis

3.1 Overlapped protein targets of GA in HeLa and K562 cells

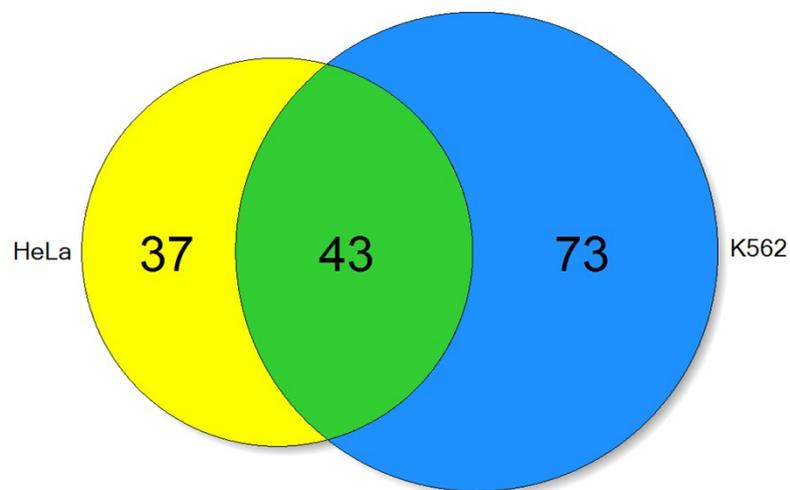


Figure S6 Venn diagram showed 80 proteins in HeLa cells and 116 proteins in K562 cells were identified as high-confidence protein targets of gambogic acid, with 43 overlapping proteins identified in both cell lines.

3.2 IPA analysis of protein targets of GA

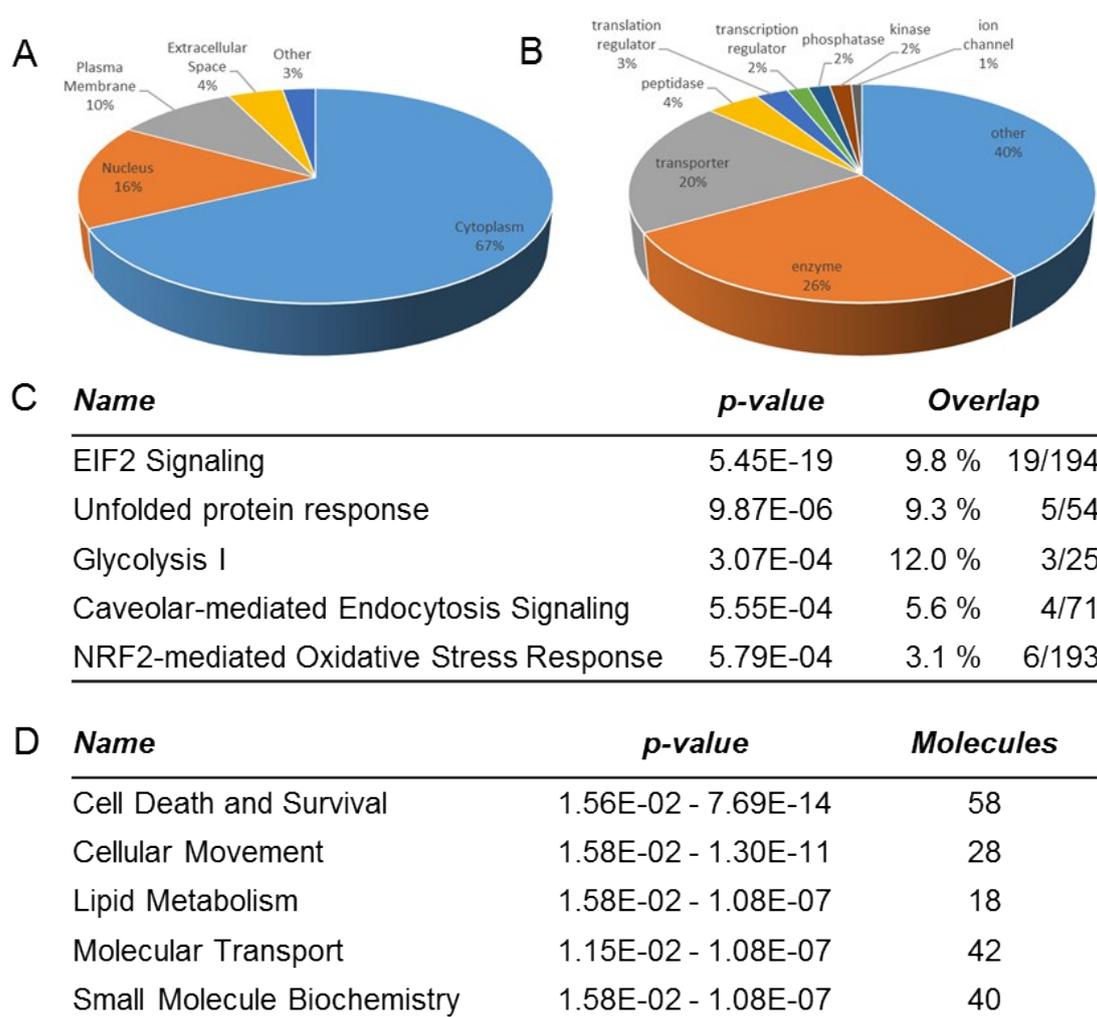
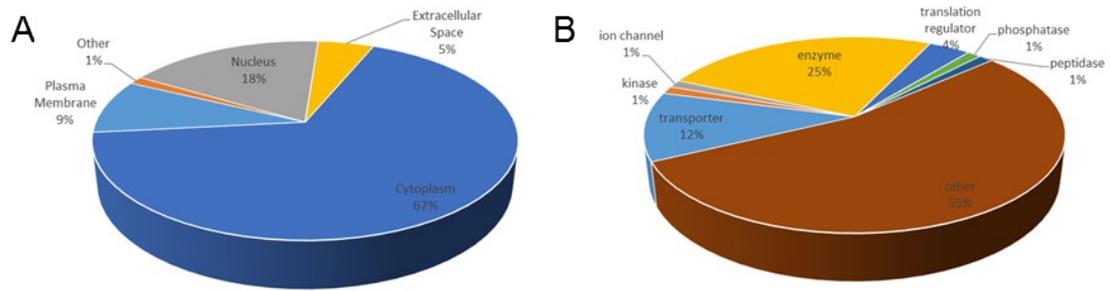


Figure S7 Ingenuity Pathway Analysis (IPA) of 116 GA targets in K562 cells. (A) Subcellular localization; (B) Protein type; (C) Top 5 canonical pathways; (D) Top 5 molecular and cellular functions.



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<i>Name</i>	<i>p-value</i>	<i>Overlap</i>
EIF2 Signaling	1.28E-20	9.30% 18/194
Regulation of eIF4 and p70S6K Signaling	1.37E-11	7.00% 11/157
mTOR Signaling	3.18E-09	5.00% 10/199
Glycolysis I	2.05E-06	16.00% 4/25
Gluconeogenesis I	1.05E-04	12.00% 3/25

D

<i>Name</i>	<i>p-value</i>	<i>Molecules</i>
Cell Death and Survival	2.84E-02 - 5.45E-09	44
RNA Post-Transcriptional Modification	1.47E-02 - 9.81E-07	10
Cellular Movement	2.20E-02 - 9.28E-06	17
DNA Replication, Recombination, and Repair	2.56E-02 - 4.03E-05	6
Gene Expression	1.83E-02 - 8.26E-05	8

Figure S9 Ingenuity Pathway Analysis (IPA) of 80 GA targets in HeLa cells. (A) Subcellular localization; (B) Protein type; (C) Top 5 canonical pathways; (D) Top 5 molecular and cellular functions.

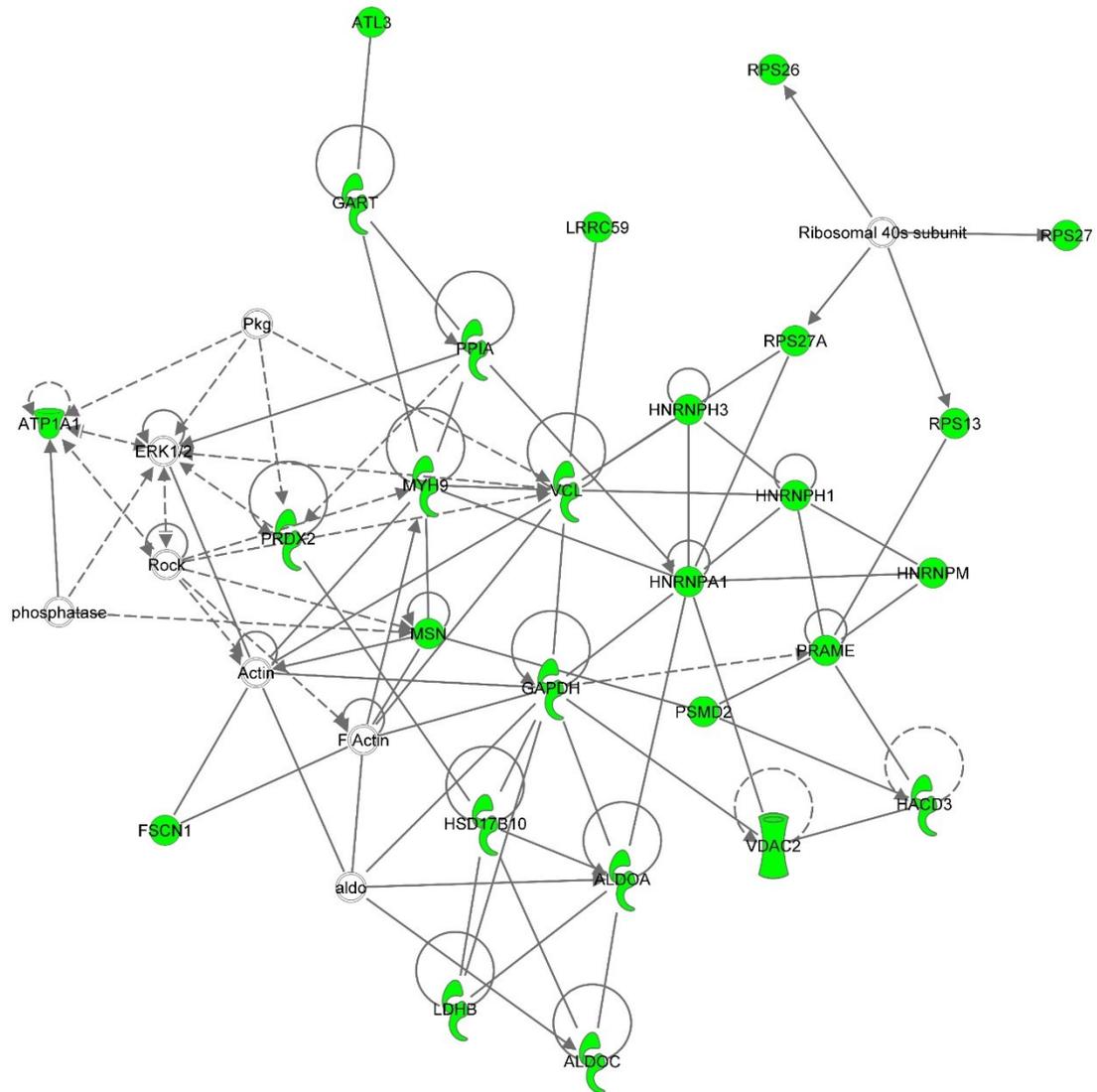


Figure S10 Ingenuity Pathway Analysis (IPA) of 80 targets in HeLa cells revealed that **GA** affects the network regarding *carbohydrate metabolism, small molecule biochemistry and cellular movement*, where 27 proteins are involved in this network with a score of 63. Proteins shown in green nodes were significantly enriched and identified as specific targets of **GA** in HeLa cells.

4. Protein lists of identified targets of **GA**

Table S1. Full lists of protein targets of **GA** in K562 and HeLa cells.

(Please see accompanying EXCEL file)