Supporting Information

for

Integrative chemoproteomics reveals anticancer mechanisms of silver(I) targeting proteasome regulatory complex

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Supplementary Experimental Section/Methods

Chemicals and consumables:

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dithiothreitol, iodoacetamide, anhydrous acetonitrile, 50% hydroxylamine, triethylammonium bicarbonate (TEAB), TCEP-HCl, tris(2-carboxyethyl)phosphine hydrochloride, desthiobiotin polyethyleneoxide Iodoacetamide (DBIA), size-exclusion chromatography (NAP-10 column), biotin, trypsin from bovine pancreas, HEPES, urea, sodium orthovanadate, sodium pyrophosphate, β-glycerophosphate, disodium, and dichloro-dihydro-fluorescein diacetate (DCFH-DA) were all purchased from Sigma-Aldrich. High-capacity streptavidin agarose, rhodamine-azide, iodoacetamide alkyne, TMT10plex™ Isobaric Label Reagent Set, CDS Empore™ C8 and C18 Extraction Disks, Annexin V-Alexa488 and propidium iodide (PI), MitoTracker-orange, Hoechst 33342 Solution, and Fluo-4-AM were all from ThermoFisher. Trypsin, mass spectrometry grade, was purchased from Promega Corporation. Mono- and poly-ubiquitinylated conjugates monoclonal primary antibody (FK2), ER-tracker-Red were purchased from Enzo Life Sciences. LC3 primary antibody was from NovusBio. Sequestosome-1/p62 primary antibody was from BD Biosciences. PTMScan® Ubiquitin Remnant Motif (K-ε-GG) Kit was purchased from Cell Signaling Technology. Solid-phase extraction column, HLB column (3cc), was purchased from Waters. Suc-LLVY-AMC was purchased from MedChemExpress.

MTT assay:

Cancer cells were seeded into 96-well plates with a cell density of ~5000 cells per well, and then treated with AgTU of different concentrations from 0 to 100 μM. After 72 h treatment, MTT (5 μg/ml) was added to the wells and incubated at 37°C for 3 h. The medium was removed, and the formazan crystal was dissolved in 100 μL DMSO and the absorbance at 570 nm was measured on a plate (Thermo Varioskan Lux). Half maximal inhibitory concentration IC_{50} values were calculated from 3 independent experiments and summarized as mean ± standard deviation (range).

ICP-MS analysis of silver uptake in cancer cells:

The cellular uptake of silver was determined by measuring silver content using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500a). HeLa or NCI-H460 cells were seeded in a 6-well plate with culture medium and grown in a humidified 5% (v/v) CO_2 incubator

at 37°C overnight, and then treated with AgTU at the same concentration (10 μM) for different time periods. After removing the medium, cells were washed with ice-cold PBS three times, followed by harvesting by trypsinization, resuspending in H_2O and sonication to obtain a homogenous cell lysate. Cell lysates were then centrifuged to remove the undissolved residue. The supernatant was collected, and then the determination of protein concentration was carried out by using Bradford protein assay. And cell lysates were digested in 68% HNO_3 at 70°C for over 4 h. Digested cell lysates were further diluted in H_2O to lower the concentration of HNO_3 to less than 5%. The cellular silver contents were quantified with an ICP-MS by measuring the most abundant isotope of silver at *m/z*=107 and 109 and corrected with respect to silver nitrate standards. The cellular uptake silver was expressed as μg of silver per g of protein. The silver uptake amount in both cells was obtained from 3 readers and summarized as mean ± standard deviation.

LC-MS analysis of AgTU and its metabolite TU ligand in cells:

HeLa cells were seeded into 6-well plates and allowed for growth to reach confluence of ~40-60%. Then cells were treated with AgTU (5 μM) for 12 h. Then the cells were washed 3 times with PBS to remove residual drug, followed by trypsin digestion, suspension in cell culture medium, and getting pelleted and resuspended in ice-cold 0.9% saline buffer. The cell numbers were normalized in different treatment groups, and cells were pelleted and then re-suspended in cold methanol/water (4:1) before undergoing freeze-thaw cycles for 5 times to extract metabolites. The cells were then centrifuged at 20,000 g for 10 min at 4°C to pellet cell debris. The supernatant was collected and dried in SpeedVac. The dried samples were reconstituted in 50% methanol and analyzed using LC-MS. AgTU was monitored with its *m/z* = 735.1223 while the released TU was monitored with *m/z* =315.1162. The signal intensities of all metabolites were obtained in 4 independent experiments and calculated as mean ± standard deviation (range).

High-content microscopy analysis of cell proliferation and death:

HeLa or NCI-H460 cells were sub-cultured into 96-well plates with a cell density of ~5000 cells per well, followed by incubation with AgTU or TU of serial concentrations, respectively. After incubation for 0, 24, 48, and 72 h, the cells were stained with 20 μM Hoechst 33342 (blue fluorescence) to count total cells and 1 μg/mL propidium iodide to count dead cells before incubation for 30 min. The fluorescence was then determined with ImageXpress micro-confocal

High-Content Imaging System (Molecular Devices) to count both the total and dead cell number.

Flow cytometry analysis of cell death using Annexin V and PI staining:

Cells were treated for 24 h with 5 μM AgTU or 0.2% DMSO as vehicle control. The samples were washed with PBS and then labeled with 2 μg/ml propidium iodide and 1× Annexin V in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) before flow cytometry analysis.

In-vivo anti-tumor study:

All animal experiments were conducted under the guidelines approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong and the animal license was issued by Department of Health, the government of Hong Kong. Each BALB/c Nude mouse was inoculated subcutaneously with 3×10^6 cells of HeLa cell. Mice were subjected to the injection of AgTU complex when the tumor volumes reached around 50 mm^3. The mice were randomly divided into the following three groups as follows:

1) Vehicle control group (MCE solution)
2) treatment group 1 (AgTU 10 mg/kg body weight)
3) treatment group 2 (AgTU 20 mg/kg body weight)

(MCE solution = 10% DMSO + 40% PEG300 + 5% Tween-80 + 45% saline)

Nude mice bearing HeLa xenograft received intraperitoneal injection of silver thiourea compound or solvent control at 3 doses weekly. The length and width of tumor were measured by Vernier caliper and the body weight was monitored every two or three days. At the end of the studied period (11 days), the mice were scarified and decapitated. The tumors were weighted and photographed.

Tumor sizes and body weight were recorded before the drug injection every time and tumor volume (V) was calculated by the following formula:

$V = LW^2/2$,

(where L is tumor length and W is tumor width)

The tumor growth inhibition effect was calculated by the following equation, and data in each group was expressed in mean ± standard deviation.

$$\text{Inhibition}\% = \left(1 - \frac{V - V_o}{V' - V'_o}\right) \times 100$$

(V_0 is the initial tumor size before AgTU treatment; V is the final tumor size after AgTU treatment; V'_0 is the initial tumor size in the solvent control; and V' is the final tumor size in the solvent control.)

Histological analyses

Tissues were collected and cut into small pieces and fixed in 10% neutral buffered formalin solution for 48 h before transferring to 75% ethanol for long- term storage at 4 °C. The paraffin blocks were prepared for sectioning at 5 μm thickness. The tissue slides were stained with hematoxylin and eosin (H&E) solution. All slides were examined under Olympus biological microscope BX53, and images were captured using an Olympus DP72 color digital camera (Olympus, Tokyo, Japan).

TPP analysis:

HeLa or NCI-H460 cells (~6×10^6 cells) were sub-cultured into 9-cm petri-dishes and incubated for 24 h before 1 h treatment with AgTU (5 μM) or DMSO (0.2%). Then the cells underwent trypsin digestion and resuspension in ice-cold PBS. Then the cells were aliquoted into PCR tubes, followed by heating for 3 min by PCR machine to 10 different temperatures (37, 41, 44, 47, 50, 53, 56, 59, 63 and 67°C), respectively. The cells then underwent lysis by 3 freeze-thaw cycles to extract proteins. After ultra-centrifugation at the speed of 100,000 g for 20 minutes, the supernatant was transferred to new tubes. The same volumes of protein samples were collected and denatured in urea buffer (6 M urea in 50 mM Tris, pH8.0). The proteins were further reduced with 5 mM DTT, carboxy-monomethylated using 25 mM IAA, and then diluted 5-fold with 50 mM Tris, pH8.0. The proteins were then digested into peptides with trypsin-LysC mixture and incubated at 37°C for 16 h. The peptides were desalted with StageTip packed with C18 materials, dried with SpeedVac, and labeled with 10-plex TMT reagents (TMT10, Thermo Fisher Scientific) as per the manufacturer's instructions. The samples from the same treatment were then mixed together and further desalted with StageTips. The peptide sample was analyzed with LC-MS/MS.

LC analysis was accomplished using an EasySpray column (75 μm i.d. × 75 cm) packed with C18 materials (2 μm 100 Å pore). The nanoLC flow allows for peptide separation with a 270-cm gradient. For LC mobile phases, mobile phase A contains 0.1% formic acid and mobile phase B is 0.1% formic acid in 80% acetonitrile. The MS was performed in positive ionization and data-dependent acquisition mode, and also FAIMSpro was incorporated to exclude singly charged ions and improve the detection of peptides with multiple charges.

Proteins were identified and quantified by searching against the Uniprot human database (downloaded on 20 May 2022, ~220,000 entries) using Proteome Discoverer (version 2.4.1). The search type was set to Reporter ion MS2, and the default TMT quantification method.[1] TMT modification is used as a static modification at N-terminal of peptide and lysine side-chain. The default Percolator filtering parameters are as below: Target/Decoy Selection of "Concatenated", Validation based on: q-Value; Input data of peptide-spectrum match is filtered using Maximum ΔCn: 0.05, Maximum Rank: 0 (not restricted); Target FDR (strict, q-Value): 0.01, Target FDR (Relaxed, q-Value): 0.05. Search the Uniprot human database (downloaded on 20 May 2022, ~220,000 entries) using Proteome Discoverer (version 2.4.1) to identify and quantify proteins; The protein abundance at 37°C was set to 1 and relative protein quantification at each temperature was calculated.

After obtaining the temperature-dependent changes, TPP R package was then applied to determine the melting curves and significant T_m shifts. The T_m shifts of all proteins were obtained by comparing T_m values in three control and AgTU treatment experiments, respectively, and the mean T_m shifts and significance level p value were calculated. To uncover the protein targets, we did data filtering as follows: Check the identified melting temperatures in all 6 experiments, all 6 melting temperatures should be obtained. Melting temperature changes should be larger than +2°C or lower than -2°C, and p value <0.05. Finally, check the fitting of melting curves, and the proteins with at least 5 of the 6 curves having R^2>0.85 were identified as possible protein targets. Based on the identified proteins, gene ontology enrichment analysis was performed.

MS analysis of reaction of AgTU with glutathione and N-acetylcysteine:

AgTU (0.1 mM) was mixed with excessive glutathione or N-acetylcysteine (0.2, 1, 5 mM, respectively) in 100 μL 10 mM ammonium bicarbonate (pH7.0) for 1 h. Then the reaction was diluted 10-fold using ddH_2O and analyzed using Agilent 6546 Q-TOF MS. For positive ion analysis, direct injection uses 80% mobile phase A (0.1% formic acid in water) and 20% mobile phase B (0.1% formic acid in methanol). For negative ion analysis, liquid flow uses 80% mobile phase A (10 mM ammonium acetate) and 20% mobile phase B (methanol). The flow rate is 0.3 mL/min. For the MS part, Dual AJS ESI ion source is used and MS1 ions were monitored in the mass range of 50-1500. The parameters are as below: gas temperature is 325°C, drying gas flow is 10L/min, nebulizer is 20 psi, sheath gas temperature is 375°C, sheath gas flow is 11 L/min. Vcap ionization voltage is 4000 V, nozzle voltage is 1000 V. Then standard mixtures were used first for analysis. Then 15 μl of sample was injected and 3 injections were used for

each sample. For positive ion mode, the *m/z* for AgTU is 735.12, TU is 315.12, GSH is 308.10. For negative ion mode, the *m/z* for Ag-GSH is 411.97 and mass window is 50 ppm.

Cysteine profiling:

HeLa cells were seeded into 9-cm petri dishes and allowed to grow for 24 h to reach 70-90% confluence before treated with 0.2% DMSO or 5 μM AgTU for 1 h. The cells were detached from the dish using trypsin and suspended in ice-cold PBS, pH7.4, with 0.1% Triton X-100. The cells were sonicated on ice for 1-2 min to extract proteins before centrifugation at 20,000 g for 20 min and collect the supernatant. For +TCEP samples, the proteins were treated with 1 mM TCEP for 30 min. To minimize spontaneous cysteine oxidation, all the extracted protein samples were soon labeled with 1 mM DBIA and reacted for 2 h in the dark. The excessive DBIA was then removed with size-exclusion column NAP-10 and eluted into 1.2 mL buffer containing 50 mM Tris, pH8.0. Then 5 mM DTT was added into the proteins followed by 20 mM IAA treatment for 15 min at darkness and 5 mM DTT for 5 min. The proteins were digested into peptides using trypsin that reacted at 37°C for 16 h. The DBIA-labeled peptides were pulled down using streptavidin agarose resin and eluted from the beads using 5 mM biotin in 10 mM PBS, pH7.4. The eluted peptides were further purified using StageTip and dried. Then the peptides were split into two equal parts, one of which was dissolved in 50 mM triethyl ammonium bicarbonate and quantified and normalized before TMT labeling while the other part was used for label-free quantification and dried before nanoLC-MS analysis. The peptides for TMT analysis were then mixed together and desalted using StageTip and dried before nanoLC-MS analysis.

The nanoLC-MS analysis had similar parameters to the above proteomics experiments. The acquired data were analyzed using Proteome Discoverer. A FASTA file was used to replace selenocysteine with cysteine to include possible selenocysteines labeled with DBIA. Then special post-translational modifications were set up as follows for both TMT and LFQ. Met: oxidation (m/z+15.995), Cys: DBIA (m/z+455.274), Cys: carbamidomethyl (m/z+57.021), Cys: U-DBIA (m/z+503.219), and Cys: Cys->Dha (m/z-33.988) were used as variable modifications in the PD software. For peptide identification using TMT, additional modifications were included as below: lysine with TMT 6-plex modification (m/z+229.163) and peptide N-terminal TMT 6-plex modification (m/z+229.163) are included as static modifications.

To identify the protein targets using TMT quantification, we performed data filtering as follows: 1) we filtered out TMT- and DBIA-modified peptides. (2) We quantified the 3 ratios

by calculating the 3 ratios below: Ratio1: AgTU-/Ctr- should be 0.011-0.7, Ratio2: AgTU+/Ctr+ should be 0.5-3, Oxidation ratio = (Ratio2 -Ratio1)/Ratio2 should be >30%. In the above calculations, AgTU- and Ctr- are AgTU treatment and control treatment without following TCEP treatment, respectively. AgTU+ and Ctr+ mean AgTU treatment and control treatment with TCEP treatment, respectively. Peptides that met the requirements were thought to possibly get oxidized by AgTU treatment. These oxidized peptides were used for GO enrichment analysis.

For ABPP analysis using label-free quantification, we analyzed peptides with cysteines modified with DBIA and then quantified their abundance in Proteome Discoverer using default LFQ parameters. We also calculate the following 3 ratios: Ratio1: AgTU-/Ctr- should be 0.011-0.75, Ratio2: AgTU+/Ctr+ should be 0.6-3, Oxidation ratio = (Ratio2 - Ratio1)/Ratio2 should be >30%. The peptides that met the criteria are thought to oxidize by AgTU treatment and then used for GO enrichment analysis.

Proteome profiling (AgTU treatment and CHX pre-treatment):

To investigate the proteome changes after AgTU treatment HeLa cells were seeded into 9-cm petri-dishes and allowed to grow for 24 h. Cells were grouped into 3 groups: Group 1 is vehicle control (without AgTU without CHX treatment, only 0.2% DMSO for 6 h), Group 2 is AgTU treatment group, treated with 5 μM AgTU for 6 h; Group 3 is treated with CHX for 2 h before treatment with 5 μM AgTU for 6 h. Then the cells were harvested and scraped into urea lysis buffer (20 mM HEPES , pH8.0, 8 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate). The cells were then lysed using sonicator on ice and the centrifuged at 20,000 g for 15 min to collect protein in the supernatant. The proteins were reduced and alkynylated using 5 mM DTT and 10 mM iodoacetamide, respectively. The proteins were diluted 4-fold in 20 mM HEPES (pH8.0) to dilute urea to < 2 M before trypsin digestion. The peptides were then purified using solid-phase extraction, eluted into 80% acetonitrile and dried before nanoLC-MS analysis.

Ubiquitome profiling:

HeLa cells were seeded into 9-cm petri-dishes and allowed to grow for 24 h. Cells were treated with AgTU (10 μM) or 0.2% DMSO for 6 h. Then the cells were harvested and scraped into urea lysis buffer (20 mM HEPES , pH8.0, 8 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate). The proteins were extracted using sonication before centrifugation at 20,000 g for 15 min to collect the supernatant. The proteins

in the lysis buffer were reduced in 5 mM DTT for 60 min and alkylated in 10 mM iodoacetamide for 15 min in the dark. The proteins were diluted 4-fold in 20 mM HEPES pH8.0 and digested into peptides using trypsin at 37°C for 16 h. Then the peptides were desalted using solid-phase extraction (Waters Oasis HLB PRiME 3 cc columns) and eluted into 80% ACN, and the eluates were dried. The peptides were further resuspended in immunoaffinity purification buffer (IAP buffer) and mixed with K-ε-GG motif antibody-beads to incubate on a rotator for 2 h at room temperature to pull down the ubiquitinated peptides. The beads were further washed twice with IAP buffer and three times with water, finally eluted with 0.2% TFA. The eluted peptides were desalted using StageTip before nanoLC-MS analysis. The parameters for nanoLC-MS analysis were similar to the TPP analysis. Data processing was performed on Proteome Discoverer and label-free quantification was performed. K-ε-GG modification (i.e. lysine with a modification of mass shift of +114.043) is configured as a dynamic modification. Dynamic modifications include oxidation of cysteine and methionine, carbamidomethylation of cysteine, N-terminal methyl loss, protein's N-terminal methionine loss + K-ε-GG, and protein's N-terminal methionine loss + acetylation. Normalization between samples was achieved by using total peptide amount. Unique and Razor peptides were used for quantification. We identified the peptides with K-ε-GG motif and at least 6 values obtained out of 8 experiments. The fold change of peptide abundance was calculated by comparing the signal intensities in AgTU versus Control group, and *t*-test was used to obtain *p* value. A peptide's ubiquitination level was thought to decrease if it has fold change within the range of 0.011-0.5 and $p < 0.05$. A peptide's ubiquitination level was considered to increase with a fold change >2 and $p < 0.05$.

Proteomics analysis of soluble and insoluble protein fractions from cells:

Cells were seeded into 9-cm petri-dishes and allowed to grow for 24 h. Cells were treated with AgTU (5 μM) or 0.2% DMSO for 12 h. Then the cells were washed before merging in soluble buffer (PBS pH7.4 containing 1% Triton X-100) and incubated on ice and shaken for 20 min. The cells were then scraped and collected into tubes before centrifugation at 15,000 g for 20 min to pellet insoluble proteins and debris. Then supernatant was collected as soluble fraction and the insoluble debris were further dissolved in 8 M urea lysis buffer in 20 mM Tris, pH8.0 and vortexed to dissolve the pellet before centrifugation to collect the insoluble protein fractions. The soluble and insoluble proteins were all treated with DTT and IAA before dilution 5-fold in 20 mM HEPES, pH8.0 and trypsin digestion at 37°C for 16 h. The peptides were then desalted using StageTip and dried before nanoLC-MS analysis. Proteome Discoverer was used to quantify protein signal intensity in soluble and insoluble fractions, respectively. The proteins'

fold changes were calculated from 3 independent experiments in both soluble and insoluble fractions, and their significance level *p* values were obtained by using *t*-test.

Autophagy assay:

HeLa cells stably expressing tandem EGFP-mRFP-LC3 fluorescent protein reporter (tfLC3) were cultured in 96-well plate and treated with vehicle control,[2] AgTU or MG132 for 24 h. Green and red fluorescent LC3 puncta (autophagosome) and red only LC3 puncta (autolysosome) were examined by ImageXpress Micro Confocal imager. The fluorescence signal was averaged from 3 images and expressed as mean ± standard deviation.

Cellular ubiquitinated protein and non-ubiquitin proteasomal protease assays:

HeLa cells stably expressing the ubiquitin fusion degradation substrate Ub^{G76V}-YFP protein,[3] and ornithine decarboxylase (ODC) degradation domain ODC-GFP fusion protein were used for monitoring ubiquitinated protein accumulation and non-ubiquitin proteolytic activity of 20S proteasome, respectively.[4] Cells were treated with 4 μM AgTU or 2 μM MG132 for 8 h. The GFP/YFP fluorescence was imaged with Nikon - CrestOptics spinning disk microscope and quantitated by Image J software.

Fluorometric proteasome activity assay:

HeLa cells were seeded into 6-well plates and allowed to grow for 24 h before 3 h of treatment with different concentrations of AgTU, or 5 mM H_2O_2, and MG132, respectively. Then the cells were detached, resuspended in buffer A (50 mM Tris pH7.4, 10 mM $MgCl_2$, 10% Glycerol, 2 mM ATP, 2 mM DTT), lysed by repeated freeze-thaw cycles, and centrifuged to collect the proteins in the supernatant. The proteins then underwent gel electrophoresis using 4% native PAGE gel. The gel was then incubated at room temperature with 50 μM suc-LLVY-AMC in buffer A for 30 min in dark. The gel was imaged with ChemiDoc MP (Bio-Rad). Then the gel was further submerged in buffer A supplemented with 0.02% SDS and 50 μM suc-LLVY-AMC before imaging.

Flow cytometry analysis of oxidative stress with DCFH-DA:

HeLa cells were sub-cultured into 6-well plates and allowed to grow for 24 h before incubation with 20 μM DCFH-DA probe for 45 min. The cells were washed with PBS to remove excessive probe. Drug treatment was performed with 5 μM AgTU or 40 μM AuTU for 3 h. The

cells were washed with PBS twice and detached from the dish with trypsin digest. The cells were washed with PBS and re-suspended in 200 μl PBS. The stained cells were then analyzed using flow cytometry with FITC filter.

Supplementary Figures

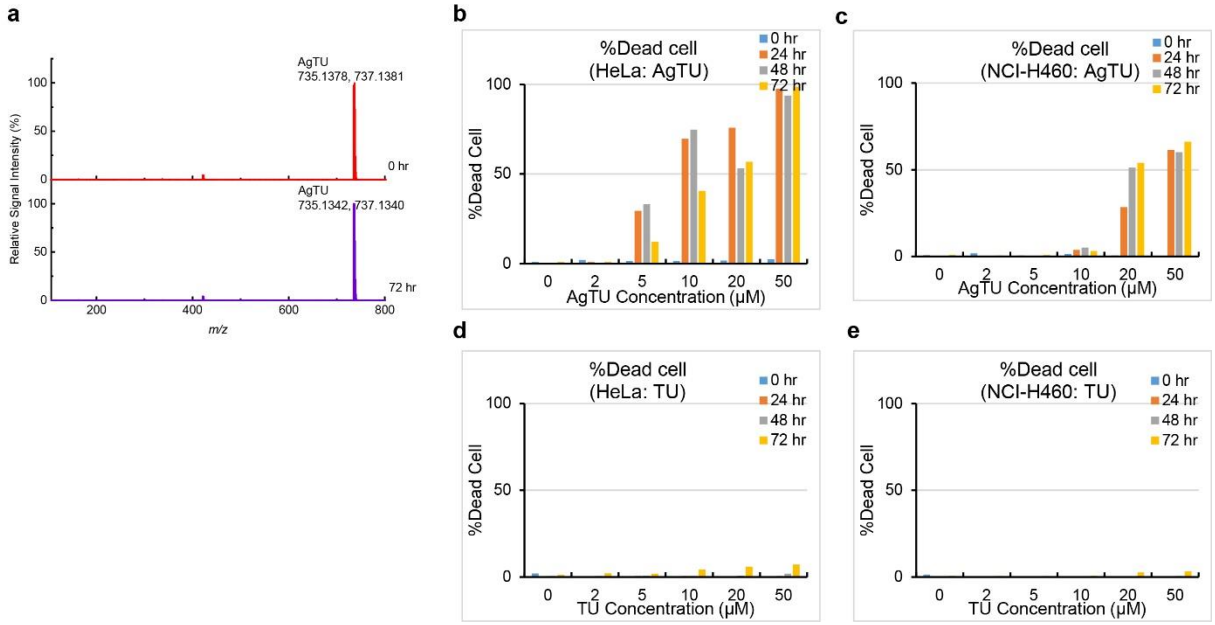

Fig. S1. (a) LC-MS analysis of AgTU stability in 10 mM ammonium bicarbonate buffer for 0 h and 72 h. (b-e) High-content analysis of AgTU-induced cytotoxicity effects in HeLa and NCI-H460 cells. (b) AgTU on HeLa cells. (c) AgTU on NCI-H460 cells. (d) TU on HeLa cells. (e) TU on NCI-H460 cells.

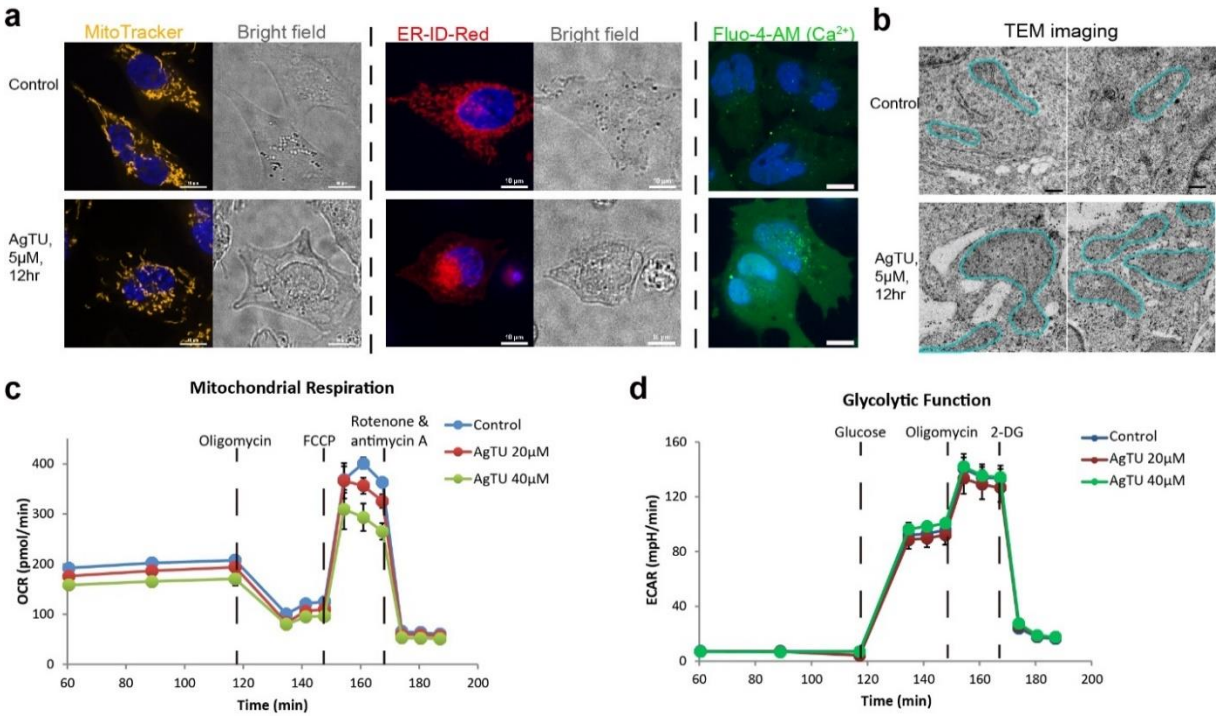

Fig. S2 AgTU treatment induces paraptosis-like cell death. (a) Confocal microscopy images showing morphological changes of HeLa cells in mitochondria and endoplasmic reticulum (ER), and Ca^{2+} release in HeLa cells treated with Fluo-4-AM and AgTU compared to control cells. (b) Transmission electron microscopy (TEM) imaging of HeLa cells in control and AgTU groups, with mitochondria shown in the blue line. (c-d) Seahorse assay for analysis of (c) oxygen consumption rate (OCR) and (d) extracellular acidification rate (ECAR) in NCI-H460 cells treated with vehicle control, AgTU of 20 μM and 40 μM, respectively.

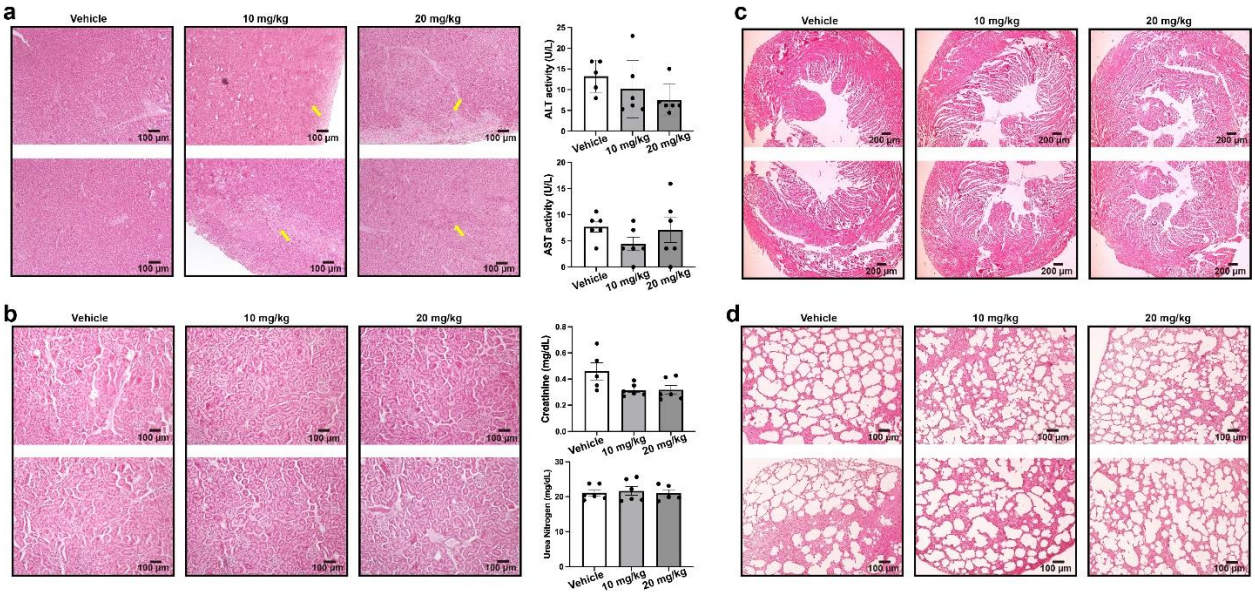

Fig. S3 Representative images of hematoxylin/eosin staining of (a) liver, (b) kidney, (c) heart and (d) lung from vehicle control and AgTU treated groups at the end point (Day 11; arrow: necrosis area), ALT/AST/Creatinine/Urea nitrogen in blood circulation was examined by biochemical assays.

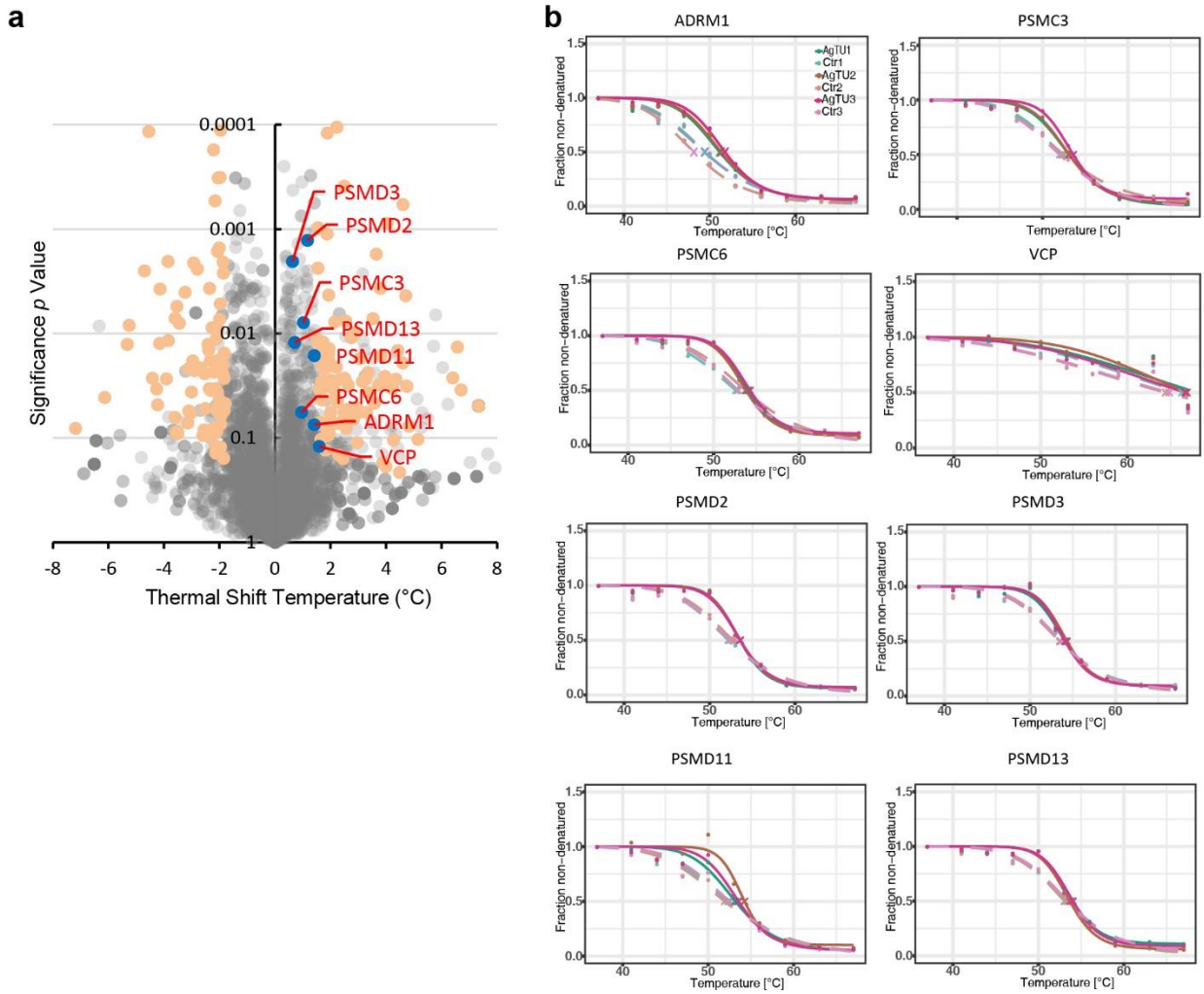

Fig. S4 TPP analysis of AgTU-targeted proteins in NCI-H460 cells. (A) Statistical diagram of the proteins whose thermal temperature was shifted after AgTU treatment, and the proteasomal proteins were highlighted with blue dots and labeled in red. (B) Thermal curve of proteasomal proteins.

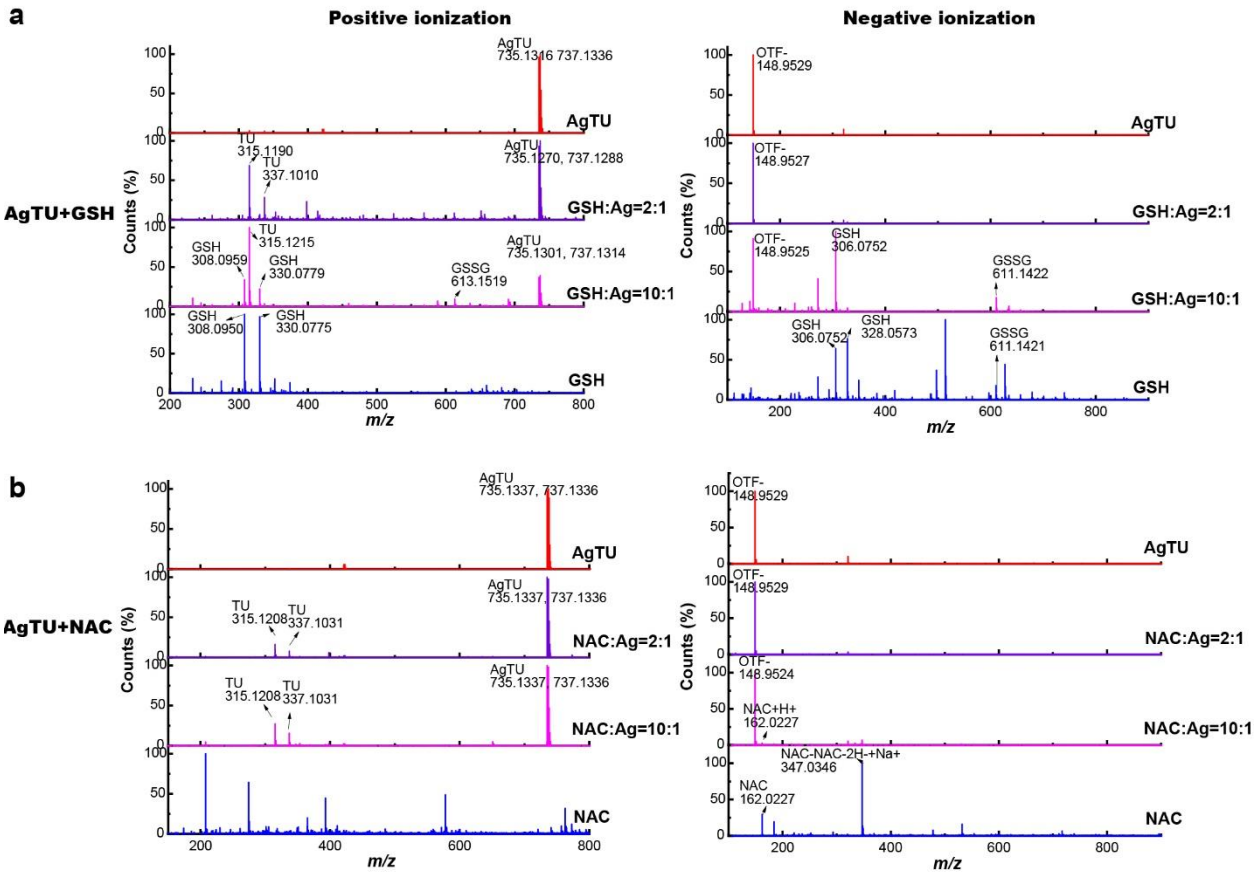

Fig. S5 LC-MS analysis of reactivity of AgTU with (A) glutathione (GSH) and (B) N-acetyl-cysteine (NAC). Reactions of AgTU with glutathione and N-acetyl-cysteine are monitored in positive ion and negative ion mode, respectively.

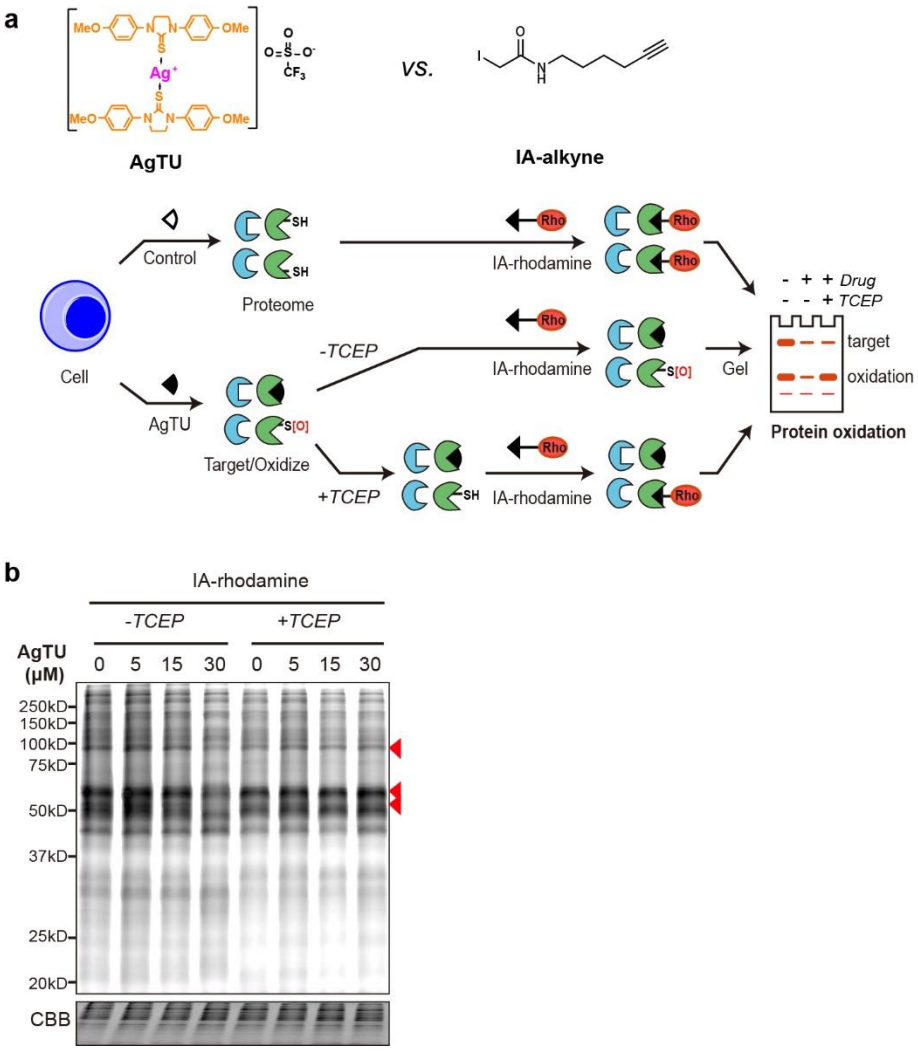

Fig. S6 Gel fluorescence analysis of the cysteine oxidation by AgTU by ligating fluorescent rhodamine group onto sulfur-targeted iodoacetamide functional groups, forming IA-rhodamine probe. (a) Scheme of the gel fluorescence assay and comparison of chemical structures of AgTU and IA-alkyne. (b) Gel fluorescence results by treating HeLa cells with AgTU of serial concentrations for 2 h before extracting the proteins from cell lysates, which were then treated with and without TCEP to reduce protein oxidation, followed by probe labeling with IA-rhodamine. The lower part depicting the corresponding gel stained with Coomassie brilliant blue (CBB).

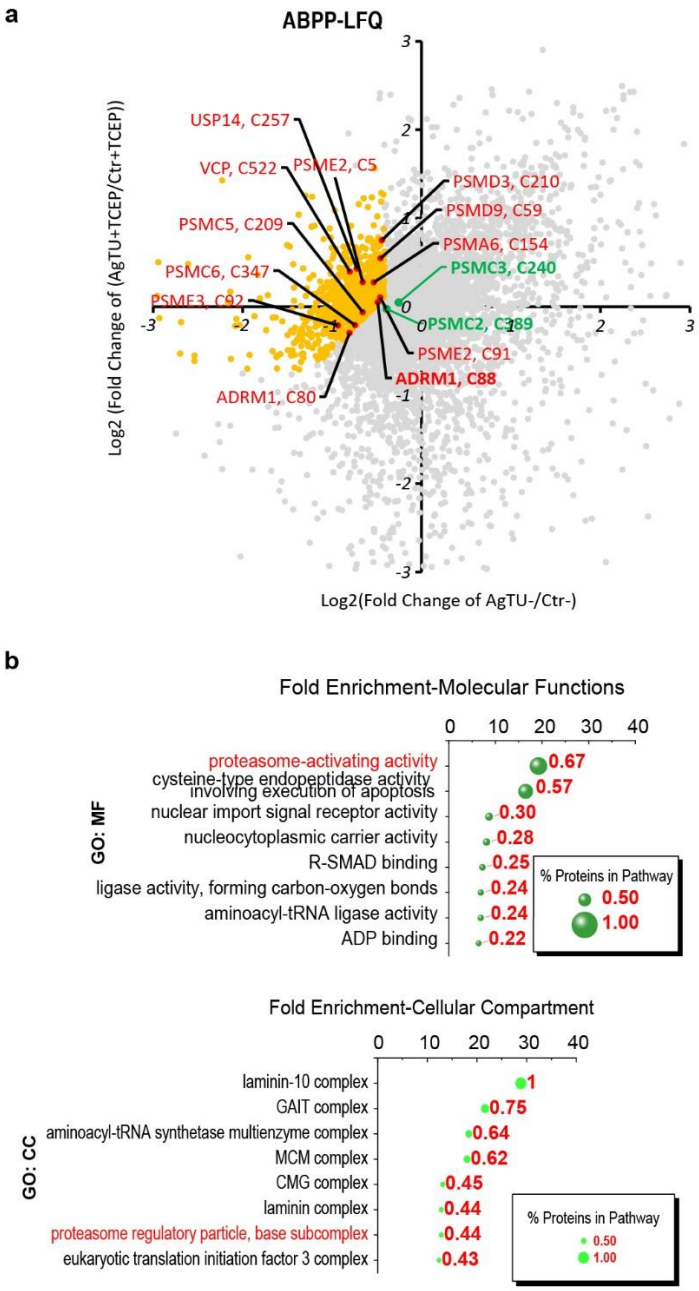

Fig. S7 Cysteine profiling using label-free quantification. (a) The volcano plot results of fold changes by comparing cysteine labeling in AgTU- with Ctr- and AgTU+ with Ctr+, respectively. (b) Gene ontology enrichment analysis of the filtered peptides that were highlighted orange in (a).

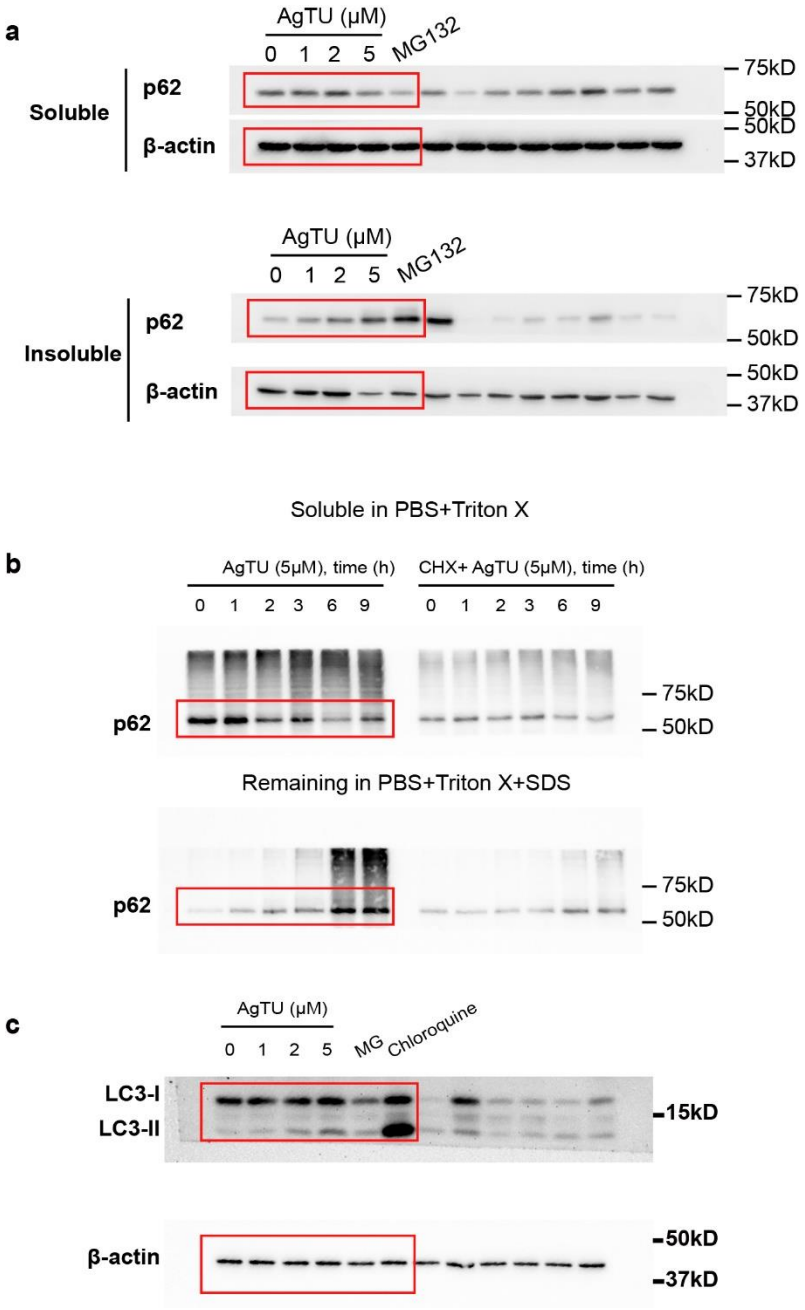

Fig. S8 (a) Western blot analysis of p62 and β-actin levels in soluble and insoluble fractions (presented in Fig. 6c) after treatment with AgTU of serial concentrations or MG132. (b) WB analysis of p62 levels in soluble and insoluble fractions (presented in Fig. 6d) after treating HeLa cells with 5 μM AgTU for different periods in the absence and presence of cycloheximide. (c) WB analysis of LC3-I, -II, and β-actin levels after treated with AgTU of different concentrations, or MG132, or chloroquine (presented in Fig. 6f). The region marked by red boxes is used in Fig. 6.

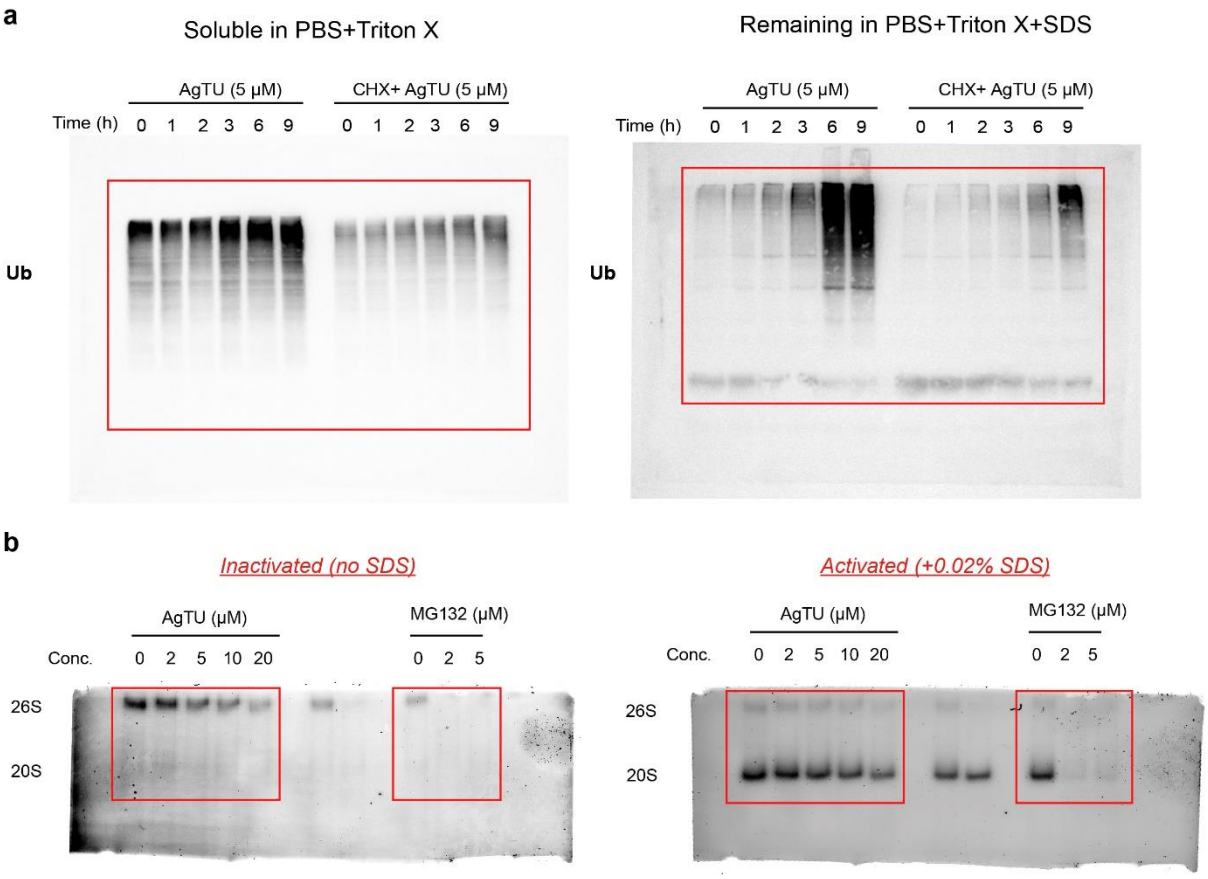

Fig. S9 (a) Western blot analysis of protein ubiquitination levles in soluble and insoluble fractions, after treatment with AgTU for 0-9 h in the absence and presence of cycloheximide, respectively (presented in Fig. 7a). (b) Gel fluorescence analysis of proteasome protease activity in inactivated buffer and activated buffer, respectively (presented in Fig. 7e). The region marked by red boxes is used in Fig. 7.

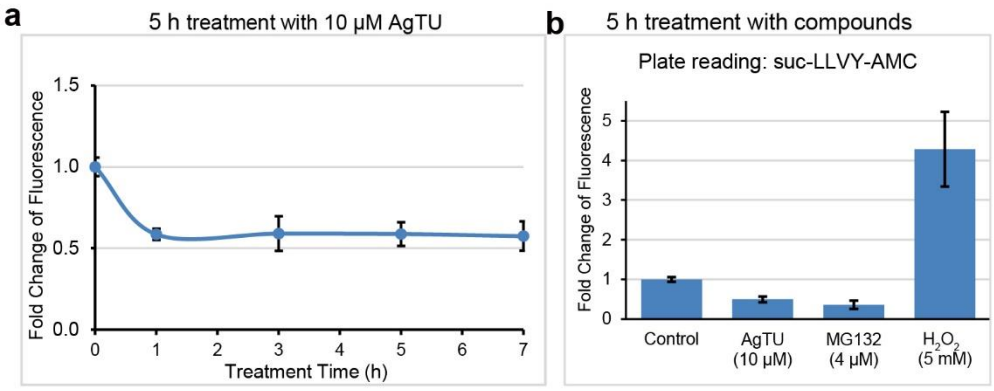

Fig. S10 Fluorometric proteasome activity assay for analysis of proteasome protease activity changes in HeLa cells treated with AgTU. (a) Time-dependent protease activity changes after AgTU treatment for 0-7 h. (b) A fluorometric assay of suc-LLVY-AMC hydrolysis using a plate reader for monitoring of protease activity changes after treatment with AgTU, MG132, and H_2O_2, respectively.

Reference

1. Franken, H. *et al.* Thermal proteome profiling for unbiased identification of direct and indirect drug targets using multiplexed quantitative mass spectrometry. *Nat. Protoc.* **10**, 1567-1593, (2015).
2. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* **3**, 452-460, (2007).
3. Menendez-Benito, V., Verhoef, L. G., Masucci, M. G. & Dantuma, N. P. Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. *Hum Mol Genet* **14**, 2787-2799, (2005).
4. Hoyt, M. A., Zhang, M. & Coffino, P. Probing the ubiquitin/proteasome system with ornithine decarboxylase, a ubiquitin‐independent substrate. *Methods Enzymol.* **398**, 399-413, (2005).