Surpporting information

Discovery of a novel NEDD8 activating enzyme inhibitor with piperidin-4-amine scaffold by structure-based virtual screening

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Materials. Cell lines K562, A549, SK-OV-3 and BXPC-3 were obtained from the American Type Culture Collection (ATCC). Cell line Caco-2 was purchased from Shanghai Institute of Biochemistry and Cell Biology (SIBCB). GES-1 cells were provided by Shanghai Gefan Biology. Cell line AGS was provided by Wuhan Procell. K562, A549, BXPC-3, and GES-1 cells were grown in RPMI 1640 (Wisent)/10% fetal bovine serum (FBS) (Wisent), Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent)/1% MEM nonessential amino acid solution (Wisent)/10% FBS, AGS cells were cultured in Ham's F12 medium (Wisent)/10% FBS, and SK-OV-3 cells were grown in DMEM/10% FBS at 37 ˚C with 5% CO_2.

The tested compound collection was obtained from ChemBridge. The database is publicly available and can be accessed free of charge. NEDD8 conjugation initiation kit (Cat. K-800, R&D), anti-Ubc12 antibody (Cat. A-655, R&D), anti-Ubc9 antibody (CAT. 4918, CST), anti-Ubc10 antibody (CAT. A-650, R&D), anti-USE1 antibody (Cat. sc-102150, Santa Cruz), anti-CDT1 antibody (Cat. 8064, CST) , anti-p53 antibody (Cat. 2527S, CST), anti-β-actin antibody (Cat. 5125S, CST), anti-NEDD8 antibody (Cat. 2745S, CST), 20×LumiGLO® reagent and 20× peroxide (Cat. 7003, CST) and FITC annexin V apoptosis detection kit (Cat. 556547, BD) were purchased from Univ-bio. Anti-p27 antibody (KF446, Nanjing Jiancheng Bioengineering Institute) was purchased from Nanjing Jiancheng Bioengineering Institute. Apoptosis and necrosis assay kit (Cat. C1056, Beyotime) was obtained from Beyotime. CCK-8 (Dojindo) was obtained from Nanjing Jianan Ltd.. Other biochemical reagents were purchased from Sigma-Aldrich.

Molecular modeling. Ligands in ChemBridge database were pretreated by *prepare ligands* protocol. In this procedure, hydrogen addition, charge standardization, duplicate removing, the largest fragment retaining and Lipinski filter were included. The published NAE structure (PDB ID: 1R4N) was used for screening. Crystallographic water, ligands and alternate conformations were removing by using the *protein preparation* protocol in DS 2.1. The molecular docking studies were performed to generate the bioactive binding poses of inhibitors in the active site (ATP binding pocket) of the enzyme using *LibDock* protocol of DS 2.1. A site sphere centered on ATP was created and the radius was set as 9. The fast conformation method for generating conformations and the Smart Minimiser for *in-situ* ligand minimization were adopted. Only 1 max fit was saved for each ligand. All other docking and consequent scoring parameters used were kept at their default settings. In native docking, the best conformation method for generating conformations was used and all docked conformations

were saved, and rescored by *Score ligand posed* protocol (Libsore, Ligscore-1, Plp-1, Jain, PMF).

AutoDock 4 which ultilized Lamarckian genetic algorithm and empirical free energy scoring function was also used for screening according to tutorials. Preparation of ligands and the protein were performed with AutoDockTools (ADT). With the edge of 60 Å, 60 Å, 60 Å, in X, Y, Z dimension respectively (a grid spacing of 0.375 Å), a grid box was created centering on the same coordinate as ATP. The grid box encompassed the whole active site (ATP pocket). Parameters for Lamarckian genetic algorithm and programming searches were kept as default settings.

After that, *Cluster ligands* protocol in DS 2.1were used to cluster the 100 top-ranked compounds into 10 classes according to ECFP_4 descriptor.

Antiproliferation assay. Cell counting kit-8 (CCK-8) was used to evaluate the proliferation of cultured cells. Cancer cells (K562, A549, SK-OV-3, BXPC-3, Caco-2 and AGS) were prepared and diluted to a concentration of 6×10^4 cells/mL. 100 μL of the cell suspension was seeded in each well of 96-well plates, totaling 6×10^3 cells/well. After 12 h incubation at 37 ˚C in 5% CO_2, the medium in the plates was replaced by 150 μL medium containing serial dilutions of compounds. Following 48 h culture, 10 μL CCK-8 was added to each well and cells were incubated for another 4 h. The light absorption value (OD) was measured at 450 nm. Inhibition rates were analyzed and the survival curves were generated using Graphpad 5 software.

Enzyme based NAE activity assay. This assay was performed using the NEDD8 conjugation initiation kit according to the instructions of the manufacturer. 2 μL NAE (2.5 μM), 2 μL NEDD8 (250 μM), 2 μL Ubc12 (50 μM), 2 μL reaction buffer and 10 μL water solution of **M22** (0 μM, 0.74 μM, 2.22 μM, 6.66 μM, 20 μM, 60 μM, 180 μM) were added into 100 μL reaction tubes in order. Reaction solutions were incubated at 37 ˚C for 10 min. The reaction was initiated by the addition of 2 μL Mg^{2+}/ATP solution (1.25 μM), and the mixture was incubated at 37 ˚C for 60 min. The reaction was quenched by the addition of 2 μL EDTA (1 M), and protein samples were electrophoresed under non-reducing conditions on a 15% SDS-PAGE gel. Ubc12-NEDD8 levels were determined by western blot analysis.

In ATP-competitive assay, 2 μL NAE (2.5 μM), 2 μL NEDD8 (250 μM), 2 μL Ubc12 (50 μM), 2 μL reaction buffer and 10 μL water solution of **M22** (6.66 μM), were added into 100 μL reaction tubes in order. Reaction solutions were incubated at 37 ˚C for 10 min. The reaction was initiated by the addition of 2 μL Mg^{2+}/ATP solution (0 μM, 1.25 μM, 10 μM, 100 μM, 1000 μM, 10000 μM), and the

mixture was incubated at 37 ˚C for 60 min. The reaction was quenched by the addition of 2 μL EDTA (1 M), and was electrophoresed under non-reducing conditions on a 15% SDS-PAGE gel. Ubc12-NEDD8 levels were determined by western blot analysis.

Cell based NAE activity assay. A549 cells were exposed to the indicated concentrations of **M22** (0.37 μM, 1.11 μM, 3.33 μM, 10 μM, 30 μM, 90 μM) or 0.1% (v/v) DMSO for 24 h. Cells were washed three times with ice-cold PBS, resuspended in RIPA lysis buffer, and incubated on ice for 30 min. Cell debris was removed by centrifugation at 13,000 rpm for 30 min at 4 ˚C. The protein concentration of the supernatant was determined with Thermo Fisher protein assay dye reagent (Thermo Fisher). Equal protein amounts were electrophoresed under non-reducing (Uba3-NEDD8, Ubc12-NEDD8, Cullins-Nedd8, Ubc9-SUMO, Ubc10-Ub and USE1-Ub) or reducing (p27, p53 and CDT1) conditions on SDS-PAGEs and subjected to western blot analysis.

Western blot analysis. Protein samples were transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) milk for 1 h at room temperature, and probed with primary antibody in 5% milk overnight at 4 ˚C. The membrane was washed with TBS/0.1% (v/v) Tween 20 (TBST) and incubated with horseradish peroxide-conjugated secondary antibody in 5% (w/v) milk for 1.5 h at room temperature. Protein bands were detected using LumiGLO® reagent and peroxide.

In this assay anti-Ubc12 antibody was used to detect Ubc12-NEDD8, and anti-NEDD8 antibody was used to measure Uba3-NEDD8 and Cullins-NEDD8. Anti-Ubc9, anti-Ubc10, anti-USE1, anti-p27, anti-CDT1 and anti-p53 antibodies were utilized to recognize Ubc9-Ub, Ubc10-SUMO, USE1-Ub, p27, CDT1 and p53 respectively.

Apoptosis/necrosis assay. Apoptosis and necrosis assay kit was used to determine cell apoptosis in this assay. A549 cells were seeded at 3×10^5 cells per well in 6-well plates and allowed to attach in 12 h. Cells were treated with different concentration **M22** (15 μM, 20 μM, 25 μM and 30 μM) for 36 h. Cells were washed with 4 ˚C PBS and 100 μL staining buffer was added to each well. After addition of 10 μL hoechst staining solution and 10 μL PI staining solution, cells were incubated at 4 ˚C for 30 min Stained cells were washed by 4 ˚C PBS and viewed under fluorescence microscope.

Apoptosis/necrosis was also evaluated using FITC annexin V apoptosis detection kit. A549 cells were seeded at 3×10^5 cells per well in 6-well plates and allowed to attach in 12 h. Cells were treated with different concentrations of **M22** (15 μM, 20 μM, 25 μM and 30 μM) for 36 h. Cells were dissociated using trypsin. Dissociated cells were washed twice with cold PBS and resuspended in 100

μL 1× binding buffer, followed by the addition of 5 μL FITC annexin V staining solution and 5 μL PI staining solution. After incubation at room temperature in the dark for 15 min, another 400 μL 1×binding buffer was added. Stained cells were analyzed immediately by flow cytometry.

Synergistic effect of M22 with bortezomib. A549 cells (6×10^3/well) were plated in 96-well plates. After 12 h for attachment, A549 cells were treated with **M22** (5 μM), bortizomib (12.5 nM) and both of them (5 μM M22 + 12.5 nM bortizomib), respectively. Following 48 h incubation, 5 μL CCK-8 was added to each well and cells were incubated for 4 h. The light absorption value (OD) was measured at 450 nm. Inhibition rates were calculated, and the q value was calculated according to the equation:

$$q = \frac{E_{\mathbf{M22}+BTZ}}{E_{\mathbf{M22}} + E_{BTZ} - E_{\mathbf{M22}} \times E_{BTZ}}$$

Where $E_{\mathbf{M22}}$, E_{BTZ} and $E_{\mathbf{M22}+BTZ}$ are the inhibition rates of **M22**, bortezomib, and combination of **M22** and bortezomib, respectively.

Tumor xenograft growth inhibition by M22. Female nude mice were subcutaneously injected with 5×10^6 AGS cells in 100 μL PBS. After tumor induction, nude mice were divided into three groups randomly. One group was for control and the other two were for the treatment of **M22** and bortezomib respectively. Compounds were administrated by intraperitoneal injection (60 mg kg^{-1} for **M22** and 1 mg kg^{-1} for bortezomib). Tumor size was monitored every second day using vernier calipers.

Acute Toxicity Assay. The morphogenesis and function of the primary organ systems of zebrafish embryos finished by 72-hours post-fertilization (hpf), thus zebrafish embryos at 72 hpf were selected for the acute toxicity assay. Adult zebrafish were maintained at 28.5 ˚C and pH of 7 ± 0.2 in 14:10 h light/dark photoperiod. They were fed with live brine shrimp once daily and dry food twice a day. Zebrafish embryos were generated by natural pairwise mating and raised at 28.5 ˚C in embryo water (0.2 g L^{-1} of Instant Ocean® Salt in distilled water). Zebrafish embryos were arrayed in 24-well plate (20 larvae per well) and incubated with 1 mL of embryo water per well containing various concentrations of **M22** or bortezomib at 28.5 ˚C for 24 h. DMSO (0.1%, v/v) solution served as the control. The observation of zebrafish was made directly in the 24-well plate using an inverted dissecting microscope. During the acute toxicity experiment, fish were not fed and dead fish were removed from the well. Records the number of dead zebrafish of each concentration solution within 24 h, and calculated the survival rate (%).

Table S1. Native docking results using six types of Docking function.

PDB ID	RMSD					
	Libdocksore	Ligscore-1	Plp-1	Jain	PMF	Estimated binding energy
1R4N	1.94	3.04	2.13	2.54	2.54	1.09

Table S2. Physical properties of positives and decoys.

Physical properties	Positives (n=27)	Decoys (n=1260)
MolW[a]	449.0±0.9	426.9±49.5
ALogP	0.2±0.9	1.1±1.5
Num_H_acceptors[b]	8.6±0.8	6.3±1.7
Num_H_Donors[c]	4.0±0.8	3.3±0.9
Num_Rings[d]	4.5±0.7	3.1±1.0

[a] Relative molecular mass.

[b] Number of hydrogen bond acceptors.

[c] Number of hydrogen bond donors.

[d] Number of rings.

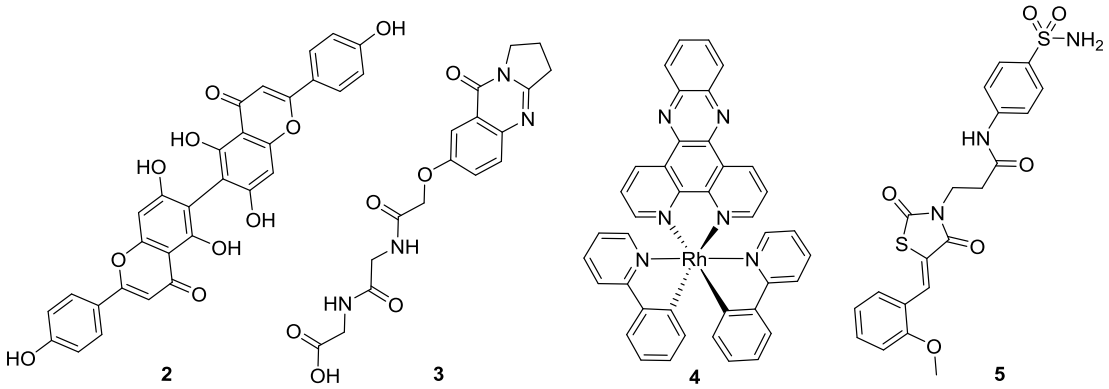

Figure S1. Structures of reported NAE inhibitor

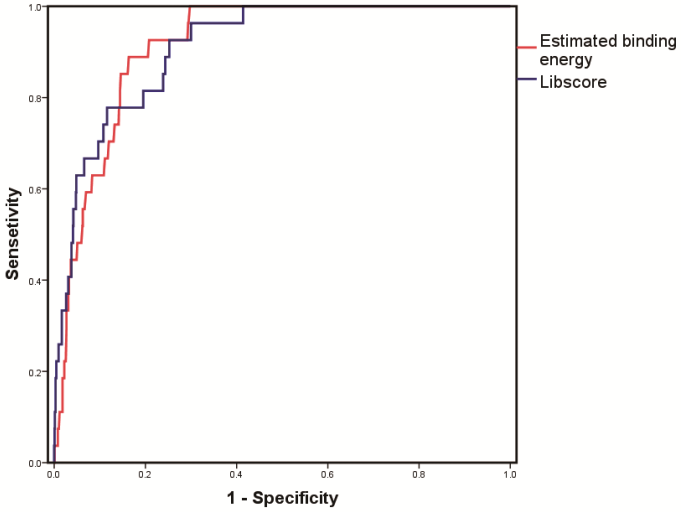

Figure S2. ROC curve for the two scoring functions. Shown in blue is the screening performance of estimated binding energy; shown in green is the screening performance of Libscore.

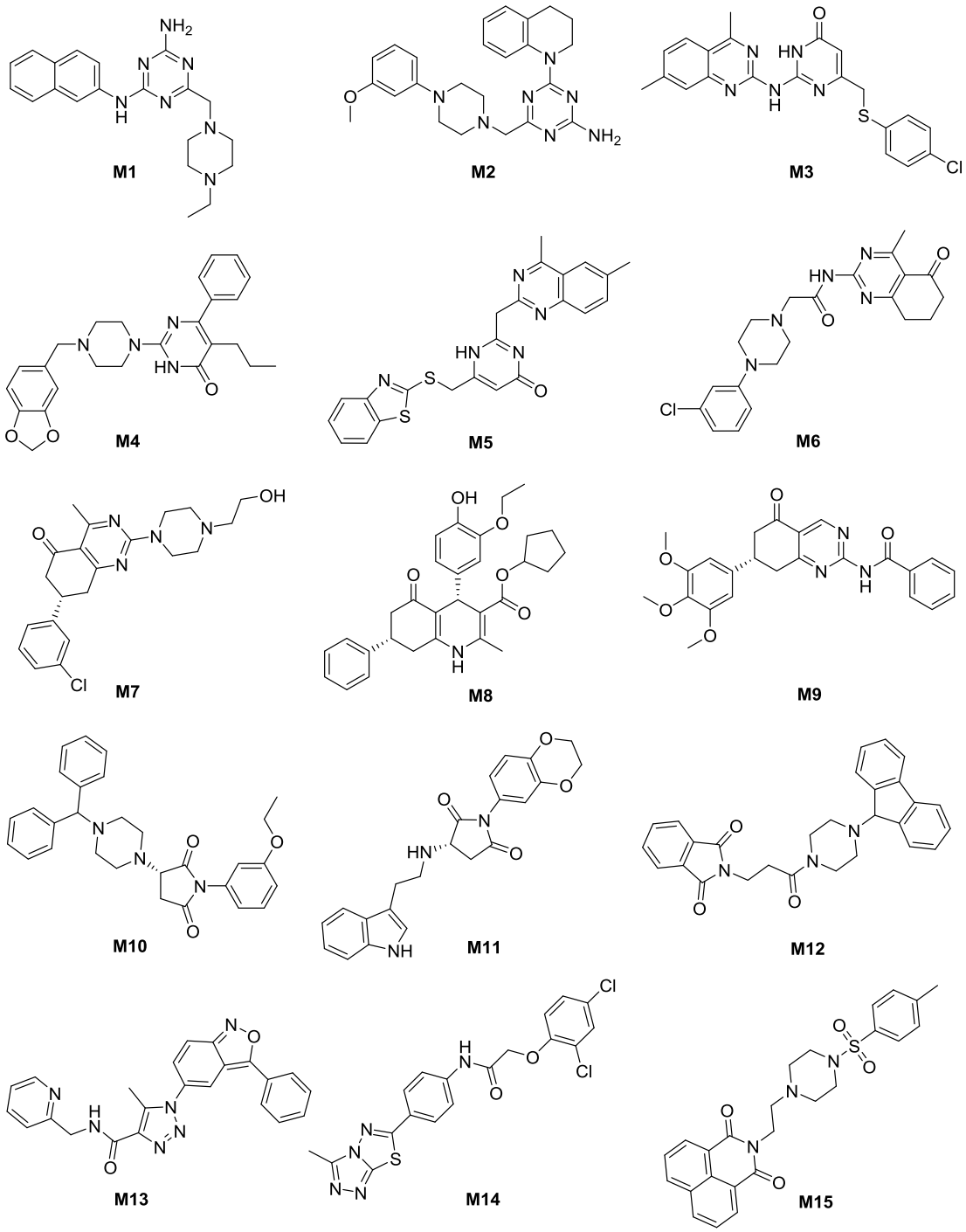

Figure S3. Structures of 23 which were selected out of 100 top-ranked compounds

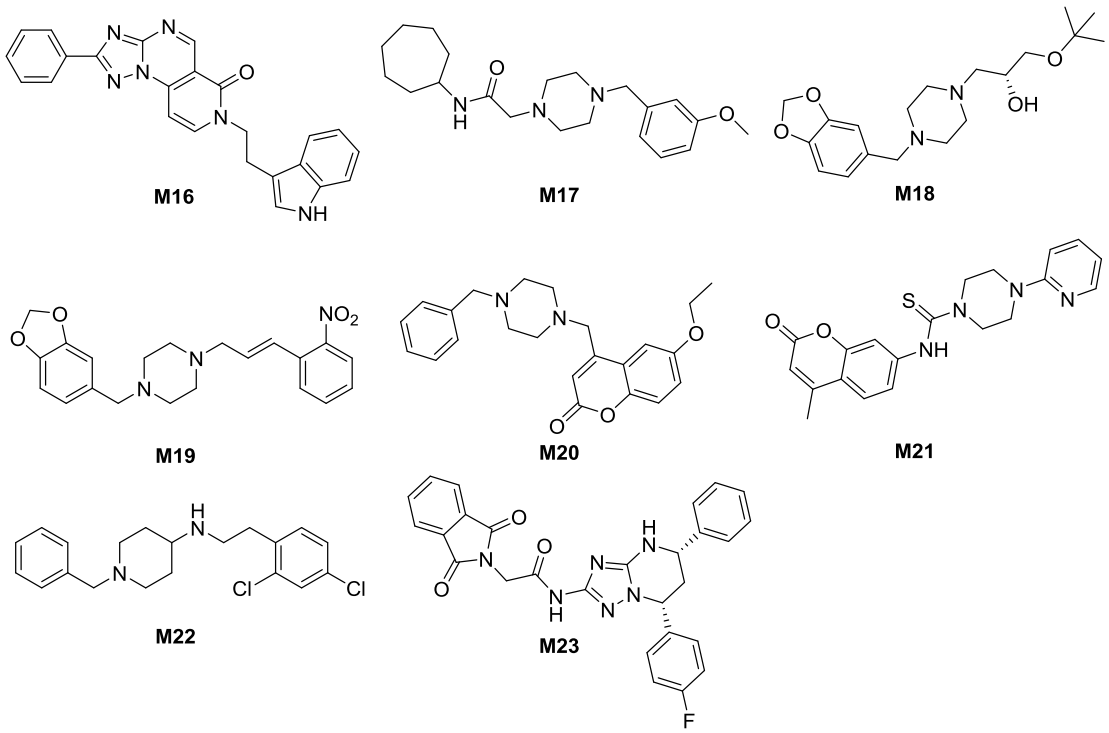

Figure S4. Structures of 23 which were selected out of 100 top-ranked compounds

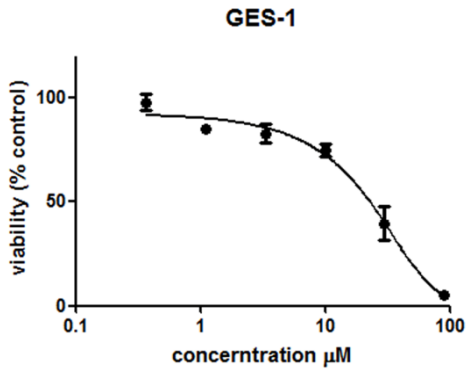

Figure S5. Survivorship curve of M22 against GES-1 (n=3).

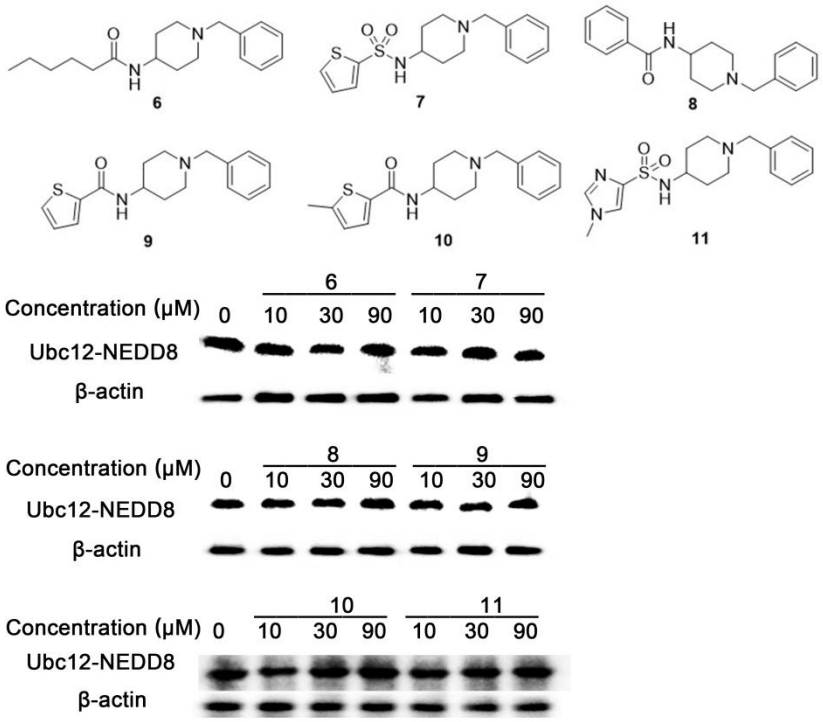

Figure S6. Structures of piperidin-4-amine based compounds which could not inhibit NAE activity and A549 proliferation.

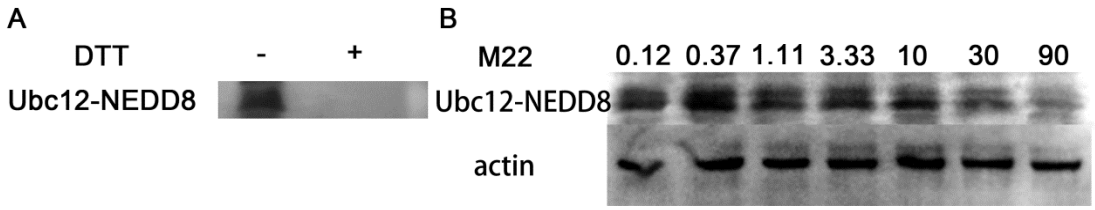

Figure S7. Ubc12-NEDD8 investigation by western blot. (A) treating A549 cells with **M22** for 24 h, extracted protein samples were analyzed by western blot with or without DTT; (B) **M22** could inhibit NAE activity in AGS cells

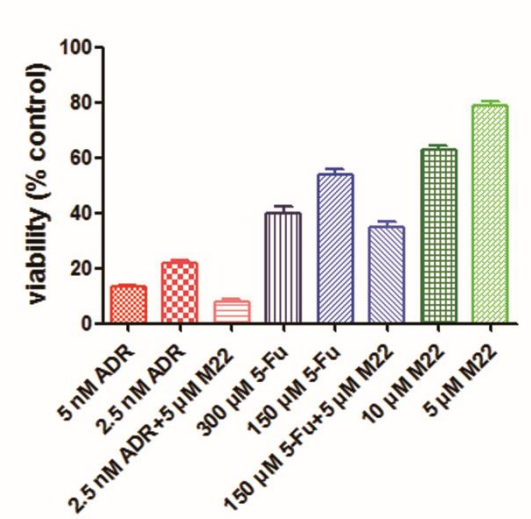

Figure S8. M22 had no synergistic effect with 5-fluorouracil or adriamycin