Electronic Supplementary Information (ESI)

Multivalency-Enhanced Enzyme Inhibitors with Biomolecule-Responsive Activity

Fan Ding,[a] Xinrui Li,[a] Xu Chen,[a] Yue Ma,[b] and Guoqing Pan[a]**

[a] Institute for Advanced Materials, School of Materials Science and Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu, 212013 China.

E-mail: panguoqing@ujs.edu.cn (Guoqing Pan).

[b] School of Chemistry and Chemical Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China. Email: yamma@ujs.edu.cn (Y.M.)

Materials and Instruments

Acrylamide (AAm, 99%, Aladdin), 3-methylacrylamide phenyl boric acid (BA, 99%, Aladdin), 4-aminobenzamidine dihydrochloride (97%, Aladdin), acryloyl chloride (97%, Aladdin), azodiisobutyronitrile (AIBN, 99%, Aladdin), 4-cyano-4-(thiobenzoyl) valeric acid (CTPA, 97%, J&K Scientific). Trypsin from bovine pancreas was purchased from Sigma Aldrich. All the other AR-grade solvents in this work were purchased from Shanghai Reagent General Factory and used as received. 0.25% Trypsin/EDTA solution, streptomycin and penicillin were purchased from Gibco BRL (USA). RPMI 1640 medium and FBS (fetal bovine serum) were purchased from HyClone (USA). FITC-phalloidin and 4'-6diamidino-2-phenylindole (DAPI) were purchased from Enzo Life Sciences, Inc. (USA). MCF-7 cells (human breast cancer cells) were derived from human breast tissue and obtained from the BeNa Culture Collection, China.

Nuclear magnetic resonance hydrogen spectroscopy (^{1}H NMR) was performed on a BRUKERAVANCEII (Bruker, Switzerland). Fourier transform infrared (FT-IR) spectroscopy was performed on a Nicolet IS50 (Thermo Fisher, USA). Dynamic lighting scattering (DLS) analysis was performed on a Zetasizer Nano ZS (Malvern Instruments). Absorbance and fluorescence intensity were obtained by microtiter plate spectrophotometer measurements (Burten Instruments Ltd., USA). Cells were cultured in a Thermo Scientific Forma Series II Water Jacketed CO2 Incubator (Shanghai, China). Fluorescence microscopy cell images were taken by a Ti-S inverted fluorescence microscope (Nikon Corporation, Japan).

Preparation of (4-acrylamidophenyl)-(amino)methaniminium chloride (AB)

(4-acrylamidophenyl)-(amino)methaniminium chloride (AB) was synthesized according to the method reported in the literature. [1] First, dissolve 34 g (0.25 mol) of sodium acetate trihydrate in 200 mL of pure water, then add 2 g (9.6 mmol) of 4-aminobenzamidine dihydrochloride, and stir to dissolve it. Then cool the solution to below 5 ℃ in an ice water bath, and slowly add 4 mL (49 mmol) of acryloyl chloride while stirring rapidly. After 1 hour of reaction, adjust the pH of the solution to 4.0 with hydrochloric acid, and precipitation can be observed. After filtration, the precipitate obtained is redissolved in 100 mL of water at 45 ℃. After dissolution, add hydrochloric acid again to adjust the pH to 1.0, and then transfer the solution to 4 ℃ for overnight crystallization. Finally, the crystals are collected by filtration and dried in an oven at 50 ℃ to obtain the final product.

Preparation of Try inhibitor

Trypsin inhibitor is prepared by polymerization of acrylamide (AAm), phenylboric acid (BA) and benzamidine (AB) through RAFT. The specific experimental steps are as follows: dissolve 355.4 mg (5 mmol) of AAm, 127.2 mg (0.625 mmol) of BA and 127.0 mg (0.625 mmol) of AB in a mixture of 7 mL of DMF and 3 mL of H_2O, and then add 8.7 mg (0.03125 mmol) of chain transfer agent CTPA and 2.6 mg (0.016 mmol) of initiator AIBN in turn. Dissolved and de-oxygenated with argon in an ice-water bath for 30 min and then transferred sealed and transferred to a constant temperature mixer. The set temperature is 65 ℃, the rotational speed is 500 r/min, and the reaction time is 48h. After the reaction is over, aerate to terminate the reaction, and then transfer the product to a 2000 D dialysis bag for dialysis for 7

days. After lyophilization, the product is obtained. The yield is calculated by weighing and the molecular weight is estimated. The synthesis of polymers with different BA and AB ratios is the same as the above method, but there are differences in the number of reactants (**Table S1**).

Test of binding property of enzyme inhibitor

The binding properties of enzyme inhibitors were tested by dynamic light scattering (DLS). Preparation of 0.5 μM Try, 200 μM ATP and 20 μM BA_mAB_n solution, and test their respective particle sizes at 25 ℃. Then mix Try with BA_mAB_n, BA_mAB_n with ATP, and test the particle size of the two mixed solutions. The change of particle size shows the binding property of the inhibitor.

The binding properties of enzyme inhibitors were tested by quartz crystal microbalance (QCM). Because the chain transfer agent CTPA has disulfide bond, BA_mAB_n can be connected to QCM chip by reducing the disulfide bond on BA_mAB_n to sulfhydryl group. The specific steps are as follows: load the chip with BA_mAB_n into the HD120-T8 QCM instrument, and then set the flow rate of the peristaltic pump to 50 μL/min. First, inject PBS solution, and then inject PBS solution containing Try (250 μg/mL) after the baseline is stable, after the baseline is stabilized again, a PBS solution containing ATP (250 μg/mL), observe the frequency change of the QCM sensor. The binding performance of the inhibitor is judged by the signal frequency change displayed by the QCM sensor.

Inhibition constant K_i test of enzyme inhibitor

The inhibition constant K_i of trypsin inhibitors was tested by double reciprocal plotting.

Because Try hydrolyzes n-α-benzoyl-DL-arginine-4-nitroaniline (BAPNA) to p-nitroaniline, which increases the absorbance at 405nm, BAPNA was chosen as the substrate for the test (**Figure S6**).

Firstly, 90 mg of BAPNA was weighed and dissolved in 20 mL of DMSO, and 4.5 mg/mL of BAPNA solution was configured. Then weigh 20 mg Try, dissolve it in 20 mL Tris-HCL, and configure 1 mg/mL trypsin solution. 4.5 mg/mL BAPNA solution of 50 μL, 75 μL, 100 μL, 250 μL and 1000 μL were added into the five centrifugation tubes respectively, and a certain amount of Tris-HCL buffer solution was added respectively to keep the solution volume to 2 mL. Then, 60 μL of Try solution (1 mg/mL) and 10μM of BA_mAB_n were added to the empty tube, buffer was added to make the solution volume 3mL, and the incubation was 1h. After incubation, take 500 μL of the mixed solution was added to five centrifuge tubes with substrates, mixed evenly, and transferred to a 37 ℃ water bath. After 5 minutes, 200 μL solution was taken out and added into the 96-well plate. The absorbance of the solution was measured at 405nm using an enzyme linked immunosorbent assay. The measured absorbance is the initial reaction rate of the system at different concentrations of substrate (0.5 mM, 0.75 mM, 1.0 mM, 2.5 mM, 10 mM) and inhibitor concentration (10μM). Then, the inhibition constant K_i can be obtained by plotting 1/v against 1/s.

Determination of absorbance and fluorescence intensity of enzyme inhibitor for Try catalytic activity

The absorbance of enzyme inhibitor to the catalytic activity of trypsin can be tested by BAPNA, because trypsin can hydrolyze BAPNA to p-nitroaniline, which increases the

absorbance at 405 nm. The specific steps were as follows: 0.5 μM of Try and 1 mM of BAPNA were added into 5 mL of Tris-HCL buffer, and then 0, 5, 10, 15, and 20 μM of BA_mAB_n were added, respectively. The absorbance (405 nm) change within 30 min was detected at 25℃, and the detection was performed every 5 min.

Use Rhodamine B to label trypsin, and test the fluorescence intensity of trypsin at the incident wavelength of 530 nm and the emission range of 560 nm-700 nm. The specific steps are as follows: add 0.5 μM of Try and different concentrations of BA_mAB_n to 5mL of Tris-HCL buffer, incubate for 1 hour, then add diluted Rhodamine B solution, and incubate for 30 minutes in a dark environment. Then measure the fluorescence intensity of the solution, set the incident wavelength at 530 nm and the emission range at 560 nm-700 nm, and observe the fluorescence intensity change of Try after adding inhibitor.

Cell culture

Human breast cancer cells (MCF-7) were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The temperature of the cell incubator was set at 37 ℃, 5% CO2.

Cell experiment of Try digestion of ECM

In the cell adhesion experiment, 0.25% trypsin/EDTA digestion solution was added to the cultured MCF-7 cells. After the completion of cell digestion, the cell suspension was put into a centrifuge (1000 r, 5 min). After the completion of centrifugation, the upper liquid was removed, and the culture medium was added to make the cell concentration 10000 cells/mL.

1mL of the configured cell suspension was added to 24-well plates, incubated for 12 h, then the culture medium was removed and 1mL of Tris-HCL buffer solution containing 10 μM Try was added. The cell adhesion status was recorded at 0,20,40, and 60 min under an inverted microscope at 25℃. The same method was used to test the changes of cell adhesion status after adding inhibitor and ATP.

To clearly observe the changes of ECM digestion and cell detachment, the cells were stained with phalloidin and DAPI and their fluorescence images were recorded. 1mL of the configured cell suspension was added into 24-well plates, incubated for 12h, the medium was removed, 1mL of Tris-HCL buffer solution containing 10μM Try was added, and the well plates were transferred to a cell incubator at 37℃ for incubation, respectively, removed after 0,15,30 min, and the buffer solution was removed. The cells were stained with phalloidin and DAPI. Specific experimental steps were as follows: 1mL of fixative containing 4% paraformaldehyde (PFA) and 1mM $CaCl_2$ were added into the pore plate and fixed for 30min. After fixation, the fixative was removed and cells were washed with PBS. Then 500μL PBS solution containing 0.4% Triton-X and 1mM $CaCl_2$ was added and incubated for 5 min to improve the permeability of cell membrane. Subsequently, the cells were washed with PBS and stained with phalloidin (phalloidin can stain the F-actin stress fibers on the cell membrane). After 3h, the fluorescent dye was removed and washed with PBS, and then anti-fluorescence quenching tablet containing DAPI was added, and the staining was finished after 30 min. The morphological changes of the cells were observed by fluorescence microscopy. The same method was used to test the morphological changes of cells after the addition of inhibitor and ATP.

Quantitative test of cell damage by Try

In order to quantitatively test the damage of Try to cells, 100 μL cultured MCF-7 was added to a 96 well plate (10000 cells/mL) and incubated in a 37 ℃ cell incubator for 24 hours. After 24 hours, the culture medium was removed and different drug groups were added. After 1 hour, remove the drug group and add 100 μL medium, and finally add CCK8 (10 μL). After incubation for 4 h, the absorbance at 450 nm was measured. Six parallel replicates were read for each set of results, and cell survival was calculated.

The cell viability was calculated as:

$$Cell\ viability(\%) = \frac{OD(sample) - OD(black)}{OD(control) - OD(black)} \times 100\%$$

Biosafety Experiments

Normal cultured MCF-7 cells (1×10^3 cells/well, 100 μL) were inoculated into 96-well plates and incubated in medium at 37 ℃ for 1, 3, and 5 days, and then 10 μL of CCK-8 solution was added. The cells were incubated at 37 ℃ for 3 h. The absorbance was measured at 450 nm using a microplate spectrophotometer, and the cell viability was calculated.

References

1. S. Nestora, F. Merlier, S. Beyazit, E. Prost, L. Duma, B. Baril, A. Greaves, K. Haupt and B. Tse Sum Bui, *Angewandte Chemie*, 2016, **128**, 6360-6364.

Tables

Table S1. Calculation results of yield and molecular weight of different proportion inhibitors.

EIs	AAm (mmol)	BA（mmol）	AB（mmol）	CTPA（mmol）	AIBN（mmol）	Yield (%)	Molecular weight (Mn)
$BA_{75}AB_{25}$	5	0.93	0.31	0.03125	0.016	78.3	15500
$BA_{66}AB_{33}$	5	0.83	0.42	0.03125	0.016	77.7	15400
$BA_{60}AB_{40}$	5	0.75	0.5	0.03125	0.016	73.6	14600
$BA_{50}AB_{50}$	5	0.625	0.625	0.03125	0.016	72.9	14500
$BA_{40}AB_{60}$	5	0.5	0.75	0.03125	0.016	76.6	15200
$BA_{33}AB_{66}$	5	0.42	0.83	0.03125	0.016	76.1	15100
$BA_{25}AB_{75}$	5	0.31	0.93	0.03125	0.016	64.8	12900

Theoretical molecular weights were calculated using the expression Mn(calc) = ([monomer]/[CTPA]) × monomer conversion (MC) × MW of the monomer + MW of RAFT agent.

Table S2. Inhibition constants of different molecular weight inhibitors.

Polymer	Monomer/Chain transfer agent (molar ratio)	Yield (%)	Molecular weight (Mn)	$K_{i(10\mu M)}$
1	50/1	37.3	2100	40.3±2.1
2	100/1	58.2	5900	21.9±0.5
3	200/1	76.6	15200	8.7±1.3
4	300/1	73.1	21600	10.1±0.9
5	400/1	79.2	31200	6.6±1.1

Theoretical molecular weights were calculated using the expression Mn(calc) = ([monomer]/[CTPA]) × monomer conversion (MC) × MW of the monomer + MW of RAFT agent.

Figures

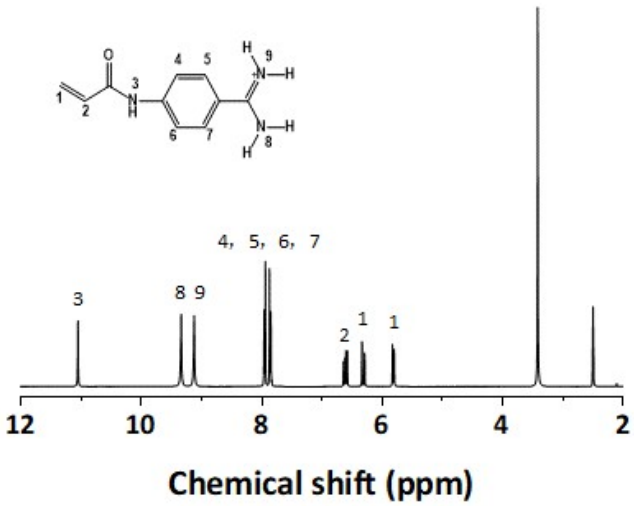

Figure S1. ^{1}H NMR spectrum of (4-acrylamidophenyl)-(amino)-methaniminium chloride.

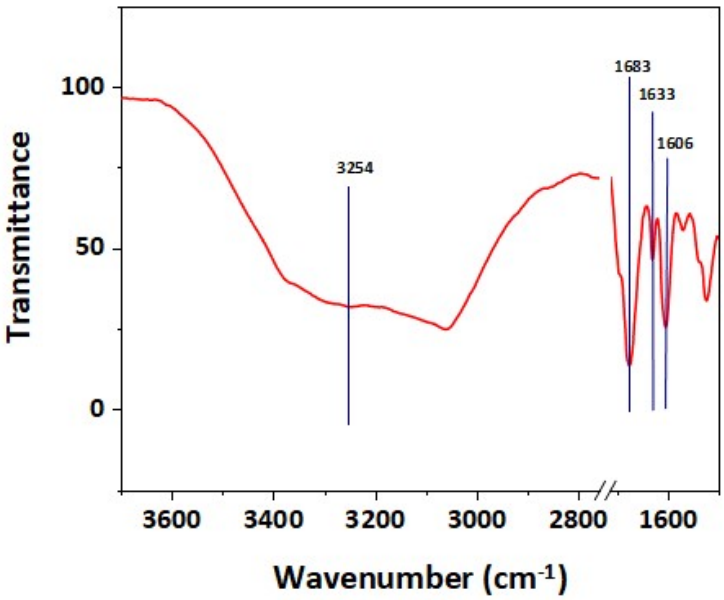

Figure S2. FTIR Spectra of (4-acrylamidophenyl)-(amino)-methaniminium chloride.

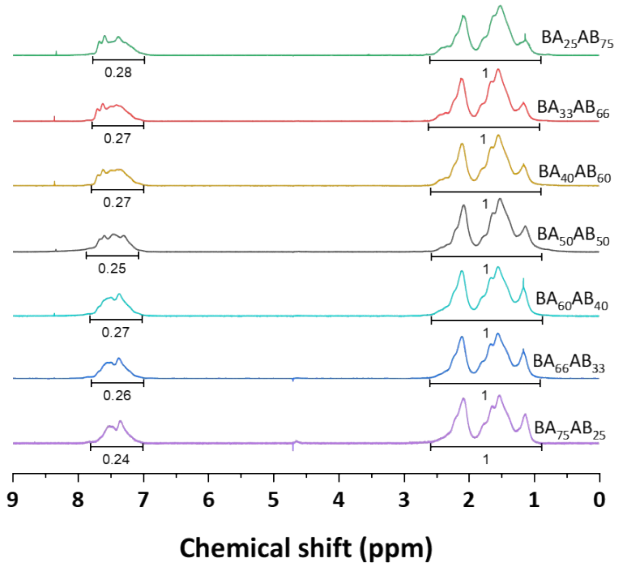

Figure S3. ^{1}H NMR spectrums of BA_mAB_n.

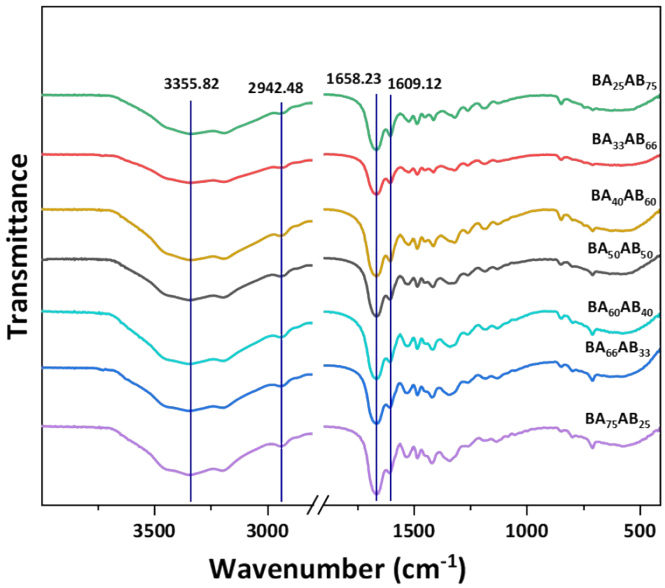

Figure S4. FTIR spectrums of BA_mAB_n.

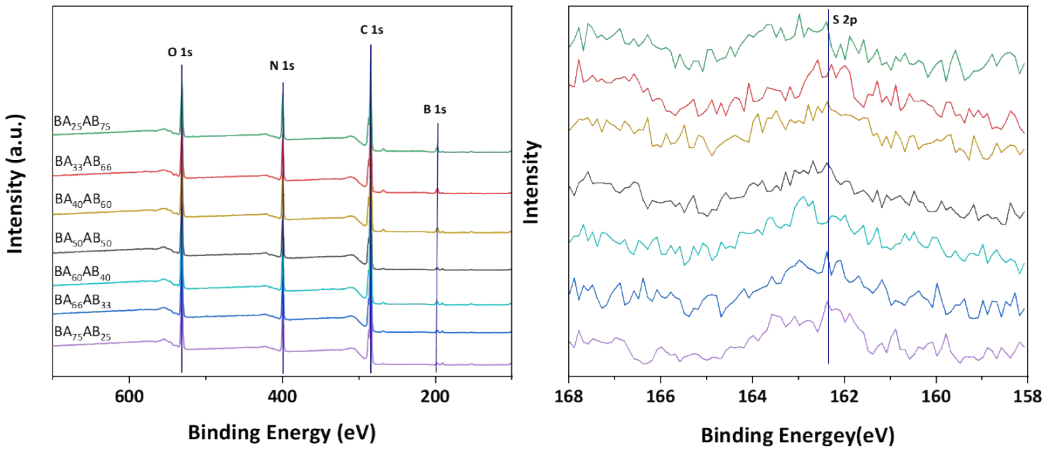

Figure S5. XPS spectrum of BA_mAB_n.

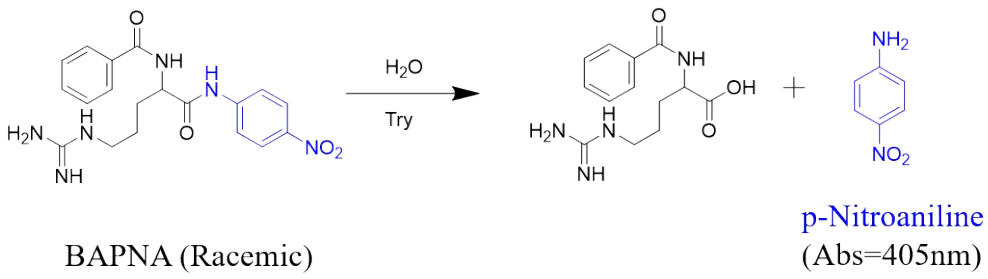

Figure S6. Schematic diagram of BAPNA hydrolysis to p-nitroaniline catalyzed by trypsin.

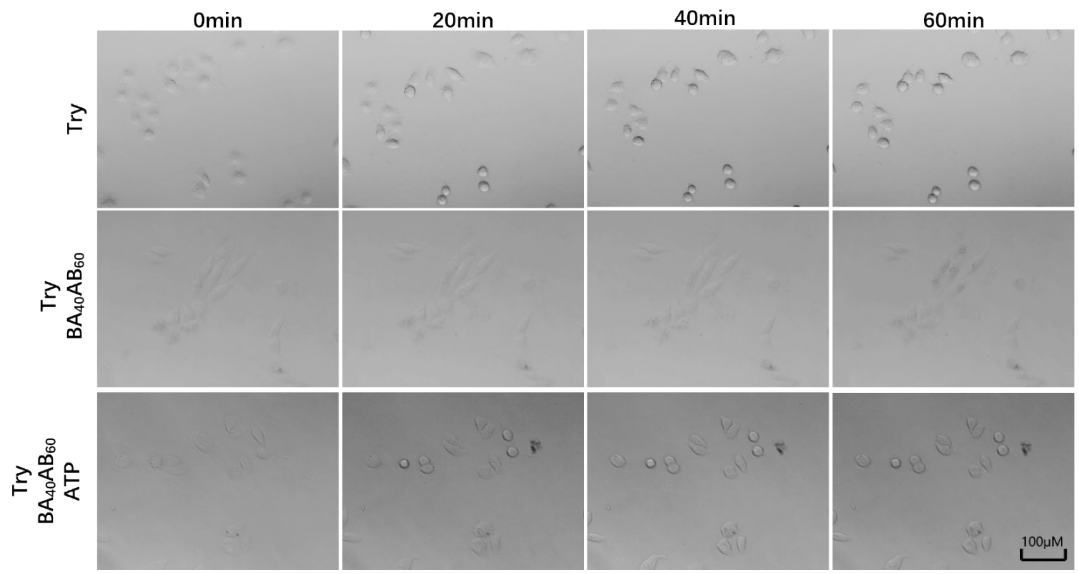

Figure S7. Bright field images of progenitor breast cancer cells with Try, $BA_{40}AB_{60}$ and ATP.

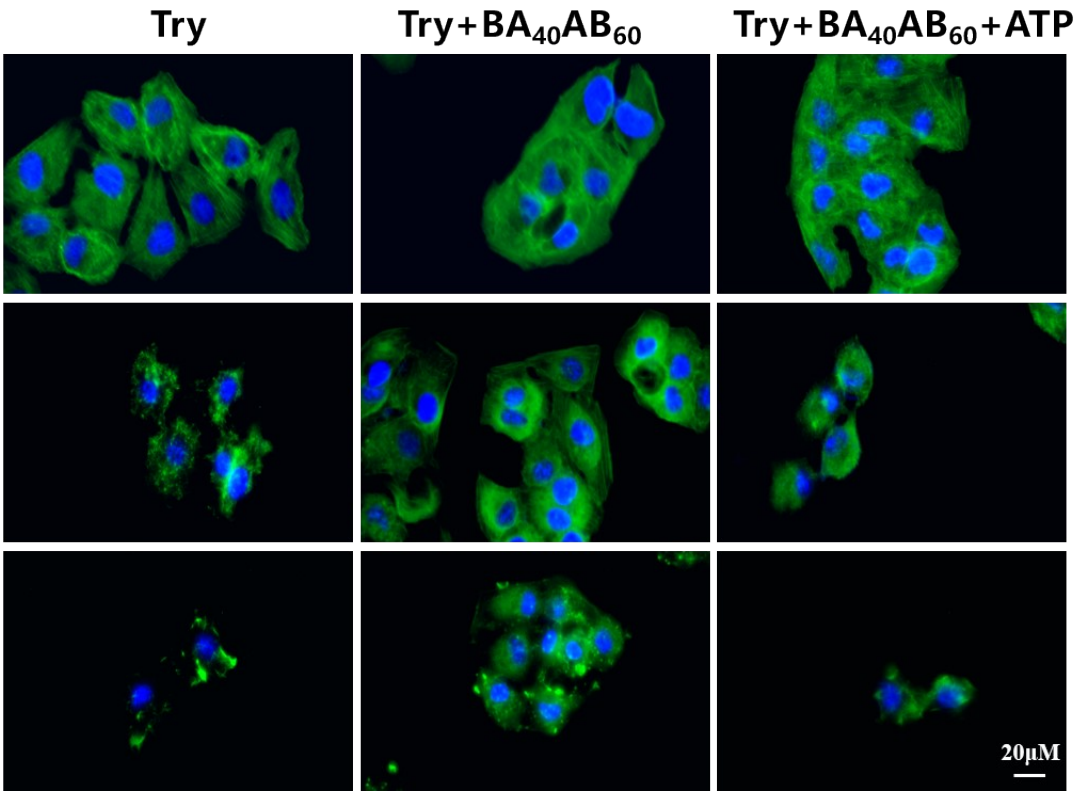

Figure S8. Fluorescent images of progenitor breast cancer cells with Try, $BA_{40}AB_{60}$ and ATP.

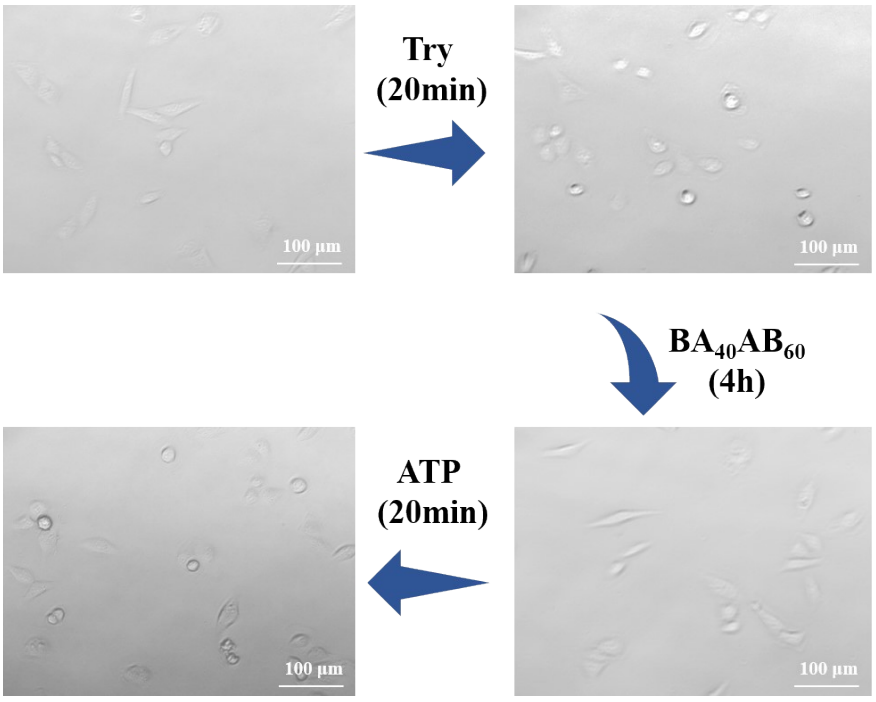

Figure S9. Morphological changes in cells after addition of Try, $BA_{40}AB_{60}$ and ATP at different times. (Try 10μM, $BA_{40}AB_{60}$ 20μM, ATP 200μM)

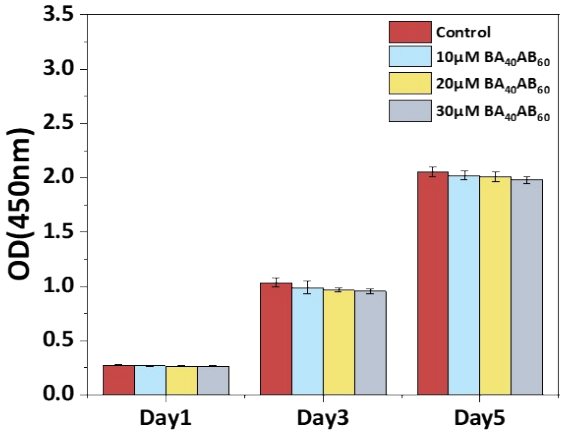

Figure S10. Proliferation profiles of cells after cultured with different drugs for 1, 3, and 5 days.