Materials and methods

Materials

Copper chloride dihydrate ($CuCl_2·2H_2O$), Sodium hydroxide (NaOH), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), and sodium phosphate dibasic (Na_2HPO_4) and 20,70-dichlorodihydrofluorescein diacetate (H_2-DCFDA) were obtained from Macklin (Shanghai, China). Aprepitant was purchased from Energy Chemical (Shanghai, China). HS-PEG-fluorescein isothiocyanate (HS-PEG-FITC; viscosity average molecular weight 5000 Da) was obtained from Xinqiao Biotechnology Co., Ltd. (Hangzhou, China). Radio immunoprecipitation assay (RIPA) lysis buffer, phenylmethanesulfonylfluoride (PMSF) and enhanced chemiluminescence (ECL) assay were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China).

Characterization

The morphology of Cu-aprepitant NPs was characterized by scanning electron microscopy (SEM, JSM-5610LV, JEOL, Tokyo, Japan) and transmission electron microscopy (TEM, JEM-1400Flash, JEOL, Tokyo, Japan). Ultraviolet and visible (UV-vis) absorbance was recorded on a UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan). Zeta potential, size distribution and polydispersity index (PDI) of Cu-aprepitant NPs in water ((25±0.1) °C) were determined by using dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Panalytical, Malvern, UK) The weight (%) of Cu in Cu-aprepitant NPs was determined by an inductively coupled plasma optical emission spectrometer (ICP-OES, 5110, Agilent, Palo Alto, USA). The characteristic basal reflection patterns of Cu-aprepitant NPs and Aprepitant were evaluated using powder X-ray diffraction (PXRD, X'Pert3 Powder, Panalytical, Alemlo, The Netherlands) analysis in the range of 10-80 (Cu Ka radiation, 40 kV, 40 mA). The in-depth chemical composition and valence state of Cu-aprepitant NPs were analyzed by X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo Fisher Scientific, Waltham, USA). The functional group confirmations were made by recording the FTIR spectra on a Nicolet iS20 spectrometer (Thermo Fisher Scientific) using a dried KBr pellet method.

Preparation of Cu-aprepitant NPs

A total of 2.5 mg of $CuCl_2·2H_2O$ was dissolved in 6.5 mL of deionized water, and 0.2 mL of NaOH (0.1 mol/L) was added to the solution under stirring. Then 1 mL of aprepitant ethanol solution (5 mg/mL) was dropwise added to the above solution, and the mixture was kept stirring for 1 h. The Cu-aprepitant NPs were collected by centrifuging at 15000 r/min for 15 min, and the supernatant was collected to measure the unloaded aprepitant by high-performance liquid chromatography (HPLC, Agilent 1260 Infinity II, Santa Clara, USA). The unloaded aprepitant was measured using an Eclipse XDB-C18 column (3.5 μm, 4.6 mm×150 mm) with a mobile phase composed of acetonitrile-water (47:53, v/v%).

Drug release

The release of aprepitant from as-prepared nanocomposites was investigated. In detail, dialysis bag of the 10 kDa molecular weight was filled with Cu-aprepitant NPs suspension, and then was immersed in 50 mL PBS buffer (pH=7.4, 6.8, or 5.7) with 2% of TWEEN-80 (w/v) to shake at 150 r/min in the dark. 2 mL PBS buffer was taken out at predetermined times and replaced with an equal volume of fresh PBS buffer. The concentration of aprepitant in the buffer solution was determined by an HPLC as the same method as in the "Preparation of Cu-aprepitant NPs" section.

Cell culture

Human breast cancer cell line (SKBR3) was purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were cultivated in the 10% FBS-containing DMEM in a humidified incubator maintained at 37 °C and 5% CO_2.

Cellular uptake

Firstly, Cu-aprepitant NPs were fluorescence-marked by mixing Cu-aprepitant NPs and HS-PEG-FITC (w/w=20:1) in aqueous solution for 12-h stirring. As-obtained FITC-Cu-aprepitant NPs were collected and washed with ethanol and deionized water for two times. Then, SKBR3 cells were cultured and seeded in a laser confocal petri dish. To investigate the cellular uptake of Cu-aprepitant NPs, cells were co-incubated with FITC-Cu-aprepitant NPs at a concentration of 10 μg/mL in a medium for 3 h. The nucleus and lysosome were stained with Hoechst 33342 (10 μg/mL) and Lysotracker Red (100 nmol/L). And cells were observed by a confocal laser scanning microscope (CLSM).

In vitro cytotoxicity and effects of cell death inhibitors

The viability of SKBR3 and MDA-MB-231 cells was assessed by using the CCK-8 assay after co-culturing them with Cu-aprepitant NPs. Cells were harvested from the culture plates and seeded (8000 cells per well) in a 96-well plate. After incubation overnight, the media in the wells were replaced with media containing Cu-aprepitant NPs at various concentrations, along with a negative control group. After 24 h of incubation, CCK-8 working solution was added, and the incubation was continued for an additional 2 h. Finally, the absorbance was measured at 490 nm in the microplate reader, and the percentage viability of cells in various treatments was calculated.

To investigate the cell death pathway induced by Cu-aprepitant NPs, SKBR3 cells were harvested from the culture plates and seeded (8000 cells per well) in a 96-well plate. After incubation overnight, the media in the wells were replaced with media containing UK5099 (5, 10, 20 μmol/L), Fer-1 (5, 10, 20 μmol/L), Nec-1 (12.5, 25, 50 μmol/L), Z-VAD (10, 20, 40 μmol/L), antimycin A (5, 10, 20 μmol/L), and bafilomycin A1 (1, 2, 4 nmol/L), respectively. After 1 h of pre-treatment, Cu-aprepitant NPs were subsequently added to the medium with a final concentration of 25 μg/mL. After another 24 h of incubation, CCK-8 working solution was added, and the incubation was continued for an additional 2 h. Finally, the absorbance was measured at 490 nm in the

microplate reader, and the percentage viability and relative viability of cells in various treatments were calculated.

In vitro apoptosis detection

The apoptosis detection was performed with the Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China). SKBR3 cells were cultured in a 6-well plate. After 24 h incubation, Cu-aprepitant NPs were added into wells (20 μg/mL per well). Afterwards, SKBR3 cells were collected after 24 h incubation and washed with PBS (4 °C). And cells were further centrifuged (2400 r/min, 10 min) and resuspended in 100 μL of binding buffer composed of 5 μL of Annexin V-FITC and 5 μL of PI for 10 min in the dark. Following, cells were centrifuged and resuspended in 100 μL of binding buffer to be analyzed by flow cytometry.

Western blot

SKBR3 cells were treated with Cu-aprepitant NPs at different concentrations for 24 h and then lysed with RIPA lysis buffer containing 1% PMSF. The protein concentration was quantified using BCA. Equal amounts of cell lysates were denatured and separated by 8%–15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After incubating in 5% skim milk powder for 2 h, the membranes were incubated overnight at 4°C with primary antibodies. Then the PVDF membranes were further incubated with the secondary antibody at room temperature for 2 h. The immunoblots were visualized by an ECL chemiluminescence substrate kit from SageBrightness (Beijing, China).

Statistical analyses

All of the data were presented as mean±standard deviation (SD). The statistical analysis was determined using one-way ANOVA analysis of variance via GraphPad Prism 9 software ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$).

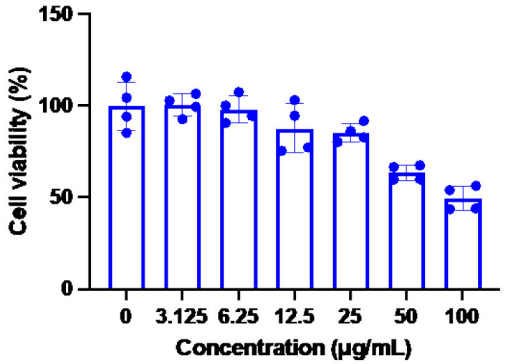

Fig. S1 Cell viability of MB-MDA-231 cells cultured with a gradient concentration of Cu-aprepitant NPs.

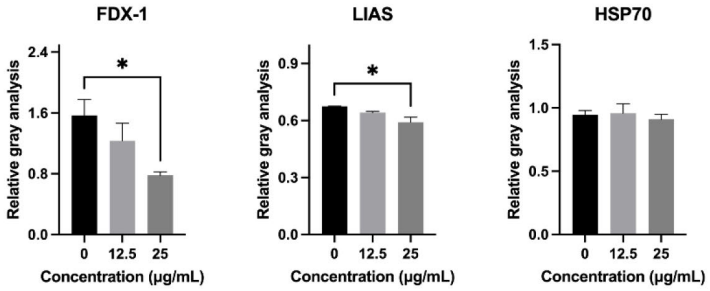

Fig. S2 Relative gray analysis of FDX-1, LIAS, and HSP70 expression based on Fig. 5e.