Eupaformosanin Induces Apoptosis and Ferroptosis Through Ubiquitination of Mutant P53 in Triple-negative Breast Cancer

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Abstract

Purpose Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype with no targeted treatment. The objective of this study was to find new therapeutic agents and appropriate therapeutic methods and overcome the limitations of TNBC treatment.

Methods P53 mutant TNBC cells were treated with eupaformosanin (Eup), a natural compound isolated from *Eupatorium cannabinum* Linn. Apoptosis and ferroptosis were detected *in vitro* and *in vivo*. RNA knockdown was used to investigate the role of mutant p53 in ferroptosis and apoptosis of Eup-induced cell death. A xenograft TNBC animal model was established to assess the anti-TNBC activity of Eup.

Results MTT assay suggested that Eup strongly inhibited the viability of TNBC cells. Meanwhile, mitochondrial apoptosis contributed to the cell death in TNBC cells while mitochondrial membrane potential (MMP; $\Delta\psi_m$) was disrupted, mitochondrial ROS (mt ROS) was enhanced, and related level of proteins was regulated. Apoptosis inhibitor Z-VAD rescued Eup-induced cell death. Afterwards, ferroptosis-induced cell death was demonstrated in TNBC cells, accompanied by lipid ROS accumulation, GSH depletion and iron increase. These events were blocked by ferroptosis inhibitors Fer-1 and DFO, indicating that ferroptosis facilitated Eup-induced cell death. Furthermore, Eup regulated mutant p53 ubiquitination, and mutant p53 signaling participated in Eup-induced apoptosis and ferroptosis, which were rescued when mutant p53 was silent in TNBC cells. Moreover, Eup exerted an anti-TNBC effect by inducing apoptosis and ferroptosis *in vivo*.

Conclusion The natural compound Eup is a potential TNBC therapeutic agent that induces apoptosis and ferroptosis through ubiquitination of mutant p53.

Introduction

Breast cancer, the most common cancer, is one of the causes of female cancer death(1). As a malignant subtype of breast cancer, TNBC lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) (2), so TNBC patients derive no benefit from endocrine therapy(3, 4). Currently, only a few patients with TNBC have demonstrated clinical improvement in response to chemotherapy (5). Therefore, it has become a hot topic to find new therapeutic targets and appropriate therapeutic approaches in TNBC treatment.

The tumor suppressor gene p53 is a transcription factor that can control senescence, cell cycle arrest and apoptosis by regulating its downstream target genes. However, as the tumor suppressor, p53 is mutant in up to 80% of TNBC(6, 7). Because of the high incidence, mutant p53 has been a biomarker and a new therapeutic target for TNBC(8).

Ferroptosis is a novel form of cell death and is distinct from apoptosis and necrosis in gene, biochemistry and morphology. It is characterized by the accumulation of lipid reactive oxygen species (ROS) and a high level of iron(9). Emerging evidences reveal that ferroptosis is implicated in various human cancers

with a tumor suppressor function. Thus, ferroptosis inducers could inhibit tumor growth and facilitate the treatment of resistance in chemotherapy(10). Also, TNBC is susceptible to ferroptosis. It has been reported that erastin is a ferroptosis inducer which selectively targets TNBC cells(11, 12). Also, targeting the MUC1-C/xCT signaling pathway and ubiquitination of GPX4 are related to trigger ferroptosis in TNBC cells(13, 14). Consequently, inducing ferroptosis has become a novel and effective treatment strategy for TNBC.

Recent studies strongly elucidate that p53 also adjusts ferroptosis under the transcriptional or posttranslational mechanism(15). Like the complex mechanism of wild-type (WT) p53 regulating ferroptosis, mutant p53 also has various functions in regulating ferroptosis. P53^{3KR} is an acetylation-defective mutant in which 3 lysine residues (in positions 117, 161 and 162) have been replaced by arginine residues. It is positive regulator of ferroptosis by reduction of expression of SLC7A11 (a specific light-chain subunit of the cystine/glutamate antiporter). By comparison, p53^{4KR98} (an acetylation-defective mutant in which an additional lysine in position 98 has been replaced) cannot restrict SLC7A11 expression(16).

Sesquiterpene lactones (SLs) have caused considerable attention due to the anti-cancer, anti-inflammatory, anti-malaria and other biological activities (17, 18). Recently, it has been demonstrated that SLs induce apoptosis and ferroptosis via ubiquitination of GPX4 in TNBC(14). SLs Eupaformosanin (Eup) is a natural product isolated from *Eupatorium cannabinum* Linn. Over the past few decades, it has been confirmed that SLs from *Eupatorium cannabinum* Linn. has diverse bioactivities, for example, SLs eupatoriopicrin could inhibit the proliferation of lung cancer, and its anti-inflammatory activity was also confirmed with suppression of a series of inflammatory factors(19–21). As a natural compound similar to eupatoriopicrin, Eup has attracted a little attention, and there are reported that Eup could restrain Ehrlich ascites cell metabolism through inhibiting deoxyribonucleic acid synthesis(22). Nevertheless, the role of Eup in TNBC remains unexplored.

In this research, we illustrated that Eup decreased cell viability and led to G2/M-phase arrest in TNBC cells (MDA-MB-231 and MDA-MB-468) for the first time. Also, Eup induced apoptosis via the mitochondria pathway. Then, ferroptosis-induced cell death was demonstrated, accompanied by lipid ROS accumulation, GSH depletion and iron increase in these TNBC cells. Interestingly, the apoptosis and ferroptosis inhibitors both rescued Eup-induced cell death. Meanwhile, we showed that Eup reduced mutant p53 protein level by inducing mutant p53 ubiquitination, importantly, mutant p53 signaling was an active participator in Eup-induced apoptosis and ferroptosis. Above all, our study shows that the natural product Eup exhibits its anti-TNBC effects by inducing apoptosis and ferroptosis through mutant p53 ubiquitination.

Materials And Methods

Chemicals and reagents

Eup was kindly provided by Dr. Bo Yang (Zhejiang Chinese Medical University, Hangzhou, China). It was analyzed by NMR and MS. The NMR spectrum of Eup can be seen in supplement materials (Fig. S1). For *in vitro* research, Eup was dissolved in DMSO at the 50 mM stock solution. For *in vivo* research, Eup was dissolved in ethanol /Cremophor EL/ saline (5: 5: 90). Cremophor EL, MTT and DAPI were purchased from Sigma-Aldrich. The PI/RNase staining cell cycle kit and Annexin V-FITC/7AAD apoptosis kit were purchased from BD Biosciences (San Diego). BODIPY C11 and phen green SK (PGSK) were obtained from Thermo Fisher Scientific. Z-VAD-FMK (HY-16658B), Deferoxamine (#HY-B0988), ferrostain-1 (#HY-100579) and glutathione (GSH) (#HY-D0187/CS-7948) were purchased from MCE. Antibodies against Bcl2 (#2870, CST), Bad (#1541, CST), caspase3 (#9662, CST), cleaved caspase 3 (#9664, CST), FTH1 (#4393, CST), p53 (#9282, CST), β -tubulin (#2128, CST), β -actin (#4970, CST), GAPDH (#2118, CST) and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly). GPX4 (ab125066, Abcam) was purchased from Abcam (Cambridge, MA, USA).

Extraction, isolation and structural identification of Eup

2 Kg *Eupatorium cannabinum* Linn. coarse powder was soaked in 20-fold 95% EtOH for 2 days, then extracted at room temperature via percolation. The crude extract was added into warm water and suspended to 1.5 L, and isolated with EtOAc (3 $\times$ 1.5 L). The EtOAc extract was evaporated. The ethyl acetate-soluble fraction (EEECL) was then subjected to silica gel column chromatography, eluting with a gradient solvent system of PE-EtOAc (20:1 - 0:1 v/v) to obtain 11 fractions (Fr.1~11). Fr. 9 (1.5 g of 19.33 g) was separated by MPLC using MeOH/H₂O as the mobile phase (45, 50 and 55%, each 600 mL), flow rate 50 mL/min, to obtained Eup (28.7 mg). The known sesquiterpenoids Eup was identified mainly by comparing NMR spectral data with literature values(23). The structure data can be seen in Fig. 1 a.

Cell culture

The TNBC cells (MDA-MB-231 and MDA-MB-468) cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. DMEM/F-12 medium, fetal bovine serum (FBS) and trypsin EDTA were purchased from Gibco. Cell lines were maintained in DMEM/F12 medium with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO₂ and 95% air atmosphere.

In vitro cell viability assay

The cell inhibition rate was evaluated using MTT assay.

In vitro GSH assay

The GSH/GSSG ratios were detected by the GSH/GSSG-Glo Assay kit (Beyotime, China) according to the manufacturer's suggestion.

Measurement of lipid ROS

The BODIPY C11 (Invitrogen) probe was used to determine lipid ROS according to the manufacturer's suggestion.

Flow cytometric analysis of cell cycle arrest and apoptosis.

For cell cycle analysis, PI/RNase was used according to the manufacturer's suggestion. Concerning apoptosis, cells were stained with Annexin-V-FITC/7AAD.

Iron assay

Intracellular chelate iron was detected by the PGSK (Thermo Fisher Scientific), and its fluorescence is quenched by iron.

Mutant p53 knockdown experiments

Short hairpin RNAs (shRNA) were used for packaging into lentivirus (LV). Cells were seeded in a six-well plate and transfected with LV-shRNA targeting mutant p53 (Genechem, China). After transfection, proteins were isolated and analyzed by western blotting.

RT-PCR

RNA was obtained by TRIZOL (Invitrogen). Then it was reverse transcribed with PrimeScript™ RT reagent Kit (Takara Bio, Inc.). Afterward, qPCR was performed with SYBR-Green (Bio-Rad Laboratories, Inc.) by the CFX96 Real-Time system (Bio-Rad Laboratories, Inc.). The relative expression levels of mRNA were calculated by $2^{-\Delta\Delta C_q}$ method. The primer sequences are exhibited as follows:

The primer sequences are shown as follows:

Human SAT1 forward: 5'-ACCCGTGGATTGGCAAGTTAT-3' and reverse 5'-TGCAACCTGGCTTAGATTCTTC-3';

Human GAPDH forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse 5'-GGCTGTTGTCATACTTCTCATGG - 3';

Western Blotting

Cell were lysed in RIPA lysis buffer. Then, equal quantity of proteins was separated on SDS-PAGE gels and transferred onto PVDF membranes (Millipore). The membranes were blocked in 5% skimmed milk, and incubated with primary antibodies at 4 °C overnight and probed with appropriate secondary antibodies. Chemiluminescent detection was performed by ECL (Bio-Rad, USA). The primary antibodies used in the study were as follows: anti-Bcl2 (#2870, CST), anti-Bad (#1541, CST), anti-caspase3 (#9662, CST), anti-cleaved caspase 3 (#9664, CST), anti-FTH1 (#4393, CST), anti-GPX4 (ab125066, Abcam), anti-p53 (#2527, CST), anti-β-tubulin (#2128, CST), anti-β-actin (#4970, CST), anti-GAPDH (#2118, CST).

Evaluation of mitochondrial membrane potential ($\Delta\psi$ m)

JC-1 (Beyotime, China) was used to determine $\Delta\psi$ m following the manufacturer's suggestion.

In vivo tumor model

The experimental procedures of the animal studies were permitted by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University. BALB/c nu/nu female mice (4-week-old; n=6) were purchased from Shanghai Experimental Animal Center (Shanghai, China). MDA-MB-231 cells (2.5×10^6) were injected subcutaneously into the flank of mice. While tumor volume reaching 50 mm³, the mice were randomly divided into vehicle control (n=6) and Eup group (15 mg/kg, n=6). Drugs were administered by intraperitoneal injection every two days. The animal health and behavior were monitored daily. Tumor growth was measured, and tumor volume was calculated with the following equation: volume = (length $\times$ width²)/2. After 14 days of drug administration, the mice were killed, and tumor tissues were obtained for TUNEL and immunohistochemical experiments.

Immunohistochemistry

Tumor tissues were fixed and paraffin-embedded. Sections were dewaxed and rehydrated. After incubation with primary antibody, the slides were then incubated in HRP horseradish peroxidase, stained with DAB (ZSGB-BIO, China), counterstained with hematoxylin.

Statistical analysis

All data are expressed as the mean $\pm$ SD of three independent experiments. Statistical significance was analyzed with a Student's t-test. The criterion of statistical significance was* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Eup inhibits cell viability and triggers G2/M cell cycle arrest in TNBC cells

The structure of Eup was presented in Fig. 1 a. To evaluate the anticancer activity of Eup on TNBC cells, the present study measured the effects of different concentrations of Eup on the viability of TNBC cells for 48 h by MTT assay. The results suggested that Eup exhibited the strong inhibitory effect on cell viability in a concentration-dependent manner (Fig 1 b). Meanwhile, the effect of Eup on cell cycle distribution was determined by flow cytometry. TNBC cells were exposed to Eup and stained with PI/RNase, the result showed that Eup could triggers G2/M cell cycle arrest while the number of cells in G0/ G1 and S phases was reduced (Fig 1 c-e). These results indicated that Eup inhibited cell viability and triggered the G2/M-phase arrest in TNBC cells.

Eup induces apoptosis by mitochondria pathway in TNBC cells

To evaluate the effects of Eup on apoptosis, an Annexin V-FITC/7AAD dual staining assay was performed using flow cytometry. With the treatment of 8 μ M Eup, the apoptosis rates of MDA-MB-231 and MDA-MB-468 cells reach 25.10 % and 36.02 %, respectively. (Fig. 2 a-c). Meanwhile, Eup reduced the protein level of caspase-3 and increased the protein levels of cleaved caspase-3 (Fig. 2 g).

Mitochondria is the main site of ROS production, (24) and the loss of mitochondrial membrane potential (MMP; $\Delta\psi_m$) is a symbol of cell apoptosis before caspase activation. As shown in Fig. 2 d-f, Eup disrupted the $\Delta\psi_m$, and the relative percentages of low $\Delta\psi_m$ were increased by 8 folds and 6 folds mediated by 8 μ M Eup in MDA-MB-231 and MDA-MB-468 cells respectively. Furthermore, Eup resulted in the downregulation of anti-apoptotic protein Bcl-2 and upregulation of pro-apoptotic protein Bad (Fig. 2 i). We also used Mito-Sox staining to detect mitochondria ROS (mtROS) production. As shown in Figure 2 h, the mtROS level was significantly enhanced with the treatment of Eup. These data suggested that apoptosis might be correlated with the effects of Eup on TNBC cells.

Eup leads to ferroptosis in TNBC cells

As a new type of cell death, the characteristics of ferroptosis are the accumulation of lipid reactive oxygen species (ROS) and the depletion of glutathione (GSH) (25). Thus, we evaluated the levels of lipid ROS and GSH in TNBC cells treated with Eup. As a result, lipid ROS accumulation (Fig. 3 a-c) and GSH depletion (Fig 3 d) were determined following treatment with Eup. Furthermore, after administration of exogenous GSH, Eup-induced cell death could be rescued in TNBC cells (Fig. 3 e). Iron is a vital reactive element in ferroptosis. Therefore, intracellular chelate iron in TNBC cells was observed using PGSK whose fluorescence is quenched by iron(26). As shown in Fig 3 f, we found that the proportions of PGSK-positive cells were decreased, which indicated that ferroptosis was induced by Eup.

Moreover, the levels of negative regulatory proteins for ferroptosis (GPX4 and FTH1) remarkably reduced under Eup treatment in TNBC cells (Fig. 3 g). Taken together, these data clarified that ferroptosis occurred in TNBC cells under the treatment with Eup.

Apoptosis and ferroptosis inhibitors rescue Eup-induced cell death

To further confirm whether apoptosis or ferroptosis was involved in the form of cell death induced by Eup, TNBC cells were incubated with Eup in the absence or presence of several inhibitors. The incubation combined with the apoptosis inhibitor Z-VAD, ferroptosis inhibitors ferrostatin-1 (Fer-1) or deferoxamine (DFO) protected against the cell death induced by Eup. (Fig. 4 a). Interestingly, Eup could induce caspase-dependent apoptosis as evidenced by the reversal effects of Z-VAD in TNBC (Fig. 4 b). Meanwhile, several critical events in ferroptosis were inhibited by Fer-1 or DFO, that Eup caused the accumulation of lipid ROS (Fig. 4 d), the increase of iron level (Fig. 4 e) and downregulation of the expression of GPX4 and FTH1 (Fig. 4 c). Taken together, these data indicated that apoptosis and ferroptosis took an active part in Eup-induced cell death in TNBC cells.

Eup induces mutant p53 ubiquitination in TNBC cells

It is widely believed that mutant p53 has become a potential biomarker and therapeutic target in TNBC due to the high prevalence of TP53 mutations(27, 28). Western blotting results showed that Eup could reduce mutant p53 protein levels in p53 mutant MDA-MB-231 cells (Fig. 5 a). Moreover, the immunofluorescence assay also identified that **Eup** reduced the level of mutant p53 (Fig. 5 c). Ubiquitination is one of the key forms of protein post-translational modifications(29), usually resulting in protein degradation by the proteasome. We treated p53 mutant MDA-MB-231 cells with Eup after pretreatment with proteasome inhibitor MG132, and the Eup-induced degradation of mutant p53 was reversed by MG132. (Fig. 5 b). This result showed that mutant p53 was modified by ubiquitination and degraded in Eup-treated MDA-MB-231 cells via the ubiquitin–proteasome system. Therefore, the ubiquitination of mutant p53 was analyzed under **Eup** treatment. The result demonstrated that **Eup** could raise the ubiquitination of mutant p53 (Fig. 5 d). To further understand the mechanism that **Eup** decreased the level of mutant p53, the interaction between **Eup** and mutant p53 was investigated. **Eup** was docked with the mutant p53 (R280K) (PDB code: 6FF9). As shown in Fig 5 e, **Eup** oriented into the hydrophobic pocket, forming strong hydrophobic interactions with Ile-162, Val-272, Lys-164 and Phe-270 as well as interacting via hydrogen bonding with Lys-164, Val-272 and Phe-270. These findings clarified that **Eup** decreased mutant p53 expression by triggering ubiquitination of mutant p53.

Eup induces apoptosis and ferroptosis through mutant p53 ubiquitination in TNBC cells

Similar to wild-type p53, mutant p53 is also a transcription factor and control apoptosis and ferroptosis. To further investigated whether mutant p53 is a key target for Eup treatment, mutant p53 protein was knocked down by shRNA (Fig 5 f). It showed that knockdown of mutant p53 by shRNA rescued Eup-induced cell death in MDA-MB-231 cells lines (Fig 5 g) as well as the rates of apoptosis (Fig 5 j), the accumulation of lipid ROS (Fig. 5 h) and the increase of iron level (Fig. 5 i). Recent studies have found that SAT1 (spermidine/Spermine N1-acetyltransferase 1) is a transcriptional target of p53, which is a regulator in apoptosis and ferroptosis(30, 31). In the present study, the expression of STA1 significantly increased after Eup treatment in MDA-MB-231, however, knockdown of mutant p53 by shRNA could reverse it (Fig 5 k). These results suggested that mutant p53 was an important target for Eup-induced apoptosis and ferroptosis.

Eup results in apoptosis and ferroptosis induction and exerts anti-TNBC effects *in vivo*

To test the anti-TNBC activity of Eup *in vivo*, MDA-MB-231 cell line xenografts in nude was established. The result showed that, compared with the control group, the tumor volume and weight were reduced evidently in the Eup-treated group (Fig. 6 a-c). In addition, there was no palpable difference in body weight among the groups, which meant that Eup was obviously non-toxic to animals (Fig. 6 d). Meanwhile, apoptosis occurred *in vivo* when these tumor tissues from the xenograft model were stained by TUNEL. The number of TUNEL-positive cells in the tumor tissues of Eup-treated mice significantly increased (Fig. 6 e). We also investigated the expression of Ki-67, mutant p53 and GPX4 by immunohistochemical staining. The expression of Ki-67 was evidently reduced under Eup treatment, showing that Eup could inhibit the growth of TNBC (Fig. 6 f). Furthermore, low mutant p53 and GPX4 expression indicated that

Eup reduced the level of mutant p53 and induced ferroptosis. Taken together, these data suggested that Eup restricted tumor growth *in vivo* by triggering apoptosis and ferroptosis as well as reducing mutant p53 levels.

Discussion

It has been demonstrated that Eup, a natural compound isolated from *Eupatorium cannabinum* Linn., has anti-tumor activity(22). Nevertheless, studies on its anti-tumor effects on TNBC have not been reported until now. In the present study, the effects of Eup were investigated on TNBC both *in vitro* and *in vivo*. The results showed that Eup exerted cell viability inhibition and G2/M-phase arrest. Meanwhile, apoptosis contributed to the cell death in TNBC cells, because the apoptotic inhibitor Z-VAD rescued it and the cleavage of caspase 3. Moreover, the mechanism of Eup-induced apoptosis was investigated that Eup disrupted the $\Delta\psi_m$ in TNBC cells, increased the level of mtROS, decreased the expression of Bcl-2, and increased the expression of Bad, those were important mitochondrial proteins in apoptosis. Furthermore, Eup could lead to the cleavage of caspase 3. These data suggested that Eup could induce mitochondrial apoptosis in TNBC cells.

As a subtype of breast cancer, TNBC lacks the expression of ER, PR and HER2, which are targets of hormonal therapy in breast cancer. Also, TNBC is a heterogeneous group of diseases with limited therapeutic options(32). So, it is urgent to find a novel therapy strategy for TNBC. Ferroptosis, a novel type of cell death, is distinct from apoptosis, necroptosis and autophagic cell death and is characterized by the accumulation of lipid reactive oxygen species (ROS) and high iron levels(33). It has been reported that ferroptosis is a tumor suppressor in various human cancers, including TNBC (34). Thus, inducing ferroptosis has become an effective treatment strategy for TNBC. Subsequently, we detected whether ferroptosis occurred in TNBC cells during Eup treatment. As expected, ferroptosis events were significantly induced following treatment with Eup, including the accumulation of lipid ROS, the depletion of GSH, the increase of iron level and downregulation of the expression of negative regulatory proteins. Interestingly, after administration of exogenous GSH, Eup-induced cell death in TNBC cells could be almost reversed. Moreover, ferroptotic inhibitor Fer-1 or DFO reduced Eup-induced cell death as well as several ferroptotic events. Therefore, ferroptosis may be related to Eup-induced growth inhibition in TNBC.

Mutant p53 has become a potential biomarker and therapeutic target in TNBC due to the high prevalence of TP53 mutations (27, 28). Our research demonstrated that Eup could induce mutant p53 ubiquitination in MDA-MB-231. Also, mutant p53 is a key target for Eup treatment because Eup-induced apoptosis and ferroptosis were inhibited in mutant p53 knockdown TNBC cell lines. Meanwhile, the expression of STA1, a transcriptional target of p53 and regulator in apoptosis and ferroptosis(30, 31), significantly increased after Eup treatment in MDA-MB-231 (R280K), however, knockdown of mutant p53 by shRNA could reverse it. Thus, these data suggested that Eup induced cell death of TNBC through p53/SAT1 pathways.

A xenograft nude mice model of MDA-MB-231 cells was established to assess whether Eup inhibited tumor growth *in vivo*. The result showed that Eup significantly suppressed the growth of MDA-MB-231-

driven tumor, induced apoptosis and ferroptosis and reduced mutant p53 level.

Conclusions

In summary, our study demonstrated that the natural compound Eup is a potential therapeutic agent for TNBC by inducing apoptosis and ferroptosis through ubiquitination of mutant p53. Moreover, the underlying mechanism of between mutant p53 and Eup-induced apoptosis and ferroptosis remained to investigate in the future.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The study was approved by the Ethics Committee of Zhejiang Chinese Medical University.

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Figures

Figure 1

Eup inhibits cell viability and induced cell cycle arrest at G2/M in TNBC cells.

a Structure of Eup. **b** The sensitivity of TNBC cells to Eup (0, 2, 4, 8, 16 μM) was determined by MTT assay for 48 h. **c** TNBC cells were exposed to Eup for 24 h. Representative results of cell cycle and quantitative analysis (**d, e**) ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Eup triggers apoptosis through the mitochondria pathway in TNBC cells.

a TNBC cells were exposed to Eup for 24 h. Representative results of Annexin V-FITC/7AAD staining. **b, c**: The quantification of apoptotic cells. ** $p < 0.01$, *** $p < 0.001$. **d** TNBC cells were exposed to Eup for 24 h. Cells was stained with JC-1 and analyzed by flow cytometry. **e, f**: The quantification of the relative

amount of green fluorescence. *** $p < 0.001$ **g, i** The expression of caspase relative proteins was detected by Western blotting. **h** Representative images of mtROS were determined by mito SOX (630×magnification).

Figure 3

Ferroptosis participates in Eup-induced cell death in TNBC cells.

a After exposed to Eup for 24 h, the cellular lipid ROS level was observed by fluorescence microscope (400×magnification) and analyzed by flow cytometry (**b, c**), $*p < 0.05$, $***p < 0.001$. **d** The GSH or GSSG was evaluated and GSH/GSSG ratios was calculated, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **e** Cell viability was determined by MTT assay after TNBC cells were pretreated with GSH (1mM) and treated with Eup (0, 8 16 μ M) for 24 h. $**p < 0.01$, $***p < 0.001$. **f** The chelate iron was determined using PGSK (green) after the treatment Eup for 24 h. **g** The levels of FTH1 and GPX4 were detected by western blotting.

Figure 4

Eup-induced cell death is rescued by apoptosis and ferroptosis inhibitors.

a TNBC cells were pretreated with Z-VAD, Fer-1 or DFO and treated with Eup (8 μ M) for 24h, and then cell viability was assayed. $**p < 0.01$, $***p < 0.001$. **b** TNBC cells were pretreated with 10 μ M Z-VAD for 2h, and treated with Eup for 24h. The expression of cleaved caspase3 were detected by western blotting in

TNBC cells. **c** TNBC cells were pretreated with 1 μ M Fer-1 or 200nM DFO for 2h and treated with Eup for 24h. The expressions of FTH1 and GPX4 were determined by western blotting in TNBC cells. **d** Representative images of lipid ROS were determined by the fluorescent probe BODIPY C11 (400 $\times$ magnification) after TNBC cells were pretreated with Fer-1 or DFO and treated with Eup. **e** The chelate iron was determined using PGSK (Green) after TNBC cells were pretreated with Fer-1 or DFO and treated with Eup.

Figure 5

Eup induces apoptosis and ferroptosis related to mutant p53 in TNBC cells

a The expression level of p53 was determined by western blotting after p53 mutant MDA-MB-231 cells were exposed to Eup for 24 h. **b** The expression level of p53 was determined by western blotting after p53 mutant MDA-MB-231 cells were pretreated with MG132 for 2h and treated with Eup for 24h. **c** Immunofluorescence staining of p53 (Red) after MDA-MB-231 cells were treated with Eup for 24 h. Nuclei were also stained with DAPI (Blue). **d** Mutant p53 MDA-MB-231 cell was treated with Eup for 3h. The interaction of mutant p53 and Ub was detected by immunoprecipitation. **e** Docking of **Eup** into mutant p53 (R280K) (PDB code: 6FF9). The protein structure is shown in cartoon drawing, with a selected label residue (upper panel). Surface representation of the protein residues (below panel). **f** Western blotting detected the level of mutant p53, which was knocked down by shRNA. **g** Mutant p53 protein was knocked down by shRNA and cell viability was evaluated. $**p < 0.01$, $***p < 0.001$. **h** The cellular lipid ROS level was observed by fluorescence microscope (400×magnification) when mutant p53 protein was knocked down by shRNA. **i** The chelate iron was detected using the PGSK (green) when mutant p53 protein was knocked down by shRNA (100×magnification). **j** Shp53-MDA-MB-231 cells were treated with Eup (4 μ M) for 24h. Representative results of Annexin V-FITC/7AAD staining. **k** The mRNA expression of STA1 was measured by RT-PCR assay. $**p < 0.01$, $***p < 0.001$.

Figure 6

Eup inhibits the growth of TNBC cells *in vivo*.

MDA-MB-231 cells were subcutaneously injected into the female BALB/c nude mice. When tumor volume reached 50 mm³, the mice were administered by i.p. injections of either control or EP (15mg/kg) on alternate days. After administration for 14 days, the mice were killed, then tumors were removed and

photographed (**a**). **b** The tumor masses were weighted. **c** Tumor volume was measured and recorded every two days. *** $p < 0.001$. **d** The body weight was recorded every two days. **e** TUNEL assay of tumor sections in control and Eup treatment group. **f** Immunohistochemical staining of Ki-67, p53 and GPX4.