Toosendanin, A Novel Late-Stage Autophagy Inhibitor Sensitizes Triple-Negative Breast Cancer to Irinotecan cChemotherapy *In Vitro* and *In Vivo*

Shuang Zhang

University of Macau Institute of Chinese Medical Sciences

Yu Dong

University of Macau Institute of Chinese Medical Sciences

Xiu-Ping Chen

University of Macau Institute of Chinese Medical Sciences

Chris Soon Heng TAN

Southern University of Science and Technology

Min Li

Hong Kong Baptist University

Jiahong Lu (✉ jiahonglu@umac.mo)

University of Macau <https://orcid.org/0000-0002-1147-125X>

Research Article

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Abstract

Background: Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer that develops resistance to chemotherapy frequently. Autophagy has been regarded as a pro-survival response to chemotherapeutic drugs in TNBC, and suppression of autophagy can be a therapeutic strategy to overcome drug resistance.

Methods: We verified the efficacy of TSN in blocking autophagy flux. The co-localization of autophagosomes and lysosomes was analyzed. Then, lysosome function was determined by pH value and the activity of hydrolytic proteases. For preclinical research, human triple-negative breast cancer MDA-MB-231 and MDA-MB-436 were used for evaluating the anti-proliferative effect *in vitro*. *In vivo*, the nude mice were intraperitoneal injection of irinotecan (10 mg/kg), TSN (0.5 mg/kg) or a combination. Autophagy activity and cell apoptosis were determined in tumor tissue. The degree of pathological injury of tissue was evaluated by liver index.

Results: Here we reported the natural autophagy inhibitor toosendanin, a triterpenoid extracted from *Melia toosenda* Sieb. et Zucc, potently inhibited late-stage autophagy in TNBC cells. This effect was achieved via elevating lysosome pH rather than blocking the fusion of autophagosomes and lysosomes. We further investigated the effects of toosendanin on the *in vitro* and *in vivo* TNBC models, in combination with chemotherapeutic drug irinotecan (or its active metabolite SN-38), a topoisomerase I (TOP1) inhibitor showing therapeutic potential for TNBC. The data showed that toosendanin blocked SN-38/irinotecan-induced protective autophagy, and significantly induced apoptosis in TNBC cells and tumor xenograft models when compared to SN-38/irinotecan or TSN treatment alone group.

1. Introduction

Triple-negative breast cancer (TNBC) is an aggressive subtype that constitutes 12–18% of breast cancer clinical cases (1, 2). Because TNBC patients lack the estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor, they are not accessible for hormone or anti-HER2 therapy. Compared to hormone receptor-positive or HER2-positive subtypes, TNBC patients displayed poorer response to anti-cancer treatment, and faster progress of tumor metastasis (2, 3). To date, therapies directed to specific molecular targets have rarely achieved clinical improvements in TNBC patients and chemotherapy remains the standard of care(1). Nonetheless, 30%~50% of TNBC patients rapidly become resistant to chemotherapy due to chromosomal instability and then, leads to the failure of treatment (4).

Autophagy is a homeostatic cellular degradation system that is responsible for degrading and recycling misfolded proteins and damaged organelles such as the endoplasmic reticulum, mitochondria, and Golgi apparatus (5). As a cellular survival mechanism, autophagy is hijacked by cancer cells to overcome metabolic stress and develop drug resistance (6, 7). Accumulation of evidence reveals that inhibition of autophagy can be an effective therapeutic strategy in combination with chemotherapy(8). At present,

only chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are tested in patients as inhibitors of autophagy, either alone or in combination with chemotherapy. However, due to the lack of specific molecular target(s), CQ is not a highly potent autophagy inhibitor and caused a wide range of side effects, such as visual impairment, gastrointestinal discomfort, electrocardiographic (ECG) changes, headache, and pruritus (5). Therefore, the development of specific and potent autophagy inhibitor(s) for the cancer treatment has considerable clinical significance.

Toosendanin (TSN), a triterpenoid extracted from the root bark of *Melia toosendan* Sieb. *et* Zucc, has been used as an insecticide in China for decades. Recent studies have revealed the anti-tumor effects of TSN on various human cancer cells such as osteosarcoma, lung cancer, and colorectal cancer (9–12). As indicated by previous reports, several mechanisms of toosendanin's anticancer effects have been reported: activating p38/MAPK pathway, regulating miR-608/Notch axis and κ -opioid receptor/ β -catenin signaling axis, inhibiting epithelial-mesenchymal transition via Akt/mTOR signaling, and targeting estrogen receptor β and p53 (9, 13–16).

In the present study, we report that TSN potently inhibited auto-lysosome maturation, leading to the accumulation of autophagosomes in human TNBC cells. This effect is due to inhibiting acidification of lysosome and impairing the lysosomal hydrolytic function rather than blocking the fusion of autophagosomes with lysosomes. Irinotecan is a topoisomerase I (TOP1) inhibitor which suppresses tumor growth by causing DNA double-strand breaks, and is indicated for the treatment of solid tumors including colorectal and lung cancer (17). Recent studies highlighted the potential of irinotecan in the TNBC treatment (18–20). By inhibiting protective autophagy, TSN sensitized TNBC cells to SN-38/irinotecan-induced cytotoxicity both *in vitro* and *in vivo*. Our findings thus demonstrate that the novel late-stage autophagy inhibitor TSN may represent a therapeutic potential for TNBC, in combination with chemotherapy drugs including irinotecan.

2. Materials And Methods

2.1 Antibodies and reagents

The chemicals used in our experiment were: toosendanin, irinotecan (SHANGHAI XIANDING BIOLOGICAL SCIENCE&TECHNOLOGY CO.LTD, HN057, 136572-09-3), Torin1 (LC Laboratories, T-7887), SN-38, chloroquine, baflomycin A1 (Sigma-Aldrich, H0165, C6628, 19-148), LysoTracker Red DND-99, LysoSensor Yellow/Blue DND-99 (Thermo Fisher, M22425, L7545).

The antibodies used in our experiments were: microtubule-associated protein 1 light chain 3 (Novus, NB100-2220), SQSTM1 (ABclonal, A11250), Lysosome-associated membrane protein, Cleaved caspase 3, and GAPDH (Cell Signaling Technology, 9091, 9661, D16H11), Cathepsin B, Cathepsin D (Santa Cruz, SC365558, SC-6486). Alexa Fluor 488-, 555- or 647-conjugated goat anti-rabbit and goat anti-mouse antibodies purchased from Invitrogen (A-11034, A-21422 and A-32733).

2.2 Cell culture

MDA-MB-231, MDA-MB-436, and BT-549 cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, 10100–147). MDA-MB-231 cells stably expressing GFP-RFP- LC3 maintained in DMEM with 10% FBS and 200 µg/ml G418. All the mediums were supplemented with 1% penicillin and streptomycin (Thermo Fisher Scientific, 12100046). Cells were incubated at 37°C in a humid 5% CO₂:95% air environment.

2.3 Cell viability (MTT) assay

Cells were seeded in 96-well plates at 5,000 and were treated for 24 to 48 h dependent on experimental conditions. Twenty µL MTT (5 mg/ml) was added in each well and incubated for 4 h. 100 µL DMSO was loaded into each well to dissolve the formazan and then OD value was measured by microplate reader at 570 nm. The cell viability was illustrated as the ratio of experimental group vs control group.

2.4 Transfections

Transfection was achieved using Lipofectamine 3000 Transfection Reagent (Invitrogen, L3000) according to the manufacturer's protocol. Cells were transfected with plasmids encoding mRFP-LC3, GFP-LC3, GFP-RFP-LC3, and mCherry-LAMP1. After 24 h incubation, the transfection mixture was removed and replaced with fresh complete medium.

2.5 Western blots

Cell proteins were extracted using ice-cold RIPA buffer (Cell Signaling Technology, 9806) with complete protease inhibitor mixture (Roche Applied Science, 04693124001). Protein was separated by gel electrophoresis in 10–15% SDS-polyacrylamide gels and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, 1,704,156). Following blocking with TBST (Tris-buffered saline with 0.1% Tween-20) buffer containing 5% (w:v) nonfat milk powder (Bio-Rad, 1706404) the blots were probed with the corresponding primary antibodies and secondary antibody. Blots were visualized using the Pierce ECL kit (Pierce, 32106) and ChemiDoc MP Imaging System (Bio-Rad, 12003154).

2.6 Immunofluorescence

After treatment, MDA-MB-231, MDA-MB-436 cells were fixed in 4% paraformaldehyde (Shanghai Sangon Biotech, E672002-0500) for 15 min at room temperature and blocked in 5% BSA for 1 h. Subsequently, cells were incubated with anti-LAMP1 (1:100) antibodies overnight at 4°C and then incubated with the appropriate secondary antibodies for 1 h at RT. Tumor tissues were snap-frozen in optimal cutting temperature (OCT) embedding medium (Tissue-Tek) and sectioned. Cryosections of tumors (5 µm thickness) were also fixed in 4% paraformaldehyde for 15 min at RT and blocked in 5% BSA for 1 h. Tumor sections were incubated with the primary antibodies: cleaved caspase 3 overnight at 4°C and then incubated with the appropriate secondary antibodies for 1 h at RT. Nuclei were stained with Hoechst 33,258 for 5 min. Fluorescence photos were taken using a confocal laser-scanning microscope. Different fields of view (> 5 regions) were analyzed on the confocal laser-scanning microscope for each labeling condition, and representative results were shown.

2.7 Lysosomal pH measurements

Lysosomal pH was measured by LysoSensor Yellow/Blue DND-160 (Invitrogen, L7545) staining according to the manufacturer's protocol. MDA-MB-231, MDA-MB-436, and BT-549 cells were plated on a 96-well culture plate. After treatment, cells were loaded with 2 μ M LysoSensor Yellow/Blue DND-160 for 30 min at 37°C and washed twice using PBS. After that, 100 μ L HBSS (containing Mg^{2+} , Ca^{2+}) was added to each well for further detection. Fluorescence emitted at 440/540 nm was measured by a microplate reader (Molecular devices FlexStation 3) in response to excitation at 329 nm and 384 nm, respectively.

2.8 Annexin-V/PI dual staining assay

A quantitative assessment of apoptosis cells was performed using the Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, C1062M). In short, the cells were cultured in a 6-well cell culture plate and treated with TSN, SN38 alone, or in combination. Then cells were collected, washed with cold PBS, and resuspended in binding buffer (1×10^6 cells/mL). After 100 μ L of cells was transferred to a tube, 5 μ L of FITC-conjugated Annexin V (Annexin V-FITC) and 2 μ L of propidium iodide (PI) were added followed by incubation for 15 min at room temperature in the dark. The stained cells were analyzed by the flow cytometer (BD LSR Fortessa™ Flow Cytometer). Data of 10,000 cells were collected in each data file. Four different populations of cells are easily distinguished: un-labelled (viable cells), Annexin V-FITC positive (early apoptotic), PI positive (necrotic), and Annexin V-FITC/PI positive (late apoptotic/necrotic cells). The fluorescence distribution was displayed as a two-color dot plot analysis, and the cells in each quadrant were determined.

2.9 Lactate dehydrogenase (LDH) release assay

LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, C0016) was used to evaluate cell death. Specifically, the LDH working solution was prepared by 10 μ L lactic acid solution, 10 μ L INT solution (1 $\times$), 10 μ L enzyme solution, with a total of 30 μ L. The cell culture 96-well plate was centrifuged at 400 g for 5 min. The supernatant of each well (60 μ L) was added into the corresponding well of a new plate. The LDH working fluid was mixed with the supernatant, incubated at room temperature for 30 min, and then the absorbance was read at 490 nm.

2.10 Xenograft assay

Nude mice aged 4-5 weeks old were obtained from the Faculty of Health Sciences, University of Macau, and fed with a standard animal diet and water. Animal research was approved by the Animal Ethics Committee in University of Macau (UMARE-036-2019). MDA-MB-231 cells stably expressing GFP-RFP-LC3 were suspended in a 1:1 ratio in serum-free DMEM medium with a matrigel basement membrane matrix (Corning, 356231) and inoculated subcutaneously (5×10^6 /site) in the right armpit. The tumor diameters were measured and the tumor volume (mm^3) was calculated with caliper as follows: Volume = (shortest diameter)² $\times$ (longest diameter)/2. The volume between 80~120 mm^3 was considered as a successful establishment of the model. Irinotecan (10 mg/kg, every 6 days) and TSN (0.5 mg/kg, every 2 days) were

dissolved in intralipid and injected into the mice intraperitoneally alone or together ($n = 7$). Intralipid was used as the vehicle control. Tumor size and body weight are recorded every two days or three days. Mice were sacrificed 28 d after medication. Organs were collected and tumors were excised, and either formalin-fixed or flash-frozen at -80°C until further use.

2.11 Statistical analysis

Each experiment was performed at least 3 times, and the results are presented as mean $\pm$ SD. One-way analysis of variance (ANOVA) was followed by Turkey as post hoc tests using the Sigma Plot 11.0 software package. A probability value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 TSN caused LC3-II accumulation and suppressed autophagic degradation.

MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) could transform from the LC3-I into its lipid counterpart LC3-II during autophagosome maturation (21). LC3-II and the cargo adaptor protein, p62/SQSTM1 (sequestosome 1) are two widely used markers to monitor autophagy. We examined the effect of TSN (Fig. 1A) on LC3-II and SQSTM1 protein levels in both MDA-MB-231 and MDA-MB-436 cells. Western blot analysis showed that TSN resulted in dose- and time-dependent accumulation of LC3B-II and SQSTM1 in both cell lines (Fig. 1B and 1C), suggesting that TSN might inhibit autophagic maturation. Furthermore, we analyzed fluorescent images of transiently GFP-LC3-expressing MDA-MB-231 cells after treatment of TSN via Leica Microscope. As shown in Fig. 1D, treatment of TSN at $1\text{ }\mu\text{M}$ increased GFP-LC3 puncta dramatically in MDA-MB-231 cells. To confirm whether TSN interrupts autophagosome maturation, we applied the tandem mRFP-GFP-LC3B reporter to monitor the autophagy flux. RFP-GFP-LC3B is comprised of two tandem fluorescent proteins: a pH-insensitive RFP and a pH-sensitive GFP. GFP fluorescence will be quenched in an acid environment, leaving an RFP signal only. The ratio of red to green fluorescence allows for a distinction between lysosomal and cytoplasmic LC3 to reflect the autophagy flux. The result showed that TSN markedly increased the number of yellow puncta in both MDA-MB-231 and MDA-MB-436 cells (Fig. 1E), similar to that induced by CQ, a classic autophagy inhibitor that impairs autophagosome fusion with lysosomes (22).. In contrast, many red puncta were observed in Torin1-treated cells. Bafilomycin A1 (BAF A1) is an inhibitor of autophagic flux by inhibiting the acidification of lysosomes. Our results showed that co-incubation of cells with TSN and BAF did not induce the further increase in LC3B-II levels compared with TSN treatment alone (Fig. 1F), confirming that TSN inhibits the autophagic degradation. Overall, these observations indicate that TSN is a potent late-stage autophagic flux inhibitor.

3.2 TSN does not inhibit the fusion process of autophagosome and lysosome.

It is essential to determine whether the inhibitory effect of TSN on autophagy is due to the blockade of autophagosome-lysosome fusion, or impairment of autolysosome degradation. To address whether TSN affects the fusion process, we examined the co-localization of RFP-GFP-LC3 and lysosomal associated membrane protein 1 (LAMP1), a marker for late endosomal and lysosomal membranes. Fig. 2A is the schematic diagram of RFP-GFP-LC3 reporter/LAMP1 to reflect the fusion of autophagosome and lysosome. As shown in Fig. 2B, both TSN and BAF treated cells showed nice colocalization of LC3 and LAMP1, indicating that TSN did not affect the fusion of autophagosomes and lysosomes.

3.3 TSN elevated lysosome pH and impaired lysosomal proteolytic function.

Lysosomes are the acidic organelles (pH 4.5) that digest macromolecules for the regeneration of basic building blocks or defending the invading pathogens. They receive their substrates by fusing with endosomes or autophagosomes. To verify whether TSN impacts the function of lysosome thereby blocking the degradation of autophagosome, we used LysoSensor™ Yellow/Blue DND-160, a ratio-metric probe that can be used to measure the pH of lysosomes by calculating the ratio of acquired fluorescent signals emitted at 440/540nm which were excited at 329/384nm. As shown in Fig. 3A, the lysosome environment was alkalized after TSN treatment in MDA-MB-231, MDA-MB-436 and BT-549 cells compared with vehicle-treated group. CQ, an autophagy inhibitor by altering the acidic environment of lysosomes, is used as a positive control. As the major lysosomal proteases, cathepsins are synthesized as inactive membrane-associated precursors and the precursors are further cleaved to generate active forms within endosomes or lysosomes (23). We next evaluated whether TSN affects protein expression and the maturation process of cathepsin B (CTSB) and cathepsin D (CTSD). As shown in Fig. 3B and 3C, TSN dramatically down-regulated the mature form of CTSB and CTSD in MDA-MB-231, and MDA-MB-436 cells, meaning that the cleavage process from inactive to the active form in lysosomes was impeded. A red-fluorescent dye LysoTracker™ Red DND-99 also used for tracking acidic organelles in live cells. As shown in Fig. 3D, cells almost lose red fluorescence signals after TSN treatment compared with control group. This result further confirmed that TSN increased the pH of lysosome. BAF, a well-known agent to inhibit acidification of lysosome, was used as a positive control. DQ™ Red BSA also used for proteases function detection. Upon hydrolysis of the DQ Red BSA to single, dye-labeled peptides by lysosomal proteases, the cell produced brightly fluorescent products. Once the function of the lysosomal proteasome is destroyed, a strong fluorescence quenching effect could observe. As shown in Fig. 3E, TSN treatment led to the red fluorescence quenching, indicating that the lysosomal proteases hydrolytic function is impaired. Taken together, these data indicated that TSN impaired the acid environment of lysosomes and prevented the maturation of lysosomal cathepsin to inhibit the lysosomal hydrolytic function.

3.4 SN-38 induces autophagy in TNBC.

In response to cytotoxic treatments, tumor cells may activate autophagy to promote survival (24). Our previous study has shown that chemotherapy compound camptothecin induced protective effects in multiple tumors (25). Irinotecan is a topoisomerase I (TOP1) inhibitor that has been shown to hold great

potential in treating TNBC (18). Irinotecan is a derivative of camptothecin and SN-38 is the active metabolite of irinotecan. We propose that SN38/irinotecan may also induce protective autophagy in TNBC cells. We, therefore, determined change of autophagy flux after SN-38 treatment in TNBC cells. As showed in Fig. 4A, SN-38 dose-dependently induced the increase of LC3-B and decrease of SQSTM1. Then we used RFP-GFP-LC3 MDA-MB-231 cells to monitor autophagy level after SN-38 treatment. We found there is a dramatic increase of red-only puncta after SN-38 treatment, implying that SN-38 activated autophagy process (Fig. 4B). These observations confirmed that TNBC cells up-regulated autophagy levels during chemotherapy.

3.5 Autophagy inhibition in combination with chemotherapy is more effective than monotherapy in reducing viability and inducing apoptosis in TNBC.

Increasing evidence indicates that inhibiting autophagy enhances the efficacy of chemotherapy by abolishing chemo-resistance and increasing cancer cell death (26). We, therefore, determined whether TSN would sensitize breast cancer cells to chemotherapy by blocking protective autophagy. Firstly, we examined the autophagy flux by the GFP-RFP-LC3 reporter. Co-treatment of SN-38 and TSN totally inhibited the maturation of autolysosome showing with yellow puncta after merge (Fig. 5A). Next, we examined whether TSN enhanced the anti-cancer activity of SN-38. Changes in cell morphology showed in Fig. 5B, and autophagy inhibition by TSN rendered TNBC cells more susceptible to chemotherapy. LDH release assay and MTT assay showed that TSN dramatically enhanced the SN-38-induced cell death and growth inhibition (Fig. 5C, D). Next, we performed an Annexin V-FITC/propidium iodide (PI) double-staining assay using flow cytometric analysis (Fig. 5F). Compared with the SN-38 treated alone cells, co-treatment cells for 48h increased the proportion of FITC+/PI+ (apoptosis) and FITC-/PI+ (necrosis) cells rate from $11.33\% \pm 2.08$ – $49.10\% \pm 4.58\%$ ($0.1 \mu\text{M}$) and $14.86\% \pm 1.32$ – $55.16\% \pm 3.01\%$ ($1 \mu\text{M}$), respectively (Fig. 5F). Consistent with these findings, combined treatment of TSN and SN-38 resulted in the increased level of cleaved caspase 3 as shown by western blot (Fig. 5E). These findings indicated that TSN was able to sensitize TNBC cells to SN-38-induced cell death probably through the suppression of autophagy flux.

3.6 TSN inhibited autophagic flux and repressed TNBC xenograft growth *in vivo*.

To determine whether our *in vitro* findings that suppression of autophagy by TSN sensitized TNBC to SN-38-induced cell death could be reproduced *in vivo*, we examined the therapeutic efficacy of TSN on mouse TNBC xenograft model. To observe the autophagy flux *in vivo*, nude mice were inoculated subcutaneously with MDA-MB-231 cells stably expressing GFP-RFP-LC3, followed by intraperitoneal administration of irinotecan (10 mg/kg) or TSN (0.5 mg/kg) alone or in combination for 4 weeks. As shown in Fig. 6C, irinotecan treatment alone group showed the effect on the growth of tumors with a 56.71% inhibition rate, whereas a combination of TSN and irinotecan led to a significant reduction in

tumor growth compared to untreated controls with an 80.56% inhibition rate. Consistently, the tumor weight in the co-treatment group led to a significant reduction ($P=0.0229$ for combination treatment vs irinotecan alone) as showed in Fig. 6D. Moreover, no significant changes in body weight were observed among vehicle control, irinotecan or TSN, and a combination of irinotecan or TSN (Fig. 6B). The appearance of tumors was consistent with the data of tumor volume and weight (Fig. 6A).

To evaluate whether the combination of TSN and irinotecan results in a change of autophagy flux in xenograft tumor tissue *in vivo*, tumor samples were excised and stained with hoechst. As showed in Fig. 6E, in the basal condition, mature autophagosomes (red-only puncta) were observed in most tumor cells, indicating that induction of autophagy flux under the hypoxia and energy-deficient environment in the TNBC mice. Moreover, in chemotherapy condition (irinotecan group), the number of mature autophagosomes was dramatically increased meaning that protective autophagy was triggered in tumor tissue. Consistent with the *in vitro* experiments, TSN treatment showed the blockage of the autophagosome maturation process as evidenced by the markedly increased number of yellow puncta, either in TSN alone or TSN combination with irinotecan group (Fig. 6E). Western blot analysis showed that irinotecan treatment increased the accumulation of LC3B-II and decreased the level of SQSTM1 (Fig. 6F). While co-treatment with irinotecan and TSN, both the levels of LC3B-II and SQSTM1 were increased compared with the irinotecan alone group, indicating irinotecan-induced protective autophagy was blocked by TSN. Excised tumor samples were also subjected to cleaved caspase 3 immunostaining. Either TSN or irinotecan treatment alone caused a minimal increase in the number of cleaved caspase 3-positive cells (Fig. 6G) compared with control. However, the irinotecan and TSN combination dramatically increased the number of cleaved caspase 3-positive cells compared with either irinotecan or TSN alone group (Fig. 6G), indicating that autophagy inhibition by TSN rendered cancer cells more vulnerable to chemotherapy. The liver index showing the ratio of organ weight to body weight was analyzed in Table 1. Compared with the control group, IRI, TSN and Co-treat did not appear hepatomegaly and had no significant difference in the liver index of mice ($P > 0.05$). Together with body weight data, the result was conceivable that at the dosage capable of inhibiting autophagy, the TSN administration was tolerant in mice. Taken together, these findings suggested that TSN potently enhanced the therapeutic activity of irinotecan *in vivo* by inhibiting autophagy flux.

Table 1
Liver mass and liver index^a in mice

Group	(Body mass)/g	(Liver mass)/g	(Liver index)/%
CTL	26.26±2.42	1.42±0.24	5.38±0.57
IRI	24.66±1.36	1.26±0.13	5.10±0.50 ^{n.s.}
TSN	25.20±1.86	1.43±0.17	5.65±0.47 ^{n.s.}
Co-treat	24.36±1.00	1.32±0.10	5.47±0.61 ^{n.s.}

^aLiver index means the ratio of organ wet weight to body mass.

^{n.s.}No significant difference among administration group and control group.

Discussion

Triple-negative breast cancer remains the most challenging breast cancer subtype to treat. Recently, targeted therapies for TNBC treatment have been requested. PI3K/AKT/mTOR pathway, RAS/MAPK pathway, EGFR, JAK/STAT pathway, and NOTCH are some potential targets in clinical trials (27). However, therapies directed to specific molecular targets have rarely achieved effective improvement during clinical remedy, and chemotherapy remains the standard of care. Nonetheless, over 30% of TNBC patients become resistant to chemotherapy and it lead to the failure of treatment (4). Drug efflux (overexpression of ATP binding cassette transporters proteins, and P-glycoprotein) (28), up- or down-regulated autophagy flux, de-regulation of distinct cell intrinsic processes (the nuclear proto-oncogene c-MYC)(29), growth factor signaling, and DNA repair have previously been explored as the mechanisms of TNBC chemo-resistance. Therefore, there is still an urgent need to develop novel molecular targets to improve the therapeutic benefit of chemotherapy given in first-line settings, including the patients with advanced, chemotherapy resistant TNBC.

Autophagy has been recognized as an effective cancer escape mechanism and the reason for drug resistance in multiple cancer types. Early clinical trials have demonstrated the feasibility and potential benefit of inhibiting autophagy in multiple cancer types, including pancreatic cancer, lung cancer, melanoma, and multiple myeloma (30). Studies have demonstrated that autophagy can be inhibited pharmacologically by targeting the different stages of the autophagic process. Early-stage inhibitors like 3-MA and LY294002, function on class III phosphatidylinositol 3-kinase (PIK3C3) and block the formation of autophagosomes (31, 32). The inhibitors that target a late stage of autophagy like chloroquine, BAF A1, leupeptin, etc. impair the fusion of the autophagosome with lysosome or hydrolyzed function of lysosome. CQ/HCQ is currently the only clinically available drug to inhibit autophagy. However, the high effective concentration of CQ may cause an off-target effect. Therefore, it is necessary to discover more potent autophagy regulators with the potential to develop into novel anti-tumor drugs.

Toosendanin (TSN), a triterpenoid extracted from *Melia toosendan* Sieb. et Zucc, has been reported to possess antioxidant, anti-inflammatory, and anti-allergic activities (33, 34). Recent studies have revealed potential anti-cancer activity of TSN in diverse cancer models, such as glioblastoma, Ewing's sarcoma, gastric cancer and hepatocellular carcinoma (15, 16, 33, 35, 36). Luo W et al. (33) reported that TSN could inhibit transforming growth factor- β 1-induced epithelial-mesenchymal transition and migration through ERK/Snail pathway in lung cancer cells. Recently, Wang H et al. provided evidence that TSN -induced apoptosis is associated with the κ -opioid receptor/ β -catenin signaling axis in colorectal cancer cells (37). Remarkably, TSN has been reported to mediate cisplatin sensitization through down-regulation of

Annexin A4 in lung cancer(38). These studies provided a possible mechanistic explanation for the anti-tumor effect of TSN. To date, only one study described the relationship between TSN and autophagy in mammal cells. However, this study claimed that TSN developed an apoptosis-sensitizing effect by inducing autophagy in lung cancer cells (39). When checking their data, no autophagy flux assay was applied in the study thus their interpretation of the results could be problematic. In our study, we provided a careful characterization of the effect of TSN on autophagy according to Autophagy Modulator Scoring System (AMSS) (40), and confirmed that TSN is a late-stage autophagy inhibitor. Considering the high potency of TSN in inhibiting autophagy both *in vitro* and *in vivo*, we believe that autophagy inhibiting activity is an important anti-cancer mechanism of TSN.

TSN has been used in clinics for treating intestinal ascariasis in China (41). However, liver toxicity of TSN in mice has been reported (42), raising the concern for the use of TSN as an anti-cancer drug. According to the mice data, TSN did not induce obvious liver toxicity at the dosage up to 40mg/kg (intragastric administration), implying there is still a safety window for the clinic use of TSN. Due to different drug administration routes, we cannot directly compare the dosage we used in the current study (0.5mg/kg, intraperitoneal administration) with that used in the previous study (40mg/kg, intragastric administration). However, our data showed that at the dosage sufficient to inhibit autophagy in tumor tissue, TSN did cause obvious liver toxicity according to the liver index. Nevertheless, extensive safety characterization is still need to evaluate the safety of TSN for long-term application.

Up-regulation of autophagy observed in tumor cells following anti-cancer treatment is regarded as a protective response (43–45), and therapeutic targeting of autophagosome formation/fusion might represent a novel molecular avenue to reduce the emergence of chemo-resistance (5). Irinotecan was initially approved for the first-line treatment of metastatic colorectal cancer and later was also approved for lung cancer treatment (17). Irinotecan can be suitable for TNBC treatment given the fact that a considerable portion of TNBC tumors harbor mutations in genes required for DNA repairment (18). Indeed, recent studies highlighted the potential of irinotecan in the BRACness TNBC treatment (18–20). In this report, we showed that irinotecan/SN-38 induced autophagy in TNBC cells as a survival mechanism, and autophagy inhibition by TSN sensitized TNBC cells to irinotecan/SN-38 chemotherapy. Taken together, the data generated from this study reveals a novel therapeutic strategy for TNBC treatment by combination of topoisomerase I inhibitor and autophagy inhibitor.

Abbreviations

BAF Bafilomycin A1

CTSB cathepsin B

CTSD cathepsin D

CQ chloroquine

ER estrogen receptor

GFP green fluorescent protein

IRI Irrinotecan

LAMP1 lysosomal associated membrane protein 1

LC3 microtubule associated protein 1 light chain 3

LDH lactate dehydrogenase

OCT optimal cutting temperature

PR progesterone receptor

RFP red fluorescent protein

SN-38 7-Ethyl-10-hydroxycamptothecin

SQSTM1 Sequestosome 1

TSN toosendanin

TNBC triple-negative breast cancer

TUNEL TdT-mediated dUTP nick-end labeling

Declarations

Author information

1. Affiliations

State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau SAR 999078, China;

Shuang Zhang, Yu Dong, Xiu-Ping Chen, Jia-hong Lu

Department of Chemistry, College of Science, Southern University of Science and Technology, Shenzhen, 518055, China.

Chris Soon Heng TAN

Mr. & Mrs. Ko Chi-Ming Centre for Parkinson's Disease Research, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China;

Min Li

2. Corresponding author

Correspondence to Jia-hong Lu.

Ethics declarations

1. Ethics approval and consent to participate

All of the procedures were reviewed and approved by the Animal Ethics Committee in University of Macau.

2. Consent for publication

All authors agree to publish this article.

3. Availability of data and material

The data analyzed during this study can be obtained from the corresponding author on reasonable request.

4. Competing interests

The authors declare no conflict of interest.

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6. Authors' contributions

Jia-hong Lu conceived and supervised the project. Shuang Zhang explored the experiments and drafted the manuscript. Yu Dong assisted to complete the *in vivo* assessment. Xiu-Ping Chen, Chris Soon Heng TAN and Min Li help with experiment design and data analysis. All authors approved the final manuscript.

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References

1. Foulkes WD, Smith IE, Reis-Filho JSJNEjom. Triple-negative breast cancer. *The New England Journal of Medicine*. 2010;363(20):1938–48.
2. Brown M, Tsodikov A, Bauer KR, Parise CA, Caggiano V. The role of human epidermal growth factor receptor 2 in the survival of women with estrogen and progesterone receptor-negative, invasive breast cancer - The California cancer registry, 1999-2004. *Cancer*. 2008;112(4):737–47.
3. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clinical Cancer Research*. 2007;13(15):4429–34.
4. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *Journal of Clinical Oncology*. 2008;26(8):1275–81.
5. Kimura T, Takabatake Y, Takahashi A, Isaka Y. Chloroquine in Cancer Therapy: A Double-Edged Sword of Autophagy (vol 73, pg 3, 2013). *Cancer Research*. 2013;73(4):1446-.
6. Amaravadi R, Kimmelman AC, White E. Recent insights into the function of autophagy in cancer. *Genes & Development*. 2016;30(17):1913–30.
7. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nature Reviews Cancer*. 2012;12(6):401–10.
8. Perez-Hernandez M, Arias A, Martinez-Garcia D, Perez-Tomas R, Quesada R, Soto-Cerrato V. Targeting Autophagy for Cancer Treatment and Tumor Chemosensitization. *Cancers*. 2019;11(10).
9. Wang HH, Wen CY, Chen SY, Wang F, He L, Li WQ, et al. Toosendanin-induced apoptosis in colorectal cancer cells is associated with the kappa-opioid receptor/beta-catenin signaling axis. *Biochemical Pharmacology*. 2020;177.
10. Luo W, Liu X, Sun W, Lu JJ, Wang Y, Chen XJPR. Toosendanin, a natural product, inhibited TGF- β 1-induced epithelial-mesenchymal transition through ERK/Snail pathway. *Phytotherapy Research*. 2018;32(10):2009–20.
11. Wang G, Feng CC, Chu SJ, Zhang R, Lu YM, Zhu JS, et al. Toosendanin inhibits growth and induces apoptosis in colorectal cancer cells through suppression of AKT/GSK-3 beta/beta-catenin pathway. *International Journal of Oncology*. 2015;47(5):1767–74.
12. Zhang T, Li J, Yin F, Lin B, Wang Z, Xu J, et al. Toosendanin demonstrates promising antitumor efficacy in osteosarcoma by targeting STAT3. *Oncogene*. 2017;36(47):6627–39.
13. Cao L, Qu DD, Wang H, Zhang SM, Jia CM, Shi ZX, et al. Toosendanin Exerts an Anti-Cancer Effect in Glioblastoma by Inducing Estrogen Receptor beta-and p53-Mediated Apoptosis. *International Journal of Molecular Sciences*. 2016;17(11).
14. Pei Z, Fu W, Wang GP. A natural product toosendanin inhibits epithelial-mesenchymal transition and tumor growth in pancreatic cancer via deactivating Akt/mTOR signaling. *Biochemical and Biophysical Research Communications*. 2017;493(1):455–60.
15. Shao SL, Li SS, Liu C, Zhang WW, Zhang ZZ, Zhu SW, et al. Toosendanin induces apoptosis of MKN-45 human gastric cancer cells partly through miR-23a-3p-mediated downregulation of BCL2. *Molecular Medicine Reports*. 2020;22(3):1793–802.

16. Wang Q, Wang Z, Hou GL, Huang P. Toosendanin Suppresses Glioma Progression Property and Induces Apoptosis by Regulating miR-608/Notch Axis. *Cancer Management and Research*. 2020;12:3419–31.
17. de Man FM, Goey AKL, van Schaik RHN, Mathijssen RHJ, Bins S. Individualization of Irinotecan Treatment: A Review of Pharmacokinetics, Pharmacodynamics, and Pharmacogenetics. *Clinical Pharmacokinetics*. 2018;57(10):1229–54.
18. Coussy F, El-Botty R, Chateau-Joubert S, Dahmani A, Montaudon E, Leboucher S, et al. BRCAness, SLFN11, and RB1 loss predict response to topoisomerase I inhibitors in triple-negative breast cancers. *Science Translational Medicine*. 2020;12(531).
19. Fenn KM, Kalinsky K. Sacituzumab govitecan: antibody-drug conjugate in triple-negative breast cancer and other solid tumors. *Drugs of Today*. 2019;55(9):575–85.
20. Lin HC, Chuang CH, Cheng MH, Lin YC, Fang YP. High Potency of SN-38-Loaded Bovine Serum Albumin Nanoparticles Against Triple-Negative Breast Cancer. *Pharmaceutics*. 2019;11(11).
21. Klionsky D. Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy (3rd edition) (vol 12, pg 1, 2015). *Autophagy*. 2016;12(2):443-.
22. Mauthe M, Orhon I, Rocchi C, Zhou XD, Luhr M, Hijlkema KJ, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*. 2018;14(8):1435–55.
23. Guha S, Padh H. Cathepsins: Fundamental effectors of endolysosomal proteolysis. *Indian Journal of Biochemistry & Biophysics*. 2008;45(2):75–90.
24. Chittaranjan S, Bortnik S, Dragowska WH, Xu J, Abeysundara N, Leung A, et al. Autophagy Inhibition Augments the Anticancer Effects of Epirubicin Treatment in Anthracycline-Sensitive and -Resistant Triple-Negative Breast Cancer. *Clinical Cancer Research*. 2014;20(12):3159–73.
25. Wu MY, Wang SF, Cai CZ, Tan JQ, Li M, Lu JJ, et al. Natural autophagy blockers, dauricine (DAC) and daurisolone (DAS), sensitize cancer cells to camptothecin-induced toxicity. *Oncotarget*. 2017;8(44):77673–84.
26. Townsend KN, Hughson LRK, Schlie K, Poon VI, Westerback A, Lum JJ. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. *Immunological Reviews*. 2012;249:176–94.
27. Garrido-Castro AC, Lin NU, Polyak K. Insights into Molecular Classifications of Triple-Negative Breast Cancer: Improving Patient Selection for Treatment. *Cancer Discovery*. 2019;9(2):176–98.
28. Calcagno AM, Fostel JM, To KK, Salcido CD, Martin SE, Chewing KJ, et al. Single-step doxorubicin-selected cancer cells overexpress the ABCG2 drug transporter through epigenetic changes. *British Journal of Cancer*. 2008;98(9):1515–24.
29. Grassilli E, Ballabeni A, Maellaro E, Del Bello B, Helin K. Loss of MYC confers resistance to doxorubicin-induced apoptosis by preventing the activation of multiple serine protease- and caspase-mediated pathways. *Journal of Biological Chemistry*. 2004;279(20):21318–26.
30. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nature Reviews Cancer*. 2017;17(9):528–42.

31. Feng Y, Gao YJ, Wang DY, Xu ZH, Sun WX, Ren P. Autophagy Inhibitor (LY294002) and 5-fluorouracil (5-FU) Combination-Based Nanoliposome for Enhanced Efficacy Against Esophageal Squamous Cell Carcinoma. *Nanoscale Research Letters*. 2018;13.
32. Wu YY, Wang X, Guo HJ, Zhang B, Zhang XB, Shi ZJ, et al. Synthesis and screening of 3-MA derivatives for autophagy inhibitors. *Autophagy*. 2013;9(4):595–603.
33. Luo W, Liu X, Sun W, Lu JJ, Wang YT, Chen XP. Toosendanin, a natural product, inhibited TGF-1-induced epithelial-mesenchymal transition through ERK/Snail pathway. *Phytotherapy Research*. 2018;32(10):2009–20.
34. Xie F, Zhang M, Zhang CF, Wang ZT, Yu BY, Kou JP. Anti-inflammatory and analgesic activities of ethanolic extract and two limonoids from *Melia toosendan* fruit. *Journal of Ethnopharmacology*. 2008;117(3):463–6.
35. Gao T, Xie A, Liu XQ, Zhan HB, Zeng J, Dai M, et al. Toosendanin induces the apoptosis of human Ewing's sarcoma cells via the mitochondrial apoptotic pathway. *Molecular Medicine Reports*. 2019;20(1):135–40.
36. He YJ, Wang J, Liu XL, Zhang L, Yi G, Li CW, et al. Toosendanin Inhibits Hepatocellular Carcinoma Cells by Inducing Mitochondria-dependent Apoptosis. *Planta Medica*. 2010;76(13):1447–53.
37. Fan HN, Chen W, Zhu JS, Zhang J, Peng SQ. Toosendanin alleviates dextran sulfate sodium-induced colitis by inhibiting M1 macrophage polarization and regulating NLRP3 inflammasome and Nrf2/HO-1 signaling. *International Immunopharmacology*. 2019;76.
38. Zheng MD, Wang ND, Li XL, Yan J, Tang JH, Zhao XH, et al. Toosendanin mediates cisplatin sensitization through targeting Annexin A4/ATP7A in non-small cell lung cancer cells. *Journal of Natural Medicines*. 2018;72(3):724–33.
39. Li X, You M, Liu YJ, Ma L, Jin PP, Zhou R, et al. Reversal of the Apoptotic Resistance of Non-Small-Cell Lung Carcinoma towards TRAIL by Natural Product Toosendanin. *Scientific Reports*. 2017;7.
40. Dong Y, Hu YJ, Sarkar S, Zong WX, Li M, Feng D, et al. Autophagy modulator scoring system: a user-friendly tool for quantitative analysis of methodological integrity of chemical autophagy modulator studies. *Autophagy*. 2020;16(2):195–202.
41. Shi Y-L, Wang W-P. [Biological effects of toosendanin, an active ingredient of herbal vermifuge in Chinese traditional medicine]. *Sheng Li Xue Bao*. 2006;58(5):397–406.
42. Zhuo Y, Zhang YD, Li M, Wu HY, Gong SL, Hu XL, et al. Hepatotoxic evaluation of toosendanin via biomarker quantification and pathway mapping of large-scale chemical proteomics. *Food and Chemical Toxicology*. 2021;153.
43. Das CK, Linder B, Bonn F, Rothweiler F, Dikic I, Michaelis M, et al. BAG3 Overexpression and Cytoprotective Autophagy Mediate Apoptosis Resistance in Chemoresistant Breast Cancer Cells. *Neoplasia*. 2018;20(3):263–79.
44. Wu M, Zhang PH. EGFR-mediated autophagy in tumourigenesis and therapeutic resistance. *Cancer Letters*. 2020;469:207–16.

45. Zheng K, He ZD, Kitazato K, Wang YF. Selective Autophagy Regulates Cell Cycle in Cancer Therapy. *Theranostics*. 2019;9(1):104–25.

Figures

Figure 1

TSN caused LC3-II accumulation stability and suppressed autophagic degradation. (A) The chemical structure of TSN with a molecular weight of 574.62 g/mol (CAS Number: 58812-37-6). (B) Western blot analysis of LC3B-II and SQSTM1 levels in MDA-MB-231 and MDA-MB-436 cells treated with the indicated concentrations (0.01–5 μ M) of TSN for 24 h. (C) Western blot analysis of LC3B-II and SQSTM1 levels in MDA-MB-231 and MDA-MB-436 cells treated with TSN (1 μ M) at the time points indicated (0, 4, 8, 12, 24h). (D) MDA-MB-231 cells were transiently transfected with GFP-LC3 plasmid. The images were captured under Leica TCS SP8 Confocal Laser Scanning Microscope after treatment of TSN (1 μ M) or DMSO for 24 h. Scale bar: 10 μ m. 20 cells in each group were counted for data analysis. (E) Cells were transfected with a tandem reporter construct (tfLC3), and were exposed to TSN (1 μ M), CQ (30 μ M) and Torin1 (100 nM) as indicated. The co-localization of GFP-LC3 and RFP-LC3 puncta was examined by confocal microscopy. Scale bars: 10 μ m. GFP or RFP puncta were counted at least in 20 cells. (F) Cells were treated without or with BAF (100 nM) in the presence or absence of 1 μ M TSN for 24 h, the expression of SQSTM1 and LC3B-II was analyzed by western blot. Comparisons of the intensities were statistically estimated and represented as mean $\pm$ SD for 3 independent experiments (* p < 0.05, ** p < 0.01, *** p < 0.001).

Figure 2

TSN did not inhibit the fusion process of autophagosome and lysosome. (A) Schematic diagram of RFP-GFP-LC3 reporter/LAMP1 reflected the fusion of autophagosome and lysosome. (B) Immunofluorescence photographs of the co-localization of LAMP1 (blue) and stably-expressing GFP-RFP-LC3 in MDA-MB-231 cells treated with TSN (1 μ M), Torin1 (100 nM), BAF (100nM) or DMSO for 12 h. Nuclei were stained with Hoechst 33,258. Scale bar: 10 μ m.

Figure 3

TSN elevated lysosome pH and impaired lysosomal proteolytic function. (A) MDA-MB-231, MDA-MB-436 and BT-549 cells were stained with LysoSensor™ Yellow/Blue DND-160 after treatment of 1 μ M TSN, 100 nM Torin1, 30 μ M CQ or DMSO for 24 h. The excited fluorescence signal at 440/535 nm was measured by 96-well plate reader. (B, C) MDA-MB-231 and MDA-MB-436 cells were treated with 1 μ M TSN, 100 nM

Torin1, 50 nM BAF or DMSO for 24 h. Total cellular extracts were prepared and subjected to western blot using antibodies against CTSB and CTSD. GAPDH was used as a loading control. (D) LysoTracker with a final concentration of 50 nM was used to label the acid compartment for 30 min. The cells were fixed and stained with Hoechst for further Confocal Scanning. (E) Cells were pre-incubated with 10 µg/ml DQ™ Red BSA for 30 min in serum-free DMEM, then treated with 1 µM TSN, 100 nM Torin1, 50 nM BAF or DMSO for 6h. The image was collected by Incucyte S3 Live-Cell System. The results are the mean ± SD values obtained from 3 independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001). One-way ANOVA with Turkey as post hoc tests.

Figure 4

SN-38 induced autophagy in TNBC. (A) Western blot analysis of LC3B-II and SQSTM1 levels in MDA-MB-231 cells treated with the indicated concentrations (0.01-1µM) of SN-38 for 24 h. (B) Cells were transfected with a tandem reporter construct (tfLC3) and exposed to SN-38 (0.1 µM) or DMSO treatment. The co-localization of GFP-LC3 and RFP-LC3 puncta was examined by confocal microscope and was counted at least 20 cells. Scale bars: 10 µm. The results are the mean ± SD values obtained from 3 independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 5

Autophagy inhibition in combination with chemotherapy is more effective than monotherapy in inducing apoptosis in TNBC. (A) Cells were transfected with a tandem reporter construct (tfLC3), and were exposed to TSN (1 µM), SN-38 (0.1 µM) or combination. The co-localization of GFP-LC3 and RFP-LC3 puncta was examined by confocal microscopy, in at least 20 cells. Scale bars: 10 µm. (B) MDA-MB-231 cells were

treated with various concentrations of SN-38 for 24h, then add TSN for another 24h. MTT assays were performed to assess cell viability. (C) MDA-MB-231 cells were treated with 0.1 μ M of SN-38 for 24h, then add 0.1 μ M TSN for another 24h. Cell morphology was collected by Incucyte S3 Live-Cell System. Scale bars: 200 μ m. (D) Cell death was measured by LDH release, and the relative LDH activity in MDA-MB-231 cells treated with the indicated concentrations of TSN (0.1 μ M) with or without SN-38 (0.1 μ M) for 24 h. (E) MDA-MB-231 cells were treated with SN-38 (0.1 or 1 μ M) for 24h. Then, TSN was added into the plate in the presence of SN-38 for another 24h. PI positive late apoptotic/necrotic cells were used for quantitative analysis. (F) Western blot analysis of cleaved caspase 3 in MDA-MB-231 cells treated with or without SN-38 (0.1 μ M), TSN (0.1 μ M) for 24 h. The results are expressed as the mean $\pm$ SD values obtained from 3 independent experiments (* p < 0.05, ** p < 0.01, *** p < 0.001).

Figure 6

TSN inhibited autophagic flux and repressed TNBC xenograft growth in vivo. (A) Images showed the tumors tissues isolated from mice receiving vehicle, irinotecan (10 mg/kg), TSN (0.5 mg/kg) or a combination of irinotecan and TSN treatment. (B) Body weight changes of mice during 28 d of exposure. (C) Average tumor volume calculation. Error bars represent means $\pm$ SD. (D) Tumor weight measurement at day 28. (E) Tumor tissues were sectioned and stained with hoechst. The fluorescent images were taken under a confocal microscope. (F) Western blot analysis of LC3B-II and SQSTM1 in tumor tissues (n=3) and the densitometric analysis of LC3B-II and SQSTM1 in tumor tissues. (G) Tumor tissues were sectioned and subjected to immunostaining of cleaved caspase 3 for evaluating apoptosis. Scale bars: 100 μ m.

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