Salvia Chinensia Benth Inhibits Triple Negative Breast Cancer Progression Through Inducing DNA Damage Pathway

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Research Article

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Abstract

Background: *Salvia chinensis* Benth (SCH) is an herb that has extensively demonstrated antitumor attributes in multiple tumors. However, there are only marginal reports about the impacts of SCH on TNBC-triple negative breast cancer tumorigenesis. Herein, our current research reported the in vitro effects as well as the in vivo antitumor effect, and the fundamental mechanisms of SCH on TNBC.

Methods: In vitro, we measured the antitumor effect of SCH on TNBC through clonogenic assay, cell viability, flow cytometry, apoptosis assay, migration and invasion assay. Meanwhile, breast cancer xenografts with the aid of MDA-MB-231 cells were established to evaluate the therapeutic effect of SCH on TNBC in vivo. To investigate the molecular mechanisms of SCH, bioinformatic analysis, specifically network pharmacology-based analysis, was performed. Then, the comet assay and western blot analyses were applied to confirm the influence of SCH on the DNA damage and repair pathways.

Results: Our results demonstrated that the application of SCH could lead to the reticence of cell proliferation, migration, invasion and the induction of cellular apoptosis caused in TNBC cells. Based on the bioinformatic analysis, we discovered two key compounds of SCH, quercetin and beta-sitosterol, and detected significant synergistic effects of the combined treatment of them on TNBC, especially on the DNA damage of TNBC cells as judged by comet assays. Moreover, substantial inductions of DNA damage were examined after SCH treatment in TNBC cells, as well as quercetin, and beta-sitosterol treatment. Apart from inducing DNA damage, SCH specifically decreased inter-strand crosslink repair pathway and the double-strand breach repair perforated by the homologous recombination pathway. Similar results were also obtained with quercetin and beta-sitosterol treatment.

Conclusion: Taken together, the current investigation elucidates the novel discernment into the therapeutic function of SCH as a DNA damage agent for treatment of TNBC.

Background

Despite tremendous progress in diagnostic and therapeutic schemes, breast cancer prevails as the foremost basis of cancer-associated mortality in women. Around 15 to 20% of the women with breast cancer are identified as TNBC-triple negative breast cancer, which are portrayed by the lack of countenance of ER, PR, or HER2. TNBC patients are characterized by an elevated risk of relapse and metastasis in the first 3 to 5 years after preliminary diagnosis [1]. Primary and acquired resistance to therapies are the major hurdles in the treatment of TNBC. The fundamental mechanism is acknowledged that cancer cells tend to rely on alternative signaling pathways when one signaling node is inhibited. Therefore, developing multi-target strategies might be more useful than targeting single node.

Herbs has been playing an important role in adjuvant therapies of TNBC, especially in advanced metastatic tumors. Previous studies demonstrated that herbal medicine, combined with chemotherapy or radiotherapy, could improve anti-tumor effects and attenuate adverse actions [2, 3]. *Salvia chinensis* Benth [SCH, also referred to as *Salvia chinensis* herbal or Chinese Sage (Shijianchuan)], a plant included

under the Labiatae family, was largely chronicled in the Compilation of Materia Medica (Ming Dynasty, A.D. 1590). SCH was availed in the treatment of ostealgia and swollen carbuncles [4]. Previous *in vitro* experiments have shown that SCH exerted anticancer activity in breast, gastric, nasopharynx, lung, colon, liver and pancreatic cancer [5, 6]. However, there are limited reports about the effects and the underlying mechanisms of SCH in TNBC.

In this study, we demonstrated that SCH could suppress TNBC cell propagation and encourage apoptosis, both *in vitro* and *in vivo*. Moreover, we identified two key ingredients of SCH, quercetin and beta-sitosterol. In addition, we revealed the potential molecular mechanisms of SCH by the methods of Network pharmacology. As for molecular mechanism, we found that the SCH exerted antitumor activity likely through regulating DNA damage related signaling networks via the synergism of quercetin and beta-sitosterol.

Methods

Cells and Reagents

Cell lines human-TNBC-MDA-MB-231, HCC1187 and the mouse TNBC cell lines 4T1 were procured from ATCC, USA, and were sustained in culture medium (MDA-MB-231 in DMEM (Gibco, C11995500BT), HCC1187 and 4T1 in RPMI-1640(Gibco, C11875500BT)) supplemented with 10% fetal bovine serum (FBS, Biological Industries, 04-001-1ACS) and 100units x ml⁻¹ per penicillin/streptomycin (Hyclone, SC30010) at 5% CO₂ and mesophilic temperature of 37°C in an incubator and were substantiated with the aid of STR (short-tandem-repeat) further they were confirmed intermittently for the absence of mycoplasma. Quercetin and Beta-Sitosterol were purchased from MedChemExpress (MCE, HY-18085 and HY-N0171A). In order to procure the aqueous extracts of SCH, shredded herb was boiled with 10X water for 2h (duplicated), subsequently with freeze-drying of the concentrate. The resultant dry-powder was preserved at -20°C. Doses used in the current investigation were aliquoted in equivalent weight of raw herb versus of that of the vehicle per ml.

Western blot analysis

RIPA-buffer augmented with protease and phosphatase inhibitors administered for the cell lysis [7]. The proteins were elucidated by SDS-PAGE and were then shifted to Nitrocellulose Blotting membranes (GE Healthcare life Science, Germany). The spots were examined with the antibodies [PARP (Cell Signaling technology, CST, 9542), vinculin (Abcam, ab129002), GAPDH (Huabio, M1310-2), ATM (Santa Cruz Biotechnology, sc135663), p-ATM(Ser1981) (CST, 5883), H2AX (Santa Cruz Biotechnology, sc517336), p-H2AX(Ser139) (Santa Cruz Biotechnology, sc517348), CHK1 (Santa Cruz Biotechnology, sc8408), p-CHK1(Ser345) (CST, 2348), P53 (Santa Cruz Biotechnology, sc126), p-P53(Ser15) (CST, 9286), ATR (Santa Cruz Biotechnology, sc515173), p-ATR(Ser428) (CST, 2853), FANCD2 (Santa Cruz Biotechnology, sc20022), RAD51 (Santa Cruz Biotechnology, sc398587)].

Migration and invasion assay

Cell migration and invasion were appraised as per the instructions of manufacturer (Corning, USA) via transwell assay kits procured. Fleetinglly, the chambers were inundated with BD Biosciences-Matrigel (California, USA) for invasion assay or without Matrigel for migration assay. The serum-restricted medium (5×10^3 cells/200ul cell suspension) was supplemented into the upper chamber with the SCH (100 mg ml^{-1}) medium holding 10% FBS in the lower chamber per well. After 2days of incubation, the non-invading cells were lightly expunged, and the invading cells stained with 0.5% crystal violet were photographed with inverted phase-contrast microscopy (Olympus corporation, Tokyo, Japan). Pictures were taken by microscope (Lecia, DMI1) at x100 magnification. Cell counts were assessed from 5 random microscope fields for every group.

Cell viability and Flow cytometry

The cells (10^3 cells/well) were incubated in 96-well plates. Then, the viability of cell was gauged with the aid of CCK-8 (Bimake, B34304). SpectraMax 190 (Molecular Devices, USA) was used to assay the optical density (OD) at 450 nm. Flow cytometric scrutiny of Apoptosis was perceived by Annexin V and PI staining via the Annexin V-APC Apoptosis-Detection-Kit (KeyGEN BioTECH, KGA1030). The examination was accomplished on BD FACSCanto™ II (BD Biosciences, USA).

Apoptosis assay

The cells were plated at 40% convergence and were permitted to proliferate overnight and then exposed to the SCH. To enumerate apoptosis, DAPI staining and fixing was tasked as delineated [8]. The images were captured with a Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Clonogenic survival assay

The cells (10^4 cells/well) were incubated in 12-well plates and were cultured continuously and every third day the media was renewed. After culturing, the cells stained with 0.5% crystal violet were subsequently washed with PBS and dried, finally dissolved with 10% acetic acid. The OD was measured at 590 nm with the aid of SpectraMax 190 (Molecular Devices, USA).

Comet assay

DNA damage was assayed by performing comet assay [9], next, using alkaline lysis buffer, the cells were lysed and electrophoresed (buffer counteracted with PBS), and stained, assessed with Gold View (Coolaber, SL2140). Ultimately, cells were documented with Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and occurrence of the DNA in tail (percentage) apportioned as a quantitative scale of DNA damage using CASP (Comet Assay Software Project Lab) software.

Establishment of tumor xenografts and in vivo treatments

Protocol of animal experiment has been endorsed by the Animal Research Committee of Dalian Medical University. To develop breast cancer xenografts, the MDA-MB-231 cells (6×10^6) were subcutaneously inoculated into female nude mice. At the culmination of the 7th day implantation, mice were administered SCH (200 mg kg^{-1} per body weight) on a daily basis or quercetin (75 mg kg^{-1}) every day or Beta-Sitosterol

(75mg kg⁻¹) each day via oral gavage for 3 weeks. The tumor volumes were calculated every 5 days with the aid of calipers and were quantified using the formula: tumor volume = (length X width²)/2. In the end, mice were treated by the method of carbon dioxide asphyxiation for euthanasia: mice were put into a clean container with carbon dioxide in a slowly increasing concentration. After about 10 minutes, mice died slowly and painlessly.

Network pharmacology analysis

The chemical ingredients and target genes of SCH were scrutinized based on the Traditional Chinese Medicine Systems Pharmacology database (TCMSP). Markers such as Oral bioavailability $\geq 30\%$ and drug likeness ≥ 0.18 were earmarked as the thresholds corresponding to the regulations of TCMSP. TNBC related genes were collected from Gene Cards (www.genecards.org) and Online Mendelian Inheritance in Man (OMIM). After collecting the data of genes of interest pertaining to SCH and TNBC, the shared genes between SCH-related genes of TNBC were analyzed by Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and the protein-protein interaction (PPI) network analyses as described [10].

Statistical analysis

All the result values are declared and denoted as mean $\pm$ SD (Standard Deviation). GraphPad-Prism software was employed to calculate the unpaired two-sided t-tests, one-way ANOVA and Tukey's multiple comparison tests (both in vitro and the in vivo animal studies). P-value < 0.05 was assumed as statistically substantial. CalcuSyn 2.0 software (Biosoft) was used for the drug interactions assessment (Chou-Talay method). Additive effect was reflected for the Combination index (CI) = 1 value, whereas synergism noted for CI < 1 ; antagonism for CI > 1 .

Results

Aqueous extract of *Salvia chinensis* Benth induces apoptosis of TNBC cells

To determine the in vitro cytotoxicity of SCH toward human TNBC cells (MDA-MB-231, HCC1187) and mouse TNBC cells 4T1, we examined the potent inhibition on the viability by clonogenic survival assay. Short-term (3 ~ 6 days) and long-term (9 ~ 15 days) inhibitory effects of SCH extract were validated on TNBC cells in a dosage dependent aspect (Fig. 1a). Supplementary to scrutinize the antitumor activity of SCH in TNBC, we conducted the migration and invasion assays. Indeed, SCH inhibited cellular migration and invasion 24 ~ 48 hours after treatment (Fig. 1b). The effect of SCH on cell survival was also noted and was established that the SCH treatment encouraged apoptotic cell death, as judged by the clear formation of nuclear fragmentation, chromatin condensation and cytoplasmic shrinkage [8] (Fig. 1c-d). Moreover, we confirmed the apoptotic effect of SCH on TNBC cells using flow cytometry (Fig. 1e-f). Altogether, these results suggested that SCH has strong antitumor activity in TNBC cells.

Analysis of Potential Mechanisms of *Salvia chinensis* Benth in TNBC

Based on TCMSP database, the quercetin and beta-sitosterol were screened as the key ingredients of SCH (Table 1), and 83 genes were established as the candidate genes of SCH. A total of 4129 genes related to TNBC were obtained from Gene Cards and OMIM database. Subsequently, we identified 65 common genes between SCH targeted genes and TNBC related genes (Fig. 2a). PPI network analysis of common genes using the STRING database showed multiple hub genes with the most interactions, including EGFR, VEGFA, IL-6, CCND1, MYC, CASP3, AR, PPARG, RELA, ERBB2, FOS, ICAM1, NOS3, PRKCA, RB1, CASP8, CYP1A1, NFE2L2, AHR, CAV1, CCNB1, PGR, VCAM1, CRP, ERBB3, GSTP1, IGF2, NQO1, CASP9, and CYP1B1 (Fig. 2b-c). GO analysis showed that the most common genes were involved in various biological processes, such as response to xenobiotic stimulus, response to steroid hormone and response to hypoxia (Fig. 2d). In addition, KEGG analysis presented the SCH-related pathways in TNBC including p53 signaling pathway, apoptosis pathway, and so on (Fig. 2e). Among these significant pathways, the p53 signaling pathway attracted our attention. Stimulation of p53-mediated transcription is a crucial-cellular reaction to DNA damage, culminating in cell cycle arrest and reticence of cell proliferation [11]. Interestingly, recent studies reported that p53 pathway was activated by quercetin and beta-sitosterol treatment [12]. Taken together, these results indicated an impact of SCH on the DNA damage and repair regulation in TNBC cells by its key compounds quercetin and beta-sitosterol.

Quercetin and Beta-sitosterol have synergistic inhibition on TNBC cells

In terms of the network pharmacology analysis, we investigated the effects of quercetin and beta-sitosterol on TNBC cells by cell viability assays. MDA-MB-231, HCC1187, and 4T1 cells were consequently administered with varying concentrations of quercetin and beta-sitosterol. This combo-treatment culminated in a significant dose-susceptible inhibition of cell viability (Fig. 3a). The combination index exposed a robust synergism of the combo-treatment, contrary to a single agent, with an index $CI < 1.0$ for the entire doses tried (Fig. 3b). In addition, the synergism of the combination was validated by the clonogenic survival assay for 3 days (Fig. 3c-d). At the molecular level, the synergistic inhibition of TNBC viability was accompanied with a substantial induction of cell apoptosis (Fig. 3g-h) and activation of cleaved-PARP (Fig. 3e-f) in combo-treatment contrary to single-agent therapy. Collectively, these results suggest a favorable scientific basis of SCH treatment for TNBC.

Salvia chinensis Benth induces DNA damage and impairs DNA damage repair in TNBC cells

Bioinformatic analysis revealed that SCH potentially influenced the DNA damage pathway. Thus, we further examined the impact of SCH on DNA damage by comet assays. We observed that the SCH treatment caused substantially elevated DNA damage in a dose-dependent manner as evaluated by the percentage of DNA in the tails of MDA-MB-231 cells (Fig. 4a-b). Accordingly, we next investigated the

effect of quercetin and beta-sitosterol on TNBC. Compared to a single agent, the combination of quercetin and beta-sitosterol was verified to cause strong synergistic induction of DNA damage in MDA-MB-231 and HCC1187 cells (Fig. 4c-e). Altogether, these data prompt us to further determine the inherent mechanism of the modification of DNA damage led by SCH in TNBC cells.

Therefore, we conducted the immunoblotting analysis to elucidate the impact of SCH, and its active ingredients, quercetin and beta-sitosterol, on the moderation of DNA damage and repair markers. Specifically, we observed the trigger of DNA damage (assessed by p-H2AX^{ser139}, p-ATM^{ser1981}, p-Chk1^{ser345}, p-ATR^{ser428}) and p53 activation in SCH-treated MDA-MB-231 cells, together with repression of DNA damage repair (gauged via Fanconi Anemia pathway-related protein FANCD2), as well as DDR proteins such as RAD51 (Fig. 4f). Conforming with these findings, we also noted that the quercetin and beta-sitosterol had strong synergistic activation of DNA damage markers and inhibition of DNA damage repair markers compared to single agent (Fig. 4f). Together, these results suggest that SCH suppresses TNBC progression by the synergism of quercetin and beta-sitosterol on the induction of DNA damage.

Salvia chinensis Benth suppresses TNBC xenograft tumor growth in vivo

Subsequent to establishing that the SCH inhibits TNBC cell proliferation and survival in vitro, scrutiny of in vivo efficacy of SCH was explored with human-xenograft mouse-model with the aid of MDA-MB-231 cells. After tumor establishment, the mice were cured with vehicle, quercetin, beta-sitosterol and SCH as described in methods. We found that SCH treatment led to substantially attenuated tumor growth compared to mice recipients of the vehicle or with a single agent (quercetin or beta-sitosterol) as shown (Fig. 5a-c). Furthermore, immunoblotting examination of cell lysates from the procured tumors verified the effects on DNA damage and repair pathway (Fig. 5d). Together, these results suggest that SCH inhibited TNBC cell growth through regulating DNA damage and repair pathways in vivo.

Discussion

Most recently, the antitumor activity of SCH has been reported in literature for different types of tumors, in vivo as well in vitro experiments have exposed the effects of SCH on proliferation, angiogenesis, apoptosis and chemotherapy resistance of cancer cells, including hepatocellular carcinoma, gastric cancer, nasopharyngeal carcinoma, luminal breast cancer and so on [4, 6, 13–16]. Here, for the first time, we uncover a noteworthy antitumor activity of SCH in TNBC. In the present work, we confirmed the inhibitory properties of SCH on TNBC cell proliferation, migration and invasion, compared with the induction of cell apoptosis, in dose- and time-dependent manners. Additionally, in the TNBC xenograft mouse models, SCH significantly suppressed neoplasm growth, which is consistent with in vitro results.

Limited studies have investigated the underlying mechanism of SCH for oncotherapy. Interestingly, our work first demonstrates that application of SCH resulted in significant DNA damage as evidenced by the elevated levels in comet tails, which is consistent with bioinformatics analysis. Based on TCMSP

database, we identified the key compounds of SCH, quercetin and beta-sitosterol. Strikingly, the combination treatment of quercetin and beta-sitosterol indeed led to a synergistic suppression of cell viability, as well as triggering of apoptosis and caspase activation. The common pathways for quercetin to hinder tumor progression are via antioxidative action, curtailing of growth, trigger apoptosis, declining in inflammation and deterrence of angiogenesis [17–24]. Meanwhile, beta-sitosterol is a plant sourced metabolite with anticancer activity against breast, prostate, colon, lung cancer and so on. Recent literature reported that beta-sitosterol interfered with the cell cycles, impacted various signaling pathways, and had a role in apoptosis, proliferation, survival, invasion, angiogenesis, metastasis and inflammation [25–29]. However, the effects on DNA damage of quercetin or beta-sitosterol has not been thoroughly investigated yet. In the current study, a more substantial trigger of DNA damage together with curtail of DNA repair in TNBC was established, after the combined treatment of quercetin and beta-sitosterol than single-agent therapies.

To inspect the molecular mechanics intrinsic via SCH lethality in TNBC, bioinformatics tools were engaged. We demonstrated that p53 pathway could be the candidate target of SCH. The activation of tumor suppressor p53 is renowned to be the chief mechanism of apoptosis triggered due to DNA damage [30]. The proteins, transmitting signals from DNA damage and cell cycle checkpoints to DNA repair pathways, are consist of ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), CHK1, H2AX, RAD51 and FANCD2. ATM is recruited to double-strand break (DSB) and execute checkpoint signaling. ATR is activated by replication stress, where it facilitates fork stabilization and restart. CHK1 and H2AX are effector kinases that function at downstream of ATR and ATM. RAD51 is directly involved in DSB repair processes of homologous recombination. The activation of FANCD2 marks as the major activation switch for the FA pathway in the process of inter-strand crosslink repair [31, 32]. In line with our hypothesis, a significant increase has been observed in the phosphorylation of CHK1, H2AX, ATM and ATR, culminating in the stimulation of p53 in cells treated with SCH. Consequently, SCH pointedly impacted the expression of genes in FANCD2-related and RAD51-related DDR pathways bolstering the trigger of DNA damage in a dose-dependent manner. Indeed, our additional data indicate a strong synergistic activity in the phosphorylation of the genes involved in DNA damage and the inhibition of the genes related to DNA repair following the combination of quercetin and beta-sitosterol versus single-agent therapies. Concordantly, the changes of proteins following SCH treatment observed in vitro were shown in tumor tissues of TNBC xenograft mouse models.

Conclusion

In summary, our findings highlight the anti-proliferative activity of SCH treatment in TNBC, with the trigger of apoptosis, DNA-damage reaction and DNA repair pathway impairment and a strong synergistic role displayed via the combination of quercetin and beta-sitosterol. The current study provides a rationale for the future application of SCH in TNBC. Nevertheless, the antitumor effects of SCH combined with other DNA damage agents such as PARP inhibitors require further studies.

Abbreviations

SCH: *Salvia chinensis* Benth, TNBC: Triple negative breast cancer, ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2, STR: Short-tandem-repeat, OD: Optical density, TCMSP: Traditional Chinese Medicine Systems Pharmacology database, OB: Oral bioavailability, DL: Drug likeness, OMIM: Online Mendelian Inheritance in Man, GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes, PPI: Protein-protein interaction, SD: Standard Deviation, CI: Combination index, DDR: DNA damage repair, ATM: Ataxia-telangiectasia mutated, ATR: ATM- and Rad3-related, DSB: Double-strand break, PARP: Poly ADP-ribose polymerase.

Declarations

Ethics approval

Protocol of animal experiment has been endorsed by the Animal Research Committee of Dalian Medical University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Research design, M.L. and Z.Z., Experiment operation, K.W. and Y.H., Data analysis, K.W., L.X. and C.S., Manuscript preparation, K.W., S.Z., S.S. and G.H., Thorough reading and final approval of the version, M.L. and J.Z. to be published.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Figure 1

SCH inhibits TNBC cell growth and activates apoptotic cell death. a. TNBC cells were subjected to varying dosages of SCH for 3, 6, 9, 12 and 15 days and stained via crystal violet (media was replenished every third day). b. Representative images of the migration and invasion after 100mg/ml SCH treatment for 24 and 48 hours with transwell assay. c. Cell apoptosis induced by 100mg/ml SCH of TNBC cells was quantified by DAPI staining. The images are shown at 48 hours. d. Cell counts for the responding groups of at least three random microscope fields (x100 magnification). Apoptotic cells are expressed as a percentage of every microscope field. Results exhibited as mean±S.D. from three separate trials. ** $P < 0.01$, *** $P < 0.001$ (Student's t test). e. Apoptotic cell death was evaluated via flow cytometric analysis subsequently DAPI and FITC-Annexin V staining in cells treated with varying dosages of SCH for 48h. f. The % of Annexin V+/PI- (early apoptotic cells, lower right), Annexin V+/PI+ (late apoptotic cells, upper right), Annexin V-/PI- (viable cells, lower left) and Annexin V+/PI+ (necrotic cells, upper left) cells are displayed. Columns epitomize the % of Annexin V positive TNBC cells. ns-not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

Figure 2

Figure 2

Bioinformatic analysis divulge latent targets of SCH. a. C-T network of SCH. The green ellipse represents target genes. The purple rectangle, orange diamond and yellow triangle stand for compounds. b. A PPI network was created with the aid of the STRING database. c. The counts of the interaction lines of each gene are shown. d. GO enrichment analysis of target genes of SCH. Pathways with attuned p -value < 0.05 are displayed. e. KEGG pathway enrichment scrutiny of target genes of SCH. Pathways with attuned p -value < 0.05 are shown.

Figure 3

Figure 3

The combination of quercetin and beta-sitosterol induces synergistic growth inhibitory effects on TNBC cells. a. TNBC cells were simultaneously treated with quercetin and/or beta-sitosterol for 48h. Cell survival was measured via CCK-8 and depicted as percentage of cell-viability versus the control. Results are presented as mean $\pm$ S.D. from three separate trials. ** P <0.01, *** P <0.001 (Student's t test). b. The interface between quercetin and beta-sitosterol was gauged with the aid of CalcuSyn software. Heatmaps depict the combination index (CI). c. TNBC cells were uninterruptedly treated with quercetin and/or beta-sitosterol for 72 hours and assayed with crystal violet staining. Depictive pictures of the plates are provided. d. The quantification of clonogenic survival assay is shown as mean $\pm$ S.D. from three independent experiments. ns, not significant, ** P < 0.01, *** P < 0.001 (Student's t test). e. TNBC cells were treated with quercetin and/or beta-sitosterol for 24 hours and then subjected to an immunoblotting analysis. f. The quantification data of relative cleaved-PARP intensities from vinculin. Results are shown as mean $\pm$ S.D. from three independent experiments. ns, not significant, * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's t test). g. Apoptotic cell death was assessed by flow cytometric analysis following DAPI and FITC-Annexin V staining in cells treated with quercetin and/or beta-sitosterol for 48 hours. h. The percentage of Annexin V+/PI- (early apoptotic cells, lower right), Annexin V+/PI+ (late apoptotic cells,

upper right), Annexin V-/PI- (viable cells, lower left) and Annexin V+/PI+ (necrotic cells, upper left) cells are shown. Columns represent the percentage of Annexin V positive TNBC cells. ns, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

Figure 4

Figure 4

SCH treatment results in induced DNA damage and suppressed DNA repair via the synergistic impacts with the combination of quercetin and beta-sitosterol on the DNA damage and repair pathway. a. Comet assay (Scale bar, 100µm) was executed to appraise DNA damage in MDA-MB-231 cells administered with varying dosages of SCH for 48h. b. The DNA-quantification in the tail from three separate trials is revealed as mean $\pm$ S.D., ns-not significant, *** $P < 0.001$ (Student's t test). c. Comet assay (Scale bar, 100µm) was performed to evaluate DNA damage in TNBC cells treated with quercetin and/or beta-sitosterol for 48 hours. DNA in the tail of MDA-MB-231 cells (d) and HCC1187 cells (e) are quantified from three independent experiments as shown as mean $\pm$ S.D. ns, not significant, *** $P < 0.001$ (Student's t test). f. MDA-MB-231-whole-cell lysates administrated with varying concentrations of SCH, quercetin and/or beta-sitosterol for 24h subsequently immunoblotting analysis and explored with designated antibodies.

Figure 5

SCH therapy induces significant tumor regression in vivo. a. NOD/SCID mice were subcutaneously injected with MDA-MB-231 cells. b. Representative images show the dissected tumors in distinct treatment groups as specified at the endpoint. c. The tumor growth curve of xenografts treated with SCH (200mg/kg body weight) daily or quercetin (75 mg/kg) daily or Beta-Sitosterol (75mg/kg) daily or vehicle via oral gavage for 3 weeks. The data presented as mean $\pm$ S.E.M. $n=5$ for each group. *** $P < 0.001$ (one-way ANOVA, with Turkey's multiple comparison tests). d. The tumor lysates were collected, and the protein profusion was established by immunoblotting analysis.

Supplementary Files

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