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Saikosaponin A Induces Apoptosis in HCT116 Colorectal Cancer Cells *via* ROS-Mediated ER Stress and p38/JNK MAPK Pathway Activation: *in Vitro* and *in Vivo* Evidence

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ABSTRACT: Colorectal cancer (CRC) is the third most prevalent cancer globally, with limited effective treatment options and significant chemotherapy resistance. Saikosaponin A (SSA), a triterpenoid saponin from *Radix Bupleuri*, exhibits anti-inflammatory and antitumor properties, but its mechanism of action in CRC remains undefined. This study aims to investigate SSA's antitumor effects against HCT116 CRC cells and elucidate the underlying molecular mechanisms through integrated omics analysis and functional validation. SSA selectively inhibited HCT116 proliferation and colony formation with minimal toxicity to HEK293 cells. SSA suppressed xenograft tumor growth by 54% (10 mg/kg) without affecting body weight or organ histology. Integrated omics revealed 6,033 DEGs and 39 DEMs, with 81 genes and 37 miRNAs significantly enriched in the MAPK pathway. SSA induced apoptosis in a dose-dependent manner, increasing ROS production 3.2-fold and decreasing MMP. Mechanistically, SSA activated p38 and JNK MAPK pathways, upregulated CHOP-mediated ER stress, and modulated apoptosis proteins (decreased Bcl-2, increased cleaved caspase-3 and -9). Network analysis identified miR-132-3p as a key regulator of MAPK hub genes (KRAS, SOS1, BRAF). SSA exerts potent antitumor activity against HCT116 CRC cells, representing a promising therapeutic candidate for CRC treatment.

Keywords: Saikosaponin A, colorectal cancer, apoptosis, endoplasmic reticulum stress, MAPK signaling

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy and second leading cause of cancer-related mortality worldwide, representing a major public health challenge^[1]. Alarming, the median age of CRC patients has decreased due to dietary and lifestyle changes, with early-onset CRC representing 10-15% of new cases^[2]. While early-stage disease is often asymptomatic, advanced CRC presents significant morbidity and metastatic spread, limiting therapeutic options^[3]. Current conventional treatments including surgery,

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chemotherapy, and immunotherapy frequently yield suboptimal outcomes due to acquired resistance, severe toxicity, and off target effects^[4]. Consequently, there is an urgent need for multi-targeted, low-toxicity therapeutic agents derived from natural sources.

Traditional Chinese Medicine (TCM) has emerged as a valuable complementary approach in oncology, demonstrating efficacy in mitigating chemotherapy side effects, reversing drug resistance, and suppressing metastasis through multi-pathway regulation^[5, 6]. *Radix Bupleuri (RB)*, a fundamental herb in TCM formulas, contains Saikosaponin A (SSA; C₄₂H₆₈O₁₃, MW 781 g/mol) as its primary bioactive triterpenoid saponin^[7]. SSA exhibits diverse pharmacological activities, including anti-inflammatory, neuroprotective, and antitumor effects across multiple cancer types^[8-10]. Notably, previous studies have established SSA-induced apoptosis in neuroblastoma through mitochondrial pathways^[11], in pancreatic cancer via EGFR/PI3K/Akt inhibition^[12], and in acute myeloid leukemia through ER stress-mediated JNK activation^[13]. However, these mechanism studies remain confined to non-CRC malignancies. Importantly, a comprehensive review by Xu et al^[14] documented that SSA exhibits cytotoxicity against HCT116 colon cancer cells yet noted that underlying molecular mechanisms remain uncharacterized. Consequently, whether SSA modulates specific miRNA-mRNA interactions, activates ROS-mediated ER stress, or converges on MAPK signaling in CRC cells remains entirely unknown, representing critical barriers to translating SSA as a mechanism-based CRC therapeutic.

The pathogenesis of CRC involves complex dysregulation of signaling networks, including hyperactivated Wnt/ β -catenin, PI3K/AKT, and MAPK pathways, alongside loss of tumor suppressors (APC, TP53)^[15]. MicroRNAs (miRNAs), small non-coding RNAs (~22 nt), critically regulate mRNA stability and translation, functioning as oncogenes or tumor suppressors in CRC progression^[16]. Specifically, oncogenic miRNAs such as miR-21 and miR-155 are frequently upregulated in CRC, promoting proliferation, invasion, and chemoresistance by targeting PTEN, PDCD4, and SOCS1. Conversely, tumor-suppressive miRNAs including miR-34a and the let-7 family are commonly downregulated, leading to derepression of oncogenic targets such as c-Myc, KRAS, and BCL2^[16]. Importantly, these miRNA alterations converge on the dysregulation of MAPK and PI3K/AKT signaling pathways, which drive malignant phenotypes in CRC^[17]. Emerging evidence indicates that food-derived bioactive components, particularly triterpenoid saponins, exert antitumor effects through miRNA-mediated regulation of MAPK and PI3K/AKT pathways in CRC^[18]. However, the specific miRNA-mRNA regulatory landscape modulated by SSA in CRC cells has not been investigated. This gap is particularly significant given that miRNA-network analysis is essential for identifying multi-targeted mechanisms and predictive biomarkers for TCM-derived therapeutics^[19].

Apoptosis is the primary pathway leading to tumor cell death, representing a finely regulated form of programmed cell death that critically influences the maintenance of cellular homeostasis^[20]. Reactive oxygen species (ROS) play a dual role in cancer, where moderate levels promote proliferation but excessive ROS triggers mitochondrial dysfunction and endoplasmic reticulum (ER) stress-induced apoptosis^[21, 22]. The unfolded protein response (UPR) mediated by PERK, IRE1 α , and ATF6 pathways initially promotes survival

but activates CHOP-mediated apoptosis under chronic ER stress^[20, 22]. Importantly, ROS production and ER stress converge on MAPK signaling (p38 and JNK) to execute apoptosis^[22]. While SSA activates ER stress-JNK signaling in leukemia cells^[13] and other saponins induce ROS-dependent MAPK activation in CRC^[22], the integration of these pathways in SSA-treated CRC cells remains uncharacterized.

This study aims to systematically investigate the anti-CRC efficacy of SSA and elucidate its underlying mechanisms through integrated miRNA-mRNA network analysis and functional validation of ROS-mediated ER stress and MAPK signaling pathways. We hypothesize that SSA induces CRC apoptosis by simultaneously modulating the miRNA-mRNA regulatory network, the ROS-mediated ER stress and MAPK pathway. By combining miRNA-mRNA integrated analysis with mechanistic validation of stress signaling, this work elucidates the multi-targeted mechanisms of a TCM-derived bioactive compound in CRC, providing a scientific basis for the development of functional food ingredients and natural health products with potential application in colorectal cancer prevention and adjuvant therapy.

2. Materials and methods

2.1. Chemicals

Saikosaponin A (SSA, 99.33% purity) was provided by Chengdu Mansite Co., Ltd. (Chengdu, China). Z-DEVE-FMK, 4-PBA, SB203580, SP600125, and NAC were supplied by MedChemExpress (Elizabeth, USA). Macklin (Shanghai, China) supplied additional chemical reagents of analytical grade.

2.2 Cell Culture

HCT116 cells were purchased from Puricella (Wuhan, China) and maintained in McCoy's 5A medium (KeyGEN BioTECH, Nanjing, China) containing 3 g/L glucose. Human embryonic kidney cell line HEK293 was obtained from Kaijie (Nanjing, China) and cultured in DMEM (BioChannel, Nanjing, China) containing 4.5 g/L glucose. Both media were supplemented with 10% fetal bovine serum (Gibco, California, USA) and 1% penicillin/streptomycin (Yuanye, Shanghai, China). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. All functional assays and drug treatments were performed using HCT116 cells in McCoy's 5A medium to ensure experimental consistency^[23].

2.3 Cell Viability Assay

HCT116 cells (1.0×10^4 cells/well) were seeded into 96-well plates and cultured overnight at 37°C before co-incubation with SSA (0 - 40 μM). Cell proliferation was measured by employing the Cell Proliferation and Toxicity Detection Kit (CCK-8) (Meilun Bio, Dalian, China)^[23].

2.4 Cell Colony Formation Assay

Cells exposed to SSA at 0, 24, 26, and 28 μM (500 cells/well) were plated into 6-well plates and maintained in continuous culture. After colony formation, wells were washed, fixed, and stained with crystal violet (Becton Dickinson)^[24]. Colony numbers were counted using ImageJ software.

2.5 Xenograft Model

Male BALB/c nude mice (4 - 6 weeks old, 14 - 21 g) were purchased from Speifu (Suzhou) Biotechnology Co., Ltd. and housed in an SPF facility (22°C, 60% humidity, and a daily 12-hour light-dark photoperiod). The HCT116 colon cancer xenograft model was created through subcutaneous administration of a cell suspension (1×10^7 cells/mL) into the right flank of nude mice^[25]. When xenograft tumors reached 100 mm³, tumor-bearing mice were randomly allocated into 3 groups with 5 animals per group. Every other day for 18 days, mice received intraperitoneal injections of SSA (10 mg/kg or 5 mg/kg) or 0.1% dimethyl sulfoxide (DMSO)^[26]. Tumor relative size and nude mouse body weight were continuously recorded throughout the experiment. Upon completion of experimental completion, blood samples were collected via cardiac puncture for serum biochemical and hematological analyses, and animals were subsequently euthanized. Serum levels of ALT, AST, creatinine, and BUN were measured using an automatic biochemical analyzer. Hematological parameters including WBC, Neu%, Lym%, RBC, HGB, and HCT were analyzed using an automated hematology analyzer. Tumors were removed, photographed, weighed, and processed for TUNEL staining. Major organs and colon tissues were harvested and prepared for H&E staining.

2.6 RNA Extraction, Library Preparation, and Sequencing

Untreated cells and cells treated with SSA (26 μ M) were completely lysed using Trizol reagent. Triplicate samples from each group were labeled as CON1, CON2, CON3, SSA1, SSA2, and SSA3. The mRNA and miRNA omics detection was performed by Hangzhou Lianchuan Biotechnology Co., Ltd. (LCBio). Sequencing libraries were constructed using the TruSeq small RNA Sample Preparation Kit (Illumina, San Diego, USA). miRNA and mRNA sequencing were performed on the Illumina HiSeq 2000/2500 and Illumina Novaseq™ 6000 platforms, respectively^[24].

2.7 miRNA-seq and mRNA-seq Expression Analysis

For mRNA-seq data, Hisat was applied to map valid reads to the UCSC genome reference (<http://genome.ucsc.edu/>). Following gene assembly and quantification with StringTie, differential expression was assessed using edgeR. Significantly differentially expressed genes (DEGs) were identified based on the threshold criteria of $|\log_2(\text{fold-change})| > 1$ and $P < 0.01$ ^[24].

For miRNA sequencing, non-miRNA sequences were filtered, and annotation was performed using the Repbase database. Initial miRNA identification was completed by defining miRNAs and miRNA precursors in HCT116 cells via the miRBase database (<http://www.mirbase.org>). The screening criteria for differentially expressed miRNAs (DEMs) were the same as above.

2.8. Enrichment Analysis

Significantly expressed target genes were predicted using TargetScan (v5.0) and miRanda (v3.3a). Gene Ontology (GO) analysis was performed to examine biological functions. The Kyoto Encyclopedia of Genes

and Genomes (KEGG) was used to analyze target gene enrichment. Both GO term and KEGG pathway analyses employed a P -value < 0.01 as the screening criterion^[24].

2.9 Protein-Protein Interaction (PPI) Network Analysis

The String tool was used to analyze protein-protein interactions (PPIs) and identify pivotal regulatory genes. The miRNA-mRNA interaction network was visualized with Cytoscape software. Core genes were selected based on indicators such as centrality, proximity, and intermediate centrality^[24].

2.10 Apoptosis Assay

HCT116 cells (6×10^5 cells/well) were dispersed in plate and treated with SSA for 24 h. Cells were harvested and randomly divided into two groups: one stained with Hoechst 33,342 dye (Bristol Myers Squibb) and analyzed for blue fluorescence intensity under UV excitation; the other stained with Annexin V-FITC and propidium iodide (PI) (Bestbio) and analyzed for apoptosis rate by flow cytometry^[23].

2.11 Immunohistochemistry and Immunofluorescence Analysis of Mouse Tumor Tissue

Fresh tumor tissue was fixed in 4% paraformaldehyde and blocked. Sections were incubated overnight with primary antibodies against Bcl-2, cleaved caspase-3, and p-JNK, followed by incubation with secondary antibodies. For immunohistochemistry, sections were stained with the DAB method and subsequently observed under a light microscope (Leica, Germany). Meanwhile, immunofluorescence sections were stained with DAPI and examined using a fluorescence microscope. (Olympus X51, Japan)^[23].

2.12 Western Blotting

Total protein was isolated from HCT116 cells using RIPA lysis buffer containing 1% PMSF (Beyotime, Shanghai, China). Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Darmstadt, Germany). After blocking with 5% non-fat milk in PBST for 2 h at room temperature, membranes were incubated with primary antibodies (1:1000) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at 37°C. Protein bands were visualized using enhanced chemiluminescence (Tanon, Shanghai, China) and quantified with ImageJ software^[24].

2.13 Mitochondrial Membrane Potential ($\Delta\psi_m$) Detection

HCT116 cells treated with SSA (0, 24, 26, and 28 μM) were harvested and incubated with JC-1 staining solution (Becton Dickinson) for 15 min. After resuspension in PBS, cellular fluorescence was observed under a fluorescence microscope and analyzed by flow cytometry^[23].

2.14 Intracellular ROS Detection

SSA-treated HCT116 cells were harvested and added to serum-free medium containing 10 μ L DCFH-DA (MedChemExpress) per group. After 1h incubation, cells were washed. Green fluorescence signals were observed using fluorescence microscopy or flow cytometry^[23].

2.15 Statistical Analysis

Data was analyzed using Origin software (Origin Software, Northampton, MA). Results were reported as mean $\pm$ SD. One-way ANOVA with Tukey's post hoc test was conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1 SSA Inhibits Growth of HCT116 Cells

To investigate the effect of SSA on the survival rate of human colon cancer cells, HCT116 and HEK293 cells were treated with different concentrations of SSA (0 - 40 μ M) for 24 h. CCK-8 assays revealed that SSA significantly reduced HCT116 cell viability (**Fig. 1A**). However, SSA exhibited low cytotoxicity toward normal HEK293 cells (**Fig. 1B**). Based on the survival rate comparison in **Fig. 1C**, subsequent experiments employed treatment concentrations of 24, 26, and 28 μ M. Subsequent colony formation assays revealed that SSA significantly reduced the colony-forming capacity of HCT116 cells (**Fig. 1D**). Following treatment with 28 μ M SSA, the colony formation rate decreased to 13% (**Fig. 1E**).

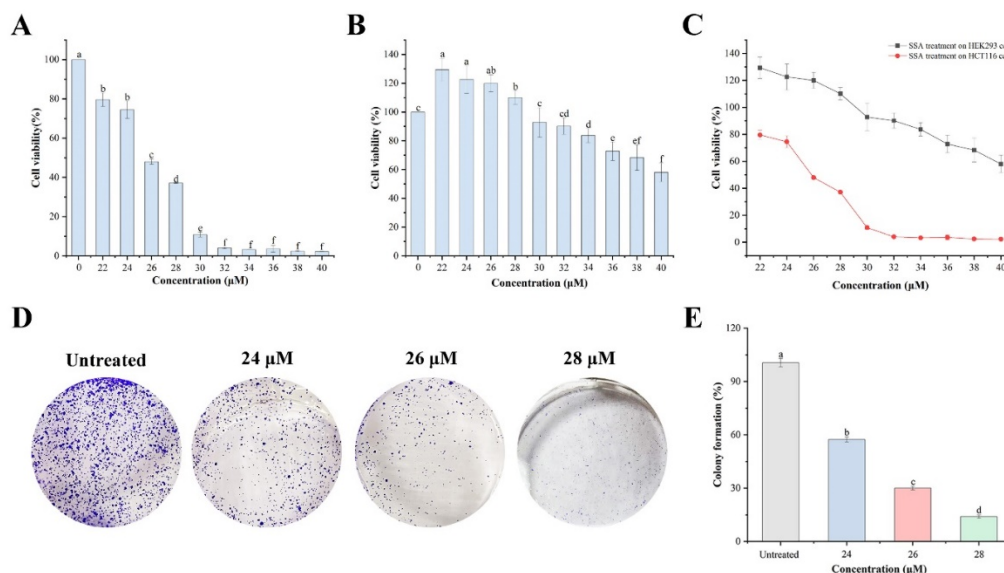


Fig. 1 SSA inhibits HCT116 cell growth. HCT116 cells (A) and HEK293 cells (B) were treated with different concentrations of SSA, and cell viability was determined using the CCK-8 assay ($n = 6$). (C) Comparison of cell viability between HEK293 and HCT116 cells treated with different concentrations of SSA ($n = 6$). (D) Image of colony formation in HCT116 cells after treatment with different concentrations of SSA. (E) Colony formation rate ($n = 3$). Data are expressed as mean $\pm$ SD. Lowercase letters indicate significant differences compared with the control group ($P < 0.05$).

3.2 SSA Inhibits Tumor Growth in Xenograft Mouse Models

After treating HCT116 cell-bearing mice with different concentrations of SSA, we measured and calculated indicators such as tumor weight, volume, and relative tumor volume (RTV). There were no

significant differences in body weight among the groups ($p > 0.05$) (**Fig. 2A**). However, tumor volume in the treatment groups exhibited a dose-dependent relative suppression (**Fig. 2B**). Furthermore, the relative tumor volumes in the three groups were 9.20 ± 1.15 (model group), 4.22 ± 0.64 (10 mg/kg SSA group), and 6.37 ± 1.34 (5 mg/kg SSA group), respectively (**Fig. 2C**). SSA treatment markedly inhibited the growth of HCT116 xenografts at the end of the treatment course (**Fig. 2F**). Furthermore, TUNEL staining of tumor xenografts revealed that more TUNEL-positive cells were detected in the SSA-treated groups at different concentrations compared to the model group, confirming that SSA treatment significantly increased apoptosis in tumor tissues (**Fig. 2D and 2E**). No statistically significant differences were observed between groups regarding liver and kidney function biomarkers, including ALT, AST, CREA, and BUN, or complete blood count parameters (**Tables S1 and S2**). No notable alterations in the organ structure or cellular morphology were detected in the heart, liver, spleen, lungs, or kidneys of HCT116-transplanted mice in any of the groups (**Fig. 2G**). H&E staining of colon tissue revealed normal intestinal structure, while the Mod group exhibited degeneration and necrosis in the colonic mucosa. All SSA-treated groups showed varying degrees of recovery (**Fig. 2H**). The above results further indicate that SSA inhibits tumor growth *in vivo* and induces apoptosis.

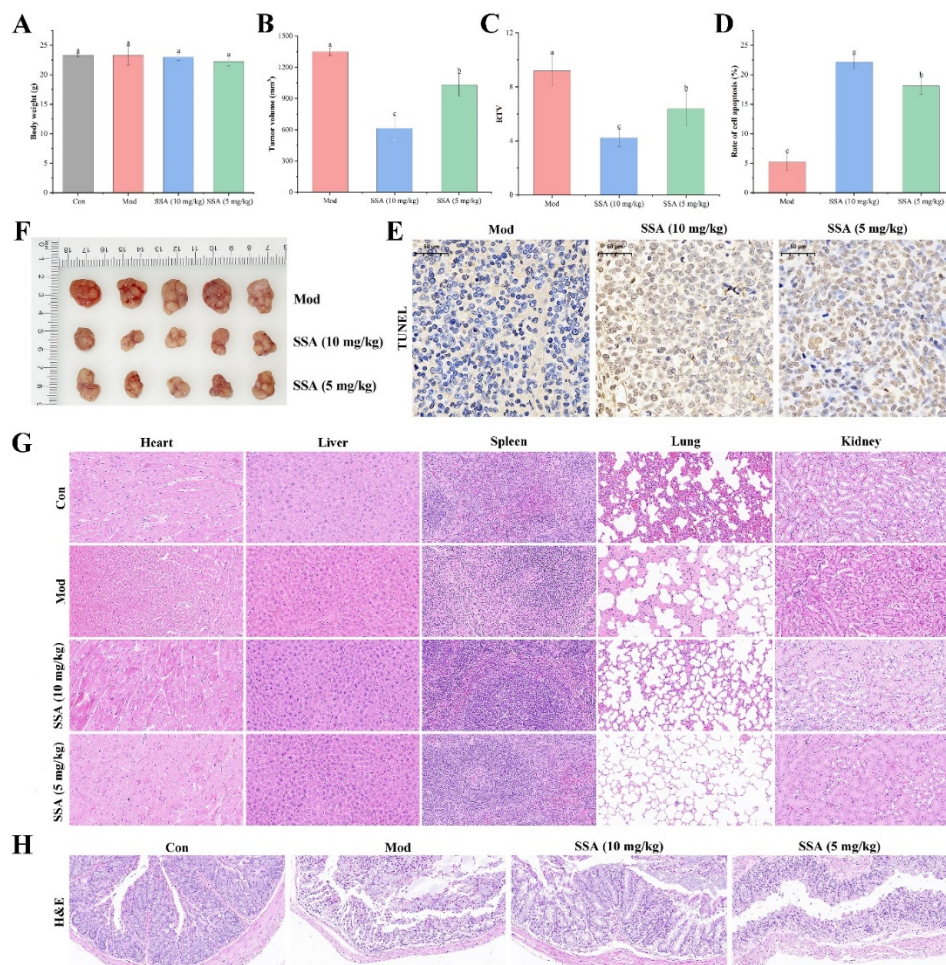


Fig. 2 SSA inhibits tumor growth in xenograft mouse models. (A) Body weights of euthanized mice in the Con group and other groups ($n = 5$). (B) Tumor volumes and (C) relative tumor volumes at euthanasia in the Mod group and SSA-treated group ($n = 5$). (D) TUNEL assay and (E) quantification of tumor tissue apoptosis ($n = 3$). (F) Images of xenografts isolated from tumors of Mod and SSA-treated mice ($n = 5$). H&E staining of organs (G) and colon tissue (H) from each group ($n = 3$). All data are expressed as mean $\pm$ SD. Lowercase letters indicate significant differences compared with the control group ($P < 0.05$).

3.3 Identification and enrichment analysis of differentially expressed miRNAs

Principal component analysis (PCA) in **Fig. 3A** indicates both intra-group similarities and inter-group differences between the Con group and SSA-treated groups. As shown in **Fig. 3B**, at $P < 0.01$, 95 miRNAs exhibited significant alterations in the SSA-treated group, with 49 miRNAs upregulated and 46 miRNAs downregulated. Further screening with $|\log_2(\text{fold change})| > 1$ identified 39 differentially expressed miRNAs (DEMs) (**Fig. 3C** and **Table 1**). miR-132-3p, miR-4485-p3_1ss1CT, and PC-3p-13345_72 showed the most significant upregulation, while miR-1277-p5, miR-95-5p_L+1, and miR-3614-3p exhibited the most pronounced downregulation. Heatmap analysis of the selected differentially expressed miRNAs (**Fig. 3D**) revealed significant differences between control and treated cell samples, while intra-group differences were relatively minor.

Fig. 3E indicated that target genes of DEMs primarily participated in biological processes (BP) such as signaling pathways, cellular components (CC) including membranes, and molecular functions (MF) involving protein binding. Additionally, they were enriched in apoptosis and mitochondria within BP and CC, respectively. KEGG analysis revealed that the target genes of 39 miRNAs were enriched in signaling pathways closely associated with apoptosis, such as PI3K/AKT and MAPK (**Fig. 3F**).

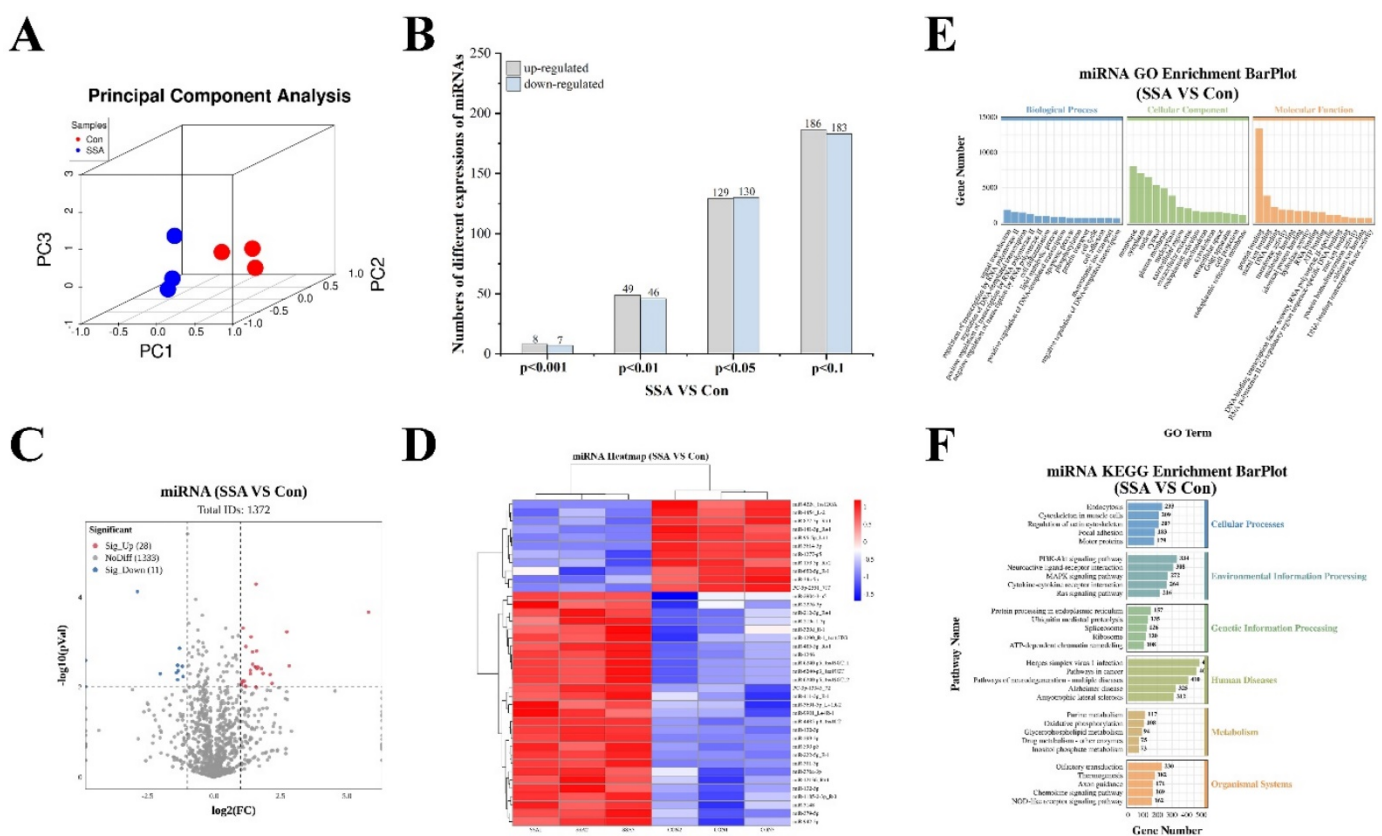


Fig. 3 miRNA sequencing analysis of HCT116 cells after SSA (26 μM) treatment. (A) Principal component analysis (PCA) of all samples ($n = 3$ per group). (B) Number of differentially expressed miRNAs. (C) Volcano plot of differentially expressed miRNAs between control and SSA-treated groups ($P < 0.01$, $|\log_2(\text{fold change})| > 1$, $n = 3$). (D) Heatmap of differentially expressed miRNAs ($n = 3$ per group). GO enrichment analysis (E) and KEGG pathway enrichment analysis (F) of 39 differentially expressed miRNAs.

Table 1 miRNA differentially expressed in HCT116 cells treated with SSA.

miRNAs	<i>P</i> value	log ₂ (fold change)	Expression level
Upregulated miRNA			
miR-132-3p	5.17×10^{-5}	1.58	middle
miR-4485-p3_1ss1CT	2.16×10^{-4}	5.81	middle
PC-3p-13345_72	4.90×10^{-4}	1.09	middle
miR-2904-3-p5	5.93×10^{-4}	2.74	middle
miR-1185-2-3p_R-3	7.67×10^{-4}	1.39	middle
miR-379-5p	1.25×10^{-3}	1.20	middle
miR-376a-3p	1.59×10^{-3}	1.60	middle
miR-222-5p_R-1	1.66×10^{-3}	1.44	middle
miR-1290_R-1_1ss13TG	3.11×10^{-3}	1.45	middle
miR-299-3p	3.39×10^{-3}	2.83	middle
miR-6240-p3_1ss19GT	3.57×10^{-3}	1.55	middle
miR-6240-p5_1ss19GT_2	3.57×10^{-3}	1.55	middle
miR-6240-p5_1ss19GT_1	3.57×10^{-3}	1.55	middle
miR-942-3p	3.63×10^{-3}	1.65	middle
miR-411-3p_R-1	3.91×10^{-3}	1.61	middle
miR-1246	3.92×10^{-3}	1.78	middle
miR-12136_R+1	4.25×10^{-3}	1.37	middle
miR-212-3p_R+1	4.99×10^{-3}	1.84	middle
miR-381-3p	4.99×10^{-3}	1.37	middle
miR-3148	5.26×10^{-3}	2.11	middle
miR-590-p5	7.36×10^{-3}	1.08	middle
miR-3691-5p_L+1R-2	7.38×10^{-3}	1.14	middle
miR-219a-1-3p	7.89×10^{-3}	1.05	middle
miR-485-5p_R+1	8.25×10^{-3}	2.18	middle
miR-9901_L+4R-1	8.76×10^{-3}	1.03	middle
miR-320d_R-1	9.50×10^{-3}	1.18	middle
miR-2276-3p	9.69×10^{-3}	1.20	middle
miR-132-5p	9.91×10^{-3}	1.58	middle
Downregulated miRNA			
miR-1277-p5	7.60×10^{-5}	-2.88	middle
miR-95-5p_L+1	1.37×10^{-3}	-1.30	middle
miR-3614-3p	2.56×10^{-3}	-inf	low
miR-4454_L-2	3.28×10^{-3}	-1.37	middle
miR-34c-5p	3.46×10^{-3}	-1.19	middle
PC-5p-2551_717	4.29×10^{-3}	-1.34	middle
miR-153-3p_R+2	4.80×10^{-3}	-1.41	middle
miR-877-3p_R+1	5.10×10^{-3}	-2.02	middle
miR-652-5p_R-1	5.79×10^{-3}	-1.16	middle
miR-141-3p_R+1	6.86×10^{-3}	-1.37	high
miR-422a_1ss12GA	9.78×10^{-3}	-inf	low

3.4 Identification and enrichment analysis of differentially expressed mRNAs

Results revealed 942 significantly upregulated and 5091 downregulated differentially expressed mRNAs (DEGs) (**Fig. 4A**). The heatmap of DEGs in **Fig. 4B** demonstrated homogeneity within groups and pronounced differences between groups. The SSA group exhibited significant gene enrichment differences across multiple GO categories. Among these, the most enriched categories were ‘Regulation of transcription by RNA polymerase II’ in BP, ‘Cytoplasm’ in CC, and ‘Protein binding’ in MF (**Fig. 4C**). Specific GO terms and the number of enriched genes were further detailed in a pie chart, providing deeper insights into SSA's impact on gene expression (**Fig. 4D**). KEGG enrichment analysis revealed the Environmental Information Processing (EIP) pathway (PI3K/AKT, MAPK) as the most significantly affected (**Fig. 4E and 4F**). These enriched signaling pathways may participate in SSA-induced apoptotic responses. These results indicate that mRNA levels in HCT116 cells undergo significant changes following SSA treatment, and a preliminary screening of significantly altered signaling pathways has been conducted.

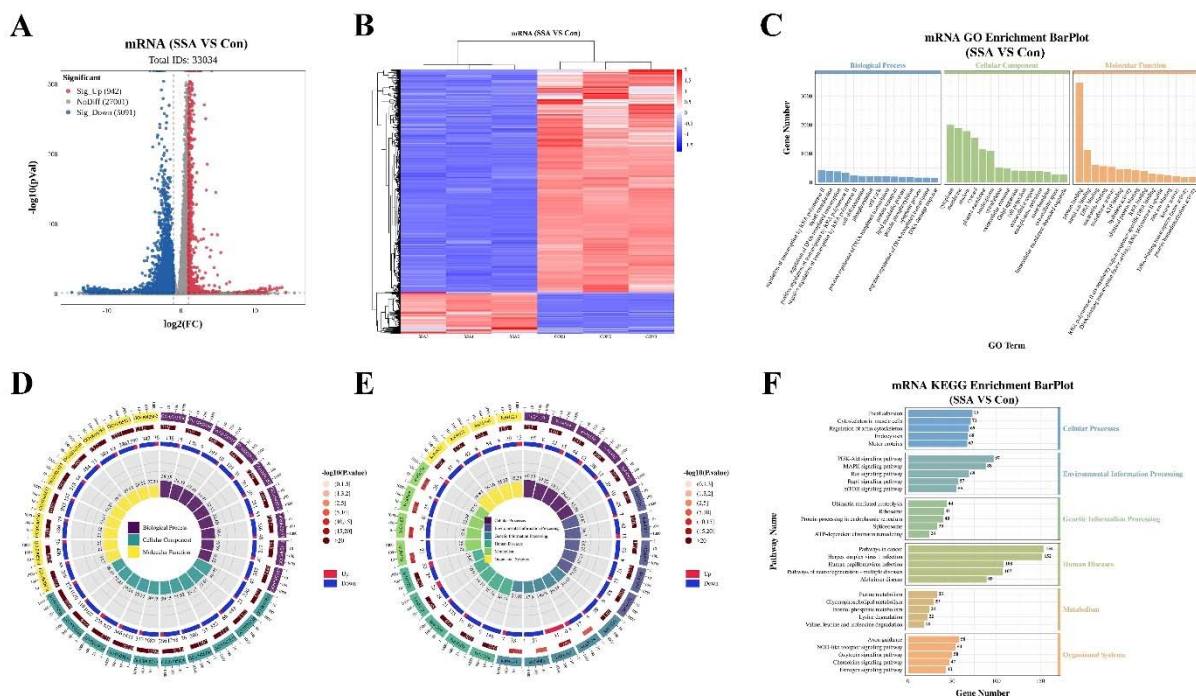


Fig. 4 mRNA sequencing analysis of HCT116 cells treated with SSA (26 μ M). Volcano plot (A), heatmap (B), GO enrichment analysis (C and D), and KEGG enrichment analysis (E and F) of differentially expressed mRNAs ($P < 0.01$, $|\text{Log}_2(\text{fold change})| > 1$, $n = 3$).

3.5 Integrated Analysis of miRNA-mRNA Correlations

Among the identified DEMs target genes, 3,974 genes corresponded to a subset of differentially expressed genes (**Fig. 5A**). Within this subset, 469 genes showed significant upregulation and 3,505 significant downregulation, exhibiting high intra-group homogeneity and significant inter-group differences (**Fig. 5B and 5C**). GO enrichment analysis revealed enrichment in apoptosis processes and mitochondria within BP and CC categories, respectively (**Fig. 5D**). Among GO terms, MF ‘protein binding’ exhibited the highest enrichment of differentially regulated genes due to SSA regulation. **Fig. 5E and 5F** provide a visual representation of gene enrichment differences between the SSA and Con groups, showing higher enrichment in terms related to ‘protein binding’, ‘cytoplasm’, and ‘nucleus’. Mapping the 3,974 significantly altered genes

to KEGG pathways revealed significant enrichment in the PI3K-AKT and MAPK pathways, critical regulators of cancer cell apoptosis. Given the direct association of the MAPK signaling pathway with apoptosis and its regulatory flexibility, we asked whether SSA triggers HCT116 apoptosis by acting on this pathway.

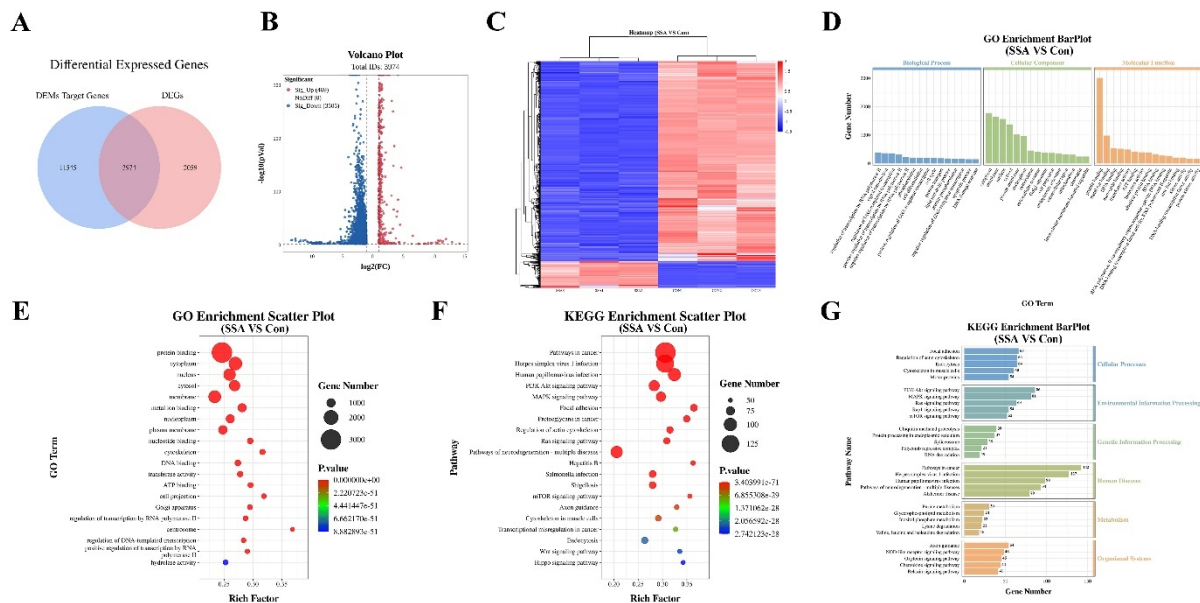


Fig. 5 GO and KEGG enrichment analysis of differentially expressed miRNAs (DEMs) and their target genes. (A) Venn diagram showing the relationship between differentially expressed genes (DEGs) and target genes associated with DEMs. Volcano plot (B) and heatmap (C) of 3,974 DEGs (n = 3). Bar chart (D) and factor plot (E) of GO enrichment analysis for 3,974 DEGs. KEGG enrichment factor plot (F) and bar chart (G) for 3,974 DEGs.

3.6 SSA Induces Apoptosis in HCT116 Cells

SSA-induced apoptosis in HCT116 cells was confirmed by Hoechst 33342 and Annexin V/PI staining. **Fig. 6A** showed that blue fluorescence intensity significantly increased after SSA treatment. Furthermore, flow cytometry revealed a dose-dependent increase in HCT116 cell apoptosis following SSA treatment (**Fig. 6B**). Additionally, immunofluorescence analysis of tumor tissues demonstrated that 10 mg/kg SSA treatment significantly downregulated Bcl-2 expression while markedly upregulating cleaved caspase3 expression (**Fig. 6C**). Western blot analysis showed that SSA dose-dependently increased BAX and cleaved caspase-9 and cleaved caspase-3, while decreasing Bcl-2, caspase-9, and caspase-3, thus corroborating mitochondrial apoptosis (**Fig. 6D and 6E**). To confirm SSA-triggered apoptosis in HCT116 cells, we employed the apoptosis inhibitor Z-DEVE-FMK. Cytometric analysis showed a marked reduction in apoptosis for cells co-treated with SSA (26 μ M) and Z-DEVE-FMK versus SSA (26 μ M) alone (**Fig. 6F**). Concurrently, Z-DEVE-FMK treatment reversed the SSA-induced increase in cleaved caspase-9 and cleaved caspase-3 (**Fig. 6G and 6H**). Together, the data indicate that SSA triggers apoptotic cell death in HCT116.

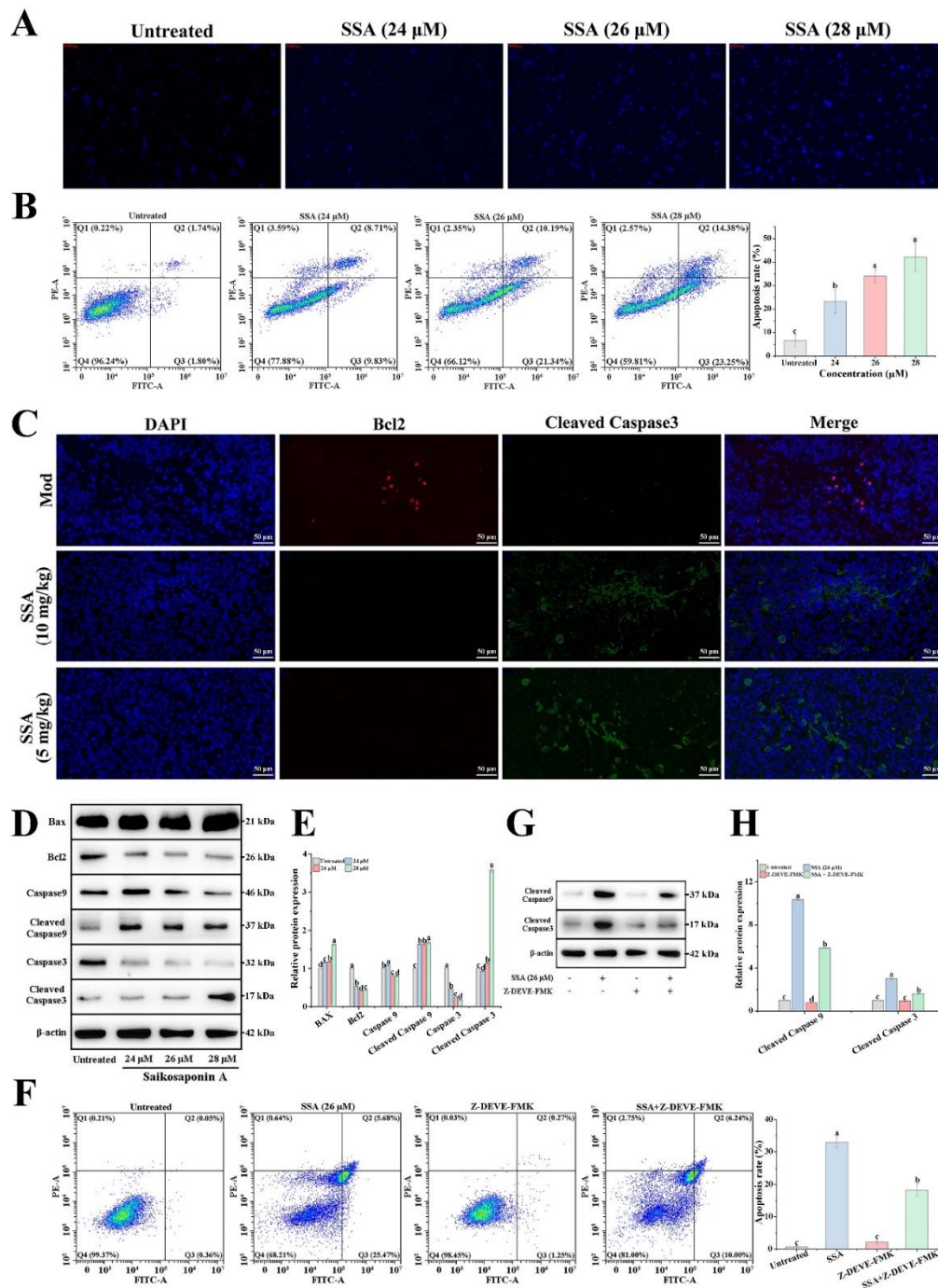


Fig. 6 SSA induces apoptosis in HCT116 cells via the mitochondrial pathway. (A) Hoechst 33,342-stained fluorescence images of SSA-treated HCT116 cells ($n = 3$). (B) Flow cytometric analysis of SSA-treated HCT116 cells ($n = 3$). (C) Immunofluorescence staining for anti-Bcl-2 and cleaved caspase-3 in tumor tissues ($n = 3$). (D) Western blotting analysis and (E) quantification of apoptosis-related proteins (Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9) from three independent experiments ($n = 3$). (F) Flow cytometry of untreated, SSA (26 μM), Z-DEVD-FMK, and SSA (26 μM) + Z-DEVE-FMK groups ($n = 3$). (G) Western blotting analysis and (H) quantification of cleaved caspase-3 and cleaved caspase-9 from three independent experiments ($n = 3$). Data are expressed as mean $\pm$ SD. Lowercase letters indicate significant differences compared with the control group ($P < 0.05$).

3.7 SSA Promotes Apoptosis by Elevating Reactive Oxygen Species Levels and Inducing ROS-Dependent JNK Pathway Activation

Next, we investigated the potential mechanisms underlying SSA's enhancement of apoptosis in HCT116 cells. First, we employed DCFH-DA reagent to detect SSA's regulatory effect on intracellular ROS levels. SSA exposure produced a progressive rise in green fluorescence within HCT116 cells (**Fig. 7A**). Flow

cytometric quantification demonstrated that 28 μM SSA treatment significantly induced intracellular ROS production in HCT116 cells (**Fig. 7B**). To validate mitochondrial involvement in SSA-induced apoptosis, we monitored mitochondrial membrane potential (MMP) with JC-1 staining. SSA exposure triggered a pronounced red-to-green fluorescence shift, signifying a drop in MMP (**Fig. 7C**). Cytometric quantification showed this depolarization intensified with SSA dose in HCT116 cells (**Fig. 7D and 7E**). Subsequently, we assessed SSA's impact on the MAPK axis. SSA dose-dependently elevated the p-p38/p38 and p-JNK/JNK ratios in HCT116 cells (**Fig. 7F and 7G**), and immunohistochemistry confirmed markedly higher p-JNK signals within tumor sections after SSA exposure (**Fig. 7H and 7I**). To functionally validate the requirement of MAPK activation in SSA-induced apoptosis, we employed specific pharmacological inhibitors targeting p38 (SB203580) and JNK (SP600125). Individual inhibition of either p38 or JNK pathway only partially attenuated SSA-triggered caspase-3 activation, whereas simultaneous blockade of both pathways almost completely abolished apoptosis (**Fig. S1**).

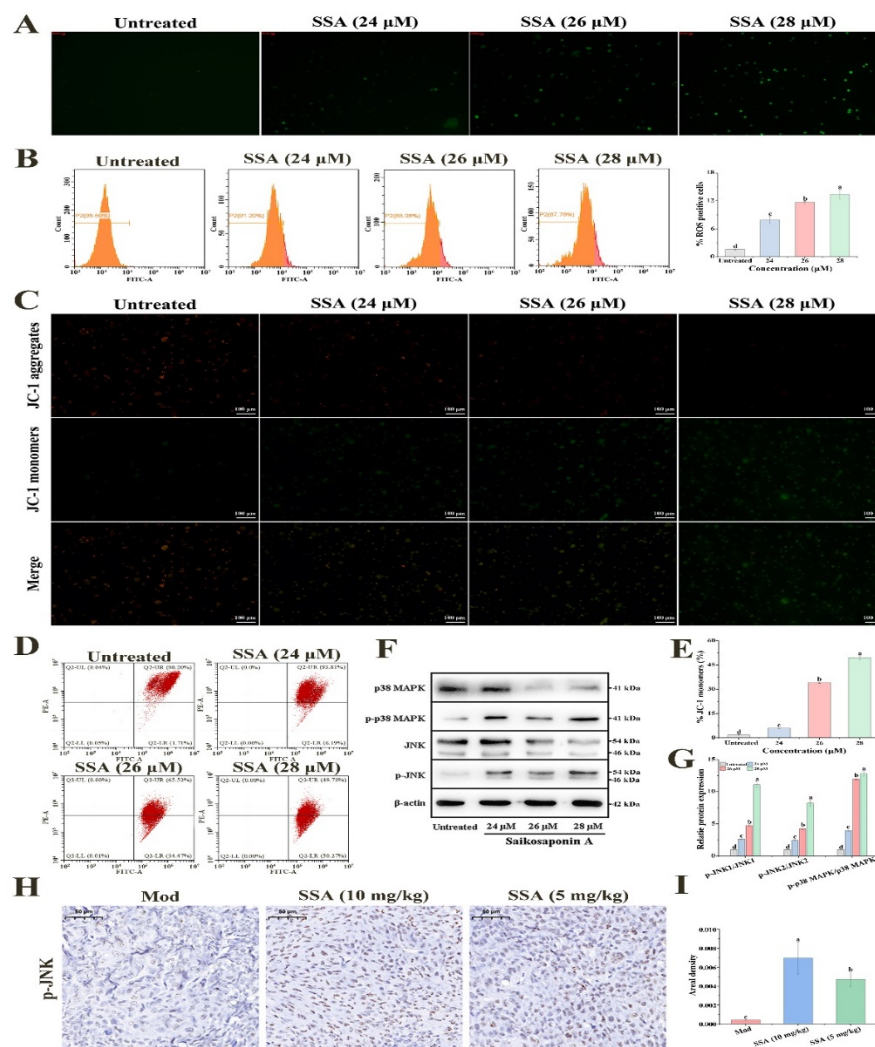


Fig. 7 SSA increases reactive oxygen species (ROS) levels and activates the MAPK signaling pathway. ROS levels were detected by fluorescence microscopy (A) and flow cytometry (B) ($n = 3$). Mitochondrial membrane potential ($\Delta\psi\text{m}$) was assessed using JC-1 dye via fluorescence microscopy (C) and flow cytometry (D), with quantitative analysis (E) ($n = 3$). (F) Western blotting analysis of JNK, p38 MAPK, and phospho-expression in HCT116 cells and (G) their quantification from three independent experiments ($n = 3$). (H) Immunohistochemistry of p-JNK in tumor tissue and (I) its quantification ($n = 3$). Data are expressed as mean $\pm$ SD. Lowercase letters indicate significant differences compared with the control group ($P < 0.05$).

3.8 SSA Promotes Apoptosis in HCT116 Cells via ROS-Mediated Endoplasmic Reticulum Stress

To validate the pro-apoptotic function of ROS, we pretreated HCT116 cells with NAC. Cytometry showed SSA + NAC cells were markedly more viable than SSA alone, confirming NAC's ROS-scavenging rescue from apoptosis (**Fig. 8A**). Furthermore, we found that NAC pretreatment reversed the SSA-triggered increases in the p-JNK/JNK ratio and cleaved caspase-3 abundance (**Fig. 8B**). Thus, SSA targets HCT116 cells through an ROS-dependent apoptotic pathway.

To further investigate the mechanism of SSA-mediated growth inhibition in HCT116 cells, we examined its link to ER stress. SSA dose-dependently raised the p-PERK/PERK and p-eIF2 α /eIF2 α ratios (**Fig. 8C**). Furthermore, NAC reversed SSA-evoked up-regulation of p-eIF2 α and CHOP (**Fig. 8D**). To validate the ER stress-mediated apoptosis mechanism induced by SSA, HCT116 cells were pretreated with the ER stress blocker 4-PBA before SSA exposure. Flow cytometry revealed that 4-PBA significantly inhibited SSA-induced apoptosis in HCT116 cells (**Fig. 8E**). Western blotting results indicated that 4-PBA significantly lowered p-eIF2 α , CHOP, Bax, cleaved caspase-9, and cleaved caspase-3 levels (**Fig. 8F**). These findings demonstrate that SSA triggers ROS-driven ER stress to propel apoptosis.

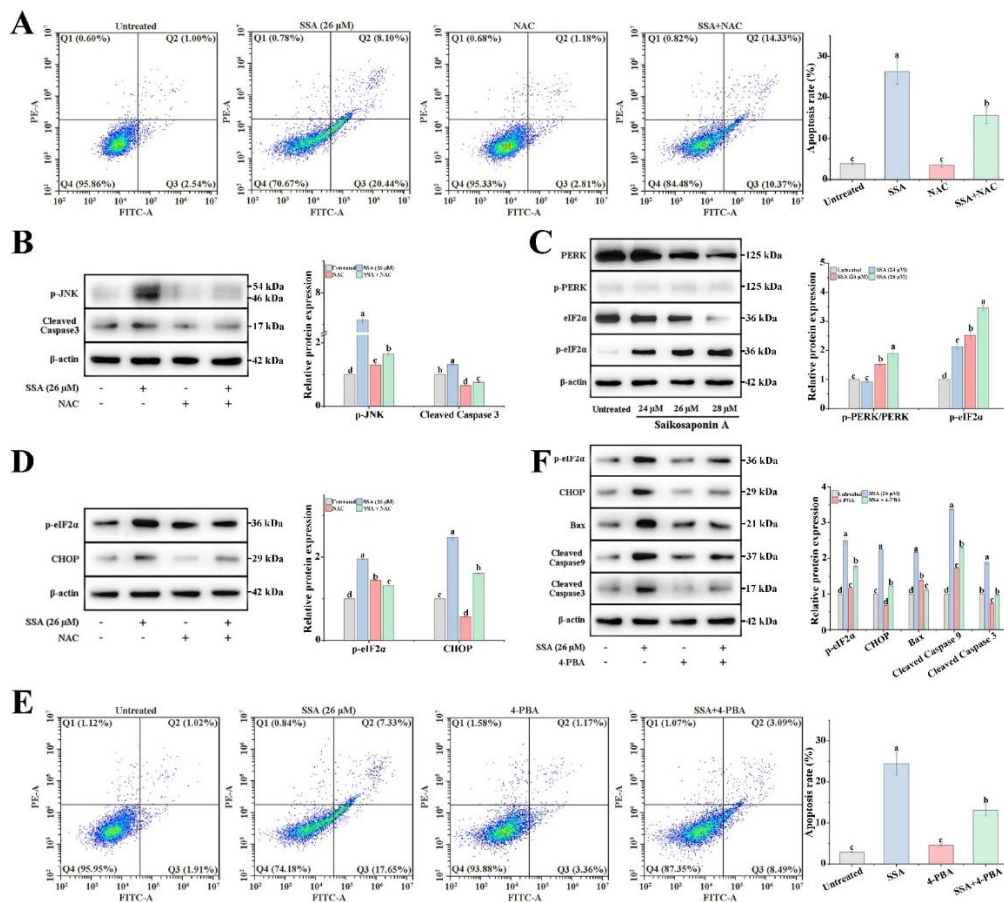


Fig. 8 SSA promotes apoptosis in HCT116 cells via ROS-mediated endoplasmic reticulum stress. (A) Apoptosis flow cytometry analysis in the untreated group, SSA (26 μ M) group, NAC group, and SSA (26 μ M) + NAC group ($n = 3$). (B) Western blotting analysis of p-JNK and cleaved caspase-3 from three independent experiments ($n = 3$). (C) Western blotting analysis of PERK, p-PERK, eIF2 α , and p-eIF2 α from three independent experiments ($n = 3$). (D) Western blotting analysis of p-eIF2 α and CHOP from three independent experiments ($n = 3$). (E) Apoptosis flow cytometry analysis in the untreated group, SSA (26 μ M) group, 4-PBA group, and SSA (26 μ M) + 4-PBA group ($n = 3$). (F) Western blotting analysis of p-eIF2 α , CHOP, Bax, cleaved caspase-9, and cleaved caspase-3 from three independent experiments ($n = 3$). Data are expressed as mean $\pm$ SD. Lowercase letters indicate significant differences compared with the control group ($P < 0.05$).

3.9 MAPK Pathway-Linked miRNA and mRNA Profiles

To validate the molecular mechanism of SSA-induced apoptosis in HCT116 cells, genes and miRNAs related to the MAPK signaling pathway were evaluated. Among 3,974 significantly differentially expressed genes, 81 DEGs mapped to the MAPK pathway (**Fig. 9A**). After excluding low-expression miRNAs, 37 DEMs were linked to the MAPK pathway (**Fig. 9B**).

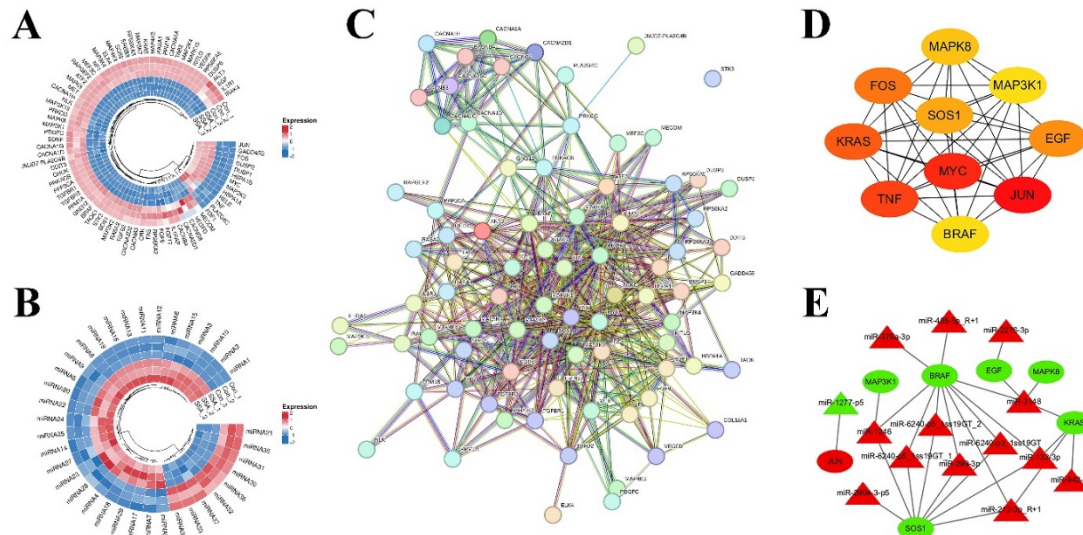


Fig. 9 Sequence data validation of mRNA-miRNA interactions in the MAPK signaling pathway in HCT116 cells. (A) Circular heatmap of differentially expressed mRNAs in the MAPK signaling pathway (n = 3). (B) Circular heatmap of differentially expressed miRNAs in the MAPK signaling pathway (n = 3). (C) Protein interaction network of differentially expressed genes. (D) Subnetwork of hub genes in the MAPK signaling pathway. (E) miRNA-mRNA negative correlation regulatory network. Node shapes: triangles represent miRNAs, circles represent mRNAs. Node colors: red indicates upregulated miRNAs/mRNAs, green indicates downregulated miRNAs/mRNAs. Gray lines denote negative regulatory relationships between miRNAs and their target mRNAs.

A PPI network of MAPK-pathway DEGs was built to probe miRNA–mRNA crosstalk, and hub genes were extracted (**Fig. 9C**). 10 hub genes were identified based on screening criteria including degree, betweenness, and closeness, including MAPK8, FOS, MAP3K1, KRAS, SOS1, TNF, MYC, EGF, JUN, and BRAF (**Fig. 9D and Table 2**). In the negative regulatory network linking these 10 key genes to DEMs, miR-3148, miR-1246, and miR-132-3p exhibited high connectivity, indicating their pivotal regulatory roles in apoptosis-related pathways (**Fig. 9E and Table 3**).

Table 2 Screened top 10 Hub genes in HCT116 cells treated with SSA.

Name	Degree	Name	Betweenness	Name	Closeness
JUN	100	JUN	933.88	JUN	64.33
MYC	80	KRAS	570.93	MYC	59
TNF	78	PRKACB	545.50	TNF	58.5
KRAS	76	MYC	519.66	KRAS	58.33
FOS	74	TNF	499.30	FOS	57.83
EGF	62	FOS	383.16	EGF	54.17
SOS1	60	PRKCG	363.17	SOS1	53.33
MAPK8	58	BRAF	245.57	MAPK8	53
MAP3K1	48	MAP3K7	241.54	BRAF	51.5
BRAF	48	SOS1	238.85	MAP3K1	50.17

Table 3 Negative regulatory relationships between Hub genes and their miRNAs with target gene relationship pairs.

Genes	Regulation	miRNA	Regulation
JUN	up	miR-1277-p5	down
KRAS	down	miR-12136_R+1	up
		miR-1290_R-1_1ss13TG	up
		miR-132-3p	up
		miR-141-3p_R+1	up
		miR-153-3p_R+2	up
		miR-212-3p_R+1	up
		miR-3148	up
		miR-381-3p	up
		miR-942-3p	up
EGF	down	miR-1290_R-1_1ss13TG	up
		miR-2276-3p	up
		miR-3148	up
SOS1	down	mir-2904-3-p5	up
		miR-12136_R+1	up
		miR-1246	up
		miR-132-3p	up
		miR-212-3p_R+1	up
		miR-299-3p	up
		miR-320d_R-1	up
		miR-379-5p	up
		miR-381-3p	up
		miR-6240-p3_1ss19GT	up
		miR-6240-p5_1ss19GT_1	up
		miR-6240-p5_1ss19GT_2	up
		PC-3p-13345_72	up
MAPK8	down	miR-1185-2-3p_R-3	up
		miR-222-5p_R-1	up
		miR-3148	up
		miR-379-5p	up
		miR-877-3p_R+1	up
MAP3K1	down	miR-1185-2-3p_R-3	up
		miR-1246	up
		miR-1290_R-1_1ss13TG	up
		PC-3p-13345_72	up
BRAF	down	miR-12136_R+1	up
		miR-1246	up
		miR-1290_R-1_1ss13TG	up
		miR-132-3p	up
		miR-212-3p_R+1	up
		miR-299-3p	up
		miR-3148	up
		miR-320d_R-1	up
		miR-376a-3p	up

miR-381-3p	up
miR-485-5p_R+1	up
miR-652-5p_R-1	up
miR-6240-p3_1ss19GT	up
miR-6240-p5_1ss19GT_1	up
miR-6240-p5_1ss19GT_2	up

4. Discussion

CRC treatment remains challenging due to chemoresistance and toxicity of conventional therapies^[27]. Risk factors include both non-modifiable (age, genetics) and lifestyle elements (diet and inactivity)^[28]. Early-stage symptoms are subtle and frequently overlooked, whereas advanced disease manifests severe complications including cachexia and multi-organ metastasis^[29]. Conventional chemotherapy and targeted therapies are limited by drug resistance, toxicity, and poor outcomes^[30]. This necessitates alternative therapeutic strategies^[27]. Molecular drivers involve hyperactivated Wnt/ β -catenin, PI3K/AKT, MAPK pathways and tumor suppressor loss (APC, TP53)^[14]. Despite these insights, traditional medicine remains underexplored^[31]. Saikosaponin A (SSA), the principal bioactive constituent of *Radix Bupleuri*, has demonstrated pro-apoptotic, cell cycle-arresting, and anti-metastatic effects in malignancies^[32], but its efficacy in CRC requires experimental validation. Here, we demonstrate that SSA exerts selective antitumor activity against HCT116 CRC cells through ROS generation, ER stress, and MAPK pathway activation. SSA exhibited sharp inhibitory effect against HCT116 cells while sparing normal HEK293 cells. This selectivity is clinically significant as it suggests SSA may spare normal tissues. *In vivo*, SSA (10 mg/kg) suppressed xenograft growth without systemic toxicity or organ damage, corroborating its safety profile.

Apoptosis eliminates dysfunctional cells to maintain homeostasis under physiological and pathological conditions^[23]. Dysregulated apoptosis drives tumorigenesis, making it a central therapeutic target^[33, 34]. SSA dose-dependently increased Hoechst 33342 fluorescence and apoptosis rates in HCT116 cells, reducing Bcl-2 while elevating cleaved caspase-3. The apoptosis inhibitor Z-DEVE-FMK reversed SSA-induced upregulation of cleaved caspase-9 and cleaved caspase-3, confirming SSA as an apoptosis driver.

ROS regulate cancer progression and mediate anticancer drug effects^[35, 36]. Excessive ROS collapses mitochondrial membrane potential (MMP) and triggers ER stress via PERK/IRE1/ATF6 pathways, inducing apoptosis^[20, 37]. Similarly, ferulic acid, a natural phenolic compound, has been demonstrated to inhibit colon cancer cells at different Duke's stages through ROS-mediated mechanisms and cell cycle regulation^[38], highlighting the broader potential of natural products in targeting colorectal cancer progression. SSA decreased MMP while elevating ROS and p-PERK/p-eIF2 α in HCT116 cells; both NAC and 4-PBA reversed SSA-induced apoptosis, confirming ROS-dependent ER stress activation. Our data position ROS as the upstream trigger in SSA-induced apoptosis. This aligns with reports that many natural products exert antitumor effects via ROS accumulation^[20, 39]. The preferential ROS sensitivity of cancer cells likely underlies SSA's selectivity, as transformed cells exhibit elevated basal oxidative stress and reduced antioxidant capacity^[40]. This vulnerability is also exploited by ferulic acid in targeting diverse Duke's stage tumors^[38],

suggesting a common strategy among natural products. SSA activated the PERK-eIF2 α -CHOP axis, an ER stress pathway. CHOP upregulation promotes apoptosis by increasing Bax expression and suppressing Bcl-2^[21]. The ER stress inhibitor 4-PBA reduced apoptosis by 58%, validating ER stress as a required intermediate. Notably, NAC partially abrogated ER stress markers, confirming ROS acts upstream of ER stress. This ROS→ER stress→apoptosis cascade represents a mechanism for SSA's antitumor activity. Integrated omics analysis positioned the MAPK pathway as the hub. SSA specifically activated p38 and JNK, consistent with stress-induced apoptosis^[24, 41]. JNK activation is crucial as it phosphorylates c-Jun, promoting transcription of pro-apoptotic genes^[42]. Pharmacological inhibition using SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) demonstrated that individual blockade of either pathway only partially attenuated apoptosis, whereas dual inhibition almost completely abolished caspase-3 activation, establishing p38 and JNK as synergistic mediators downstream of ROS. The ROS-MAPK link was confirmed by NAC's ability to suppress JNK phosphorylation. This is consistent with studies showing piperine, ferulic acid, and other phytochemicals activate JNK/p38 via oxidative stress^[17, 38, 43-44], underscoring the conserved role of ROS-MAPK signaling in natural product-induced colon cancer cell death across different tumor stages.

miRNAs (~22 nucleotides) silence targets via 3'UTR pairing, regulating CRC initiation, progression, metastasis, and drug resistance^[44]. miRNA-mRNA network analysis identifies therapeutic targets^[45]. SSA-treated HCT116 cells exhibited 39 DEMs (28 up, 11 down) and 6,033 DEGs (942 up, 5,091 down), yielding 3,974 significant genes enriched in Hippo, PI3K/Akt, and MAPK pathways. The MAPK axis governs proliferation and ROS-driven apoptosis^[46]. SSA inhibited p38 and JNK activation, with NAC reversing JNK effects. Eighty-one DEGs and 37 DEMs were linked to MAPK, with network analysis identifying 10 hub genes (MAPK8, FOS, MAP3K1, KRAS, SOS1, TNF, MYC, EGF, JUN, BRAF) that regulate apoptosis across cancers^[24, 41, 47-51]. A miRNA-mRNA network identified four miRNAs: miR-3148, miR-1246, miR-132-3p, and miR-212-3p_R+1. miR-132-3p regulates KRAS, SOS1, and BRAF, and its upregulation inhibits CRC proliferation while enhancing apoptosis^[52]. The miR-132-3p-KRAS/SOS1/BRAF axis emerged as a regulatory circuit. miR-132-3p was upregulated 3.2-fold and has been previously implicated as a tumor suppressor in CRC^[52-54]. KRAS and BRAF are oncogenic drivers in ~50% of CRCs, and their downregulation by miR-132-3p could partially explain SSA's efficacy^[48, 51]. While our network analysis is correlative, it provides testable hypotheses for future mechanistic studies.

This study reveals both similarities and differences in the antitumor mechanisms of Saikosaponin A compared with recent reports. Our findings align with studies showing that saikosaponin D triggers mitochondrial dysfunction and apoptosis through caspase-9/3 activation in colorectal cancer cells^[55]. Notably, SSA exhibits more potent selective cytotoxicity than SSB and SSD reported in previous studies, likely due to its unique configuration facilitating ROS generation and ER stress activation. However, we demonstrate SSA's selective cytotoxicity in colorectal cancer, likely attributable to the unique metabolic dependencies of these cells, particularly regarding glutamine metabolism and redox homeostasis^[56]. The observed PERK-eIF2 α -CHOP axis activation is consistent with the current understanding that prolonged ER stress induces CHOP-

mediated apoptosis^[57], but our cross-rescue experiments with NAC and 4-PBA establish a causal ROS→ER stress→apoptosis cascade rather than parallel activation. Notably, our multi-omics analysis reveals that SSA regulates the MAPK pathway at the transcriptional level, with the miR-132-3p-KRAS/SOS1/BRAF axis expanding SSA's mechanism from kinase-substrate interactions to post-transcriptional regulation^[58, 59]. These distinct mechanisms position SSA as a promising candidate for CRC therapeutics and combination therapy: ROS-ER stress activation may synergize with conventional chemotherapeutics to overcome chemoresistance^[39]; MAPK pathway modulation and KRAS/BRAF suppression suggest combination potential with targeted therapies in RAS/RAF-mutant CRC subtypes^[41, 48]; ROS-induced immunogenic cell death may enhance immunotherapy efficacy^[36]. Furthermore, SSA's safety profile and natural origin support its development as a functional food ingredient for CRC prevention^[31]. Specifically, SSA may synergize with 5-FU or oxaliplatin through ROS-mediated mechanisms to overcome chemoresistance or enhance EGFR/BRAF inhibitor efficacy in RAS-mutant CRC via MAPK pathway modulation. Future studies should optimize dosing schedules, identify predictive biomarkers (e.g., miR-132-3p expression), and develop formulation strategies to improve bioavailability.

Several limitations must be acknowledged. First, all *in vitro* experiments utilized a single colorectal cancer cell line (HCT116, MSI-H with KRAS mutation), which may not adequately reflect the molecular heterogeneity of colorectal cancer. Second, although we identified a cross-regulatory network involving ROS-ER stress-MAPK-miRNA, the direct molecular targets of SSA remain unclear. Third, while inhibitor experiments demonstrate functional necessity of p38 and JNK activation, whether SSA directly binds to these kinases or acts through upstream kinases (e.g., MKK3/6, MKK4/7) awaits direct kinase assays and chemical proteomics. Fourth, our subcutaneous xenograft model cannot fully recapitulate the tumor microenvironment complexities; therefore, orthotopic or patient-derived xenograft models would be more appropriate^[60-62]. Finally, our single-time-point omics design precludes capture of dynamic apoptotic progression, which requires time-resolved approaches^[63]. Future studies should validate these findings in additional CRC cell lines and patient-derived organoids^[61, 62], identify direct SSA-binding proteins through chemical proteomics, characterize pharmacokinetics and develop nano-formulation strategies, employ orthotopic and PDX models^[62, 63], and utilize time-resolved multi-omics approaches^[64] to comprehensively address these limitations and advance SSA toward clinical application.

To summarize, SSA exerts selective antitumor activity against colorectal cancer through a mechanism involving ROS generation, ER stress, and MAPK pathway activation, representing a promising therapeutic candidate for further clinical development.

5. Conclusion

This study demonstrates that Saikosaponin A selectively induces apoptosis in HCT116 colorectal cancer cells through a coordinated mechanism initiated by ROS generation, which triggers mitochondrial dysfunction and ER stress activation, leading to p38/JNK MAPK pathway activation and culminating in Bcl-2 downregulation with caspase-3/-9 cleavage. Inhibitor studies establish p38 and JNK as functionally necessary

mediators of this response. *In vivo* data reveals that SSA (10 mg/kg) suppresses xenograft growth by 54% without systemic toxicity. Integrated miRNA-mRNA analysis identifies the miR-132-3p/KRAS/SOS1/BRAF regulatory axis as a key component of SSA's mechanism. These findings provide a strong preclinical rationale for developing SSA as a novel, multi-targeted CRC therapy. Future studies should validate these mechanisms in additional models, establish pharmacokinetic profiles, and explore combination strategies with standard-of-care agents.

Conflict of interest

There is none to declare.

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Ethics declarations

Animal experiments were conducted in compliance with the Regulations on the Management of Laboratory Animals issued by the China National Science and Technology Commission and were approved by the Animal Welfare and Ethics Committee of the Institute of Health Sciences, Hefei National Comprehensive Science Center (Approval No.: IHM-AP-2025-170; Approval Date: December 31, 2025).

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