Exploration of anti-osteosarcoma activity of asiatic acid based on network pharmacology and *in vitro* experiments

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Abstract. Osteosarcomas are malignant bone tumors that typically originate in the epiphyses of the long bones of the extremities in adolescents. Asiatic acid has been reported to possess anti-inflammatory, neuroprotective, antidiabetic, antitumor and antimicrobial activities. The present study used a combination of network pharmacological prediction and *in vitro* experimental validation to explore the potential pharmacological mechanism of asiatic acid against osteosarcoma. A total of 78 potential asiatic acid targets in osteosarcoma were identified using databases. Kyoto Encyclopedia of Genes and Genomes analysis indicated that the PI3K/AKT and MAPK signaling pathways are essential in the treatment of osteosarcoma with asiatic acid. Molecular docking revealed binding of asiatic acid to EGFR, Caspase-3, ESR1, HSP90AA1, IL-6 and SRC proteins. asiatic acid inhibited proliferation through G_2/M cell cycle arrest in osteosarcoma cells. In addition, asiatic acid induced mitochondria-dependent apoptosis as demonstrated by increases in Bax and VDAC1 expression, and a decrease in Bcl-2 protein expression. The increased autophagosomes, increased LC3-II/I ratios and decreased p62 expression in the treatment group indicated that asiatic acid triggered autophagy. In addition, asiatic acid decreased the levels of phosphorylated (p-)PI3K/PI3K and p-AKT/AKT, increased reactive oxygen species (ROS) and upregulated the levels of p-ERK1/2/ERK1/2, p-p38/p38 and p-JNK/JNK in osteosarcoma cells. These results demonstrated that asiatic acid inhibited osteosarcoma cells proliferation by inhibiting PI3K/AKT and activating ROS/MAPK signaling pathways, suggesting asiatic acid is a potential agent against osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignant bone tumor in adolescents, with a mean presenting age of 16 years and a male predominance (1-3). Pathologically, osteosarcomas are malignant mesenchymal tumors characterized by pleomorphic spindle-shaped cells capable of producing bone-like stroma (4-6). In addition, osteosarcomas present a high degree of malignancy, rapid growth and metastasis susceptibility and are difficult to treat, resulting in a relatively high rate of disability and mortality (7,8). Clinical treatments for osteosarcoma include surgery, adjuvant chemotherapy, targeted therapies and immunotherapy (9,10). However, the average 5-year survival rates for patients with primary osteosarcoma and distant metastases are <65 and 25%, respectively (11,12). Methotrexate, doxorubicin and cisplatin have become the standard regimens for the treatment of osteosarcoma in clinical practice. However, their clinical application is limited due to their toxicity and side effects (13). On the other hand, patients with metastases, recurrences and unresectable tumors often become resistant to current standard treatment regimens (14,15). Therefore, novel therapeutic agents for advanced osteosarcoma with less toxicity remain to be elucidated. Natural effective components of plants are potentially useful against tumors due to their multi-target and multi-pathway synergistic modulatory effects that can lead to multiple therapeutic effects at different stages of tumorigenesis, development, metastasis and immune regulation (16). Asiatic acid ($C_{30}H_{48}O_5$) is a pentacyclic triterpenoid from *Centella asiatica* with anti-inflammatory, neuroprotective, anti-diabetic, antitumor and antibacterial characteristics (17-19). The results of a phase I clinical trial of asiatic acid capsules (ECa 233) in healthy volunteers indicated a lack of adverse reactions, suggesting that asiatic acid is medicinally safe (20). Therefore, asiatic acid has the characteristics of fewer toxic side effects and antitumor effects, which can provide a reference value for

the treatment of osteosarcoma. However, the potential targets and mechanisms of the treatment of osteosarcoma with asiatic acid remain to be elucidated.

Cell cycle regulation mechanism proteins include cyclin, CDK and CDK inhibitors (21). Cancer therapy often targets the inhibition of cyclins and CDKs (22). During apoptosis, cells are stimulated by specific intracellular or extracellular signals to undergo programmed cell death (23) and the process results in chromatin pyknosis, DNA fragmentation, cell shrinkage and apoptotic body formation (24). Classical apoptosis mechanisms are triggered by intrinsic or extrinsic signaling pathways (25). The extrinsic apoptotic pathway starts with the binding of specific death receptors (such as Fas, tumor necrosis factor receptor 1 and death receptor 5) to their corresponding ligands, and recruitment of the adaptor proteins Fas associated death domain and caspase-8 to form a death-inducing signaling complex that initiates the subsequent caspase cascade to promote cell death (26). In the intrinsic apoptotic pathway, a Bcl-2 family protein imbalance leads to a decreased mitochondrial membrane potential (MMP) and the release of apoptosis-related proteins, resulting in activation of the caspase cascade and apoptosis induction (27). Autophagy is a type II cell death mechanism (28). This dynamic process is tightly regulated to allow cells to maintain their cellular nutrition and energy balance by phagocytosing and breaking down damaged or senescent proteins, organelles and harmful components (29). Autophagy mechanisms are frequently linked to apoptosis, inflammatory responses and cancer chemoresistance (30-32). The role of autophagy in tumor growth is two-sided. On the one hand, tumor cells can recover damaged or excess cytoplasmic contents in a lysosome-dependent manner through the autophagy pathway to overcome an undernourished environment and promote tumor growth. On the other hand, the autophagy regulation can induce apoptosis (33,34). Thus, cancer treatments can induce apoptosis by inhibiting autophagy in different tumor cell types and environments (35,36). Apoptosis-related proteins [Bcl-2, caspase-3 (Caspase-3) and P53] and diverse signaling pathways are activated during autophagy (37,38).

Network pharmacology can be used to predict potential targets and pathways by constructing complex networks among a drug, its targets and a disease, to improve the success rate of novel drug trials (39). Molecular docking is a theoretical simulation method that can be used to evaluate ligand-receptor interactions and find the best binding models for ligands (40). The present study used network pharmacology to predict the targets and signaling pathways associated with asiatic acid in osteosarcoma. Finally, the predicted targets and pathways were assessed via molecular docking and *in vitro* experiments to identify novel treatment strategies for osteosarcoma.

Materials and methods

Analysis of the disease-target-drug network. The molecular structure of asiatic acid was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and entered into the PharmMapper (http://www.lilab-ecust.cn/pharmmapper/), Swiss Target Prediction (http://swisstargetprediction.ch/) and Uniprot (https://www.uniprot.org/) databases to identify drug targets with scores >0 after eliminating repeated targets. In addition, the Online Mendelian Inheritance in Man (OMIM; https://omim.org/), Therapeutic Target Database (TTD; http://db.idrblab.net/ttd/) and Genecards (https://www.genecards.org/) databases were searched to identify disease targets based on the keyword 'osteosarcoma'. Duplicated disease targets were removed and Venn diagrams of common drug-disease targets were drawn using the Venny software mapping tool (https://bioinfogp.cnb.csic.es/tools/venny/). Finally, Cytoscape software (3.8.2 version) was used to draw a network diagram of 'disease-target-drug' interactions.

Protein-protein interaction (PPI) network construction. The aforementioned common targets were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/) and Cytoscape software (https://cytoscape.org/; version 3.8.2) was used to draw the resulting PPI network maps. Network analyzer was used to perform a topological analysis of the PPI networks. The core targets selected according to a degree value were mapped using R software (http://www.R-project.org).

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses. GO and KEGG functional enrichment analyses of critical target genes were performed using the Bioconductor bioinformatics package (https://www.bioconductor.org/; version 3.17) of R software (http://www.R-project.org). GO and KEGG enrichment analysis results were visualized in bar and bubble plots. P-value <0.05 was employed to identify statistically significant GO terms and KEGG pathways. The significance of GO and KEGG enrichment was represented by employing the fold change threshold=$-\log_{10}$ (P-value) for a more comprehensive depiction of the figure.

Molecular docking. The asiatic acid structure obtained from the PubChem database was imported into Chem3D software and the optimized structures were further imported into Schrodinger software (https://www.schrodinger.com/; version 2019.1) for hydrogenation and energy minimization. The protein structures of EGFR (1M17), SRC proto-oncogene, non-receptor tyrosine kinase (SRC; 1YOL), Caspase-3 (2CNK), heat shock protein 90α family class A member 1 (HSP90AA1; 4BQG), Estrogen Receptor 1 (ESR1; 7UJF) and Interleukin 6 (IL-6; 4O9H) were obtained from the RCSB database (https://www.rcsb.org/), and entered into Schrodinger Maestro's Protein Preparation Wizard for hydrogenation, peptide repair, energy minimization and structure optimization. The molecular docking was then optimized in the Glide platform of the software by determining sites on the basis of protein structure and ligands in 15 Å x 15 Å x 15 Å boxes. Finally, molecular docking and screening were conducted using the Standard Precision Glide Docking method.

Cell culture. MG63 human osteosarcoma cells (cat. no. TCHu124), HOS human osteosarcoma cells (cat. no. TCHu167) and MC3T3-E1 mouse-derived normal osteoblast cells (cat. no. GNM15) were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. 143B human osteosarcoma cells (cat. no. CRL-8303) were obtained from American Type

Culture Collection. Human osteosarcoma cells were cultured in Minimum Essential Medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) containing 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a sterile incubator containing 5% CO_2 at 37˚C. MC3T3-E1 cells were cultured in α-MEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin-streptomycin under the same controlled culture conditions. The osteosarcoma and MC3T3-E1 cells were passaged when reaching 80-90% confluency in culture flasks.

Antibodies and reagents. Asiatic acid (high-performance liquid chromatography ≥98%; Shanghai Yuanye Biotechnology Co., Ltd.) was dissolved in DMSO. An appropriate concentration of DMSO was used for the experimental controls. Antibodies against Bcl-2 (cat. no. 4223), Bax (cat. no. 2772), phosphorylated (p-)ERK1/2 (cat. no. 4370), ERK1/2 (cat. no. 4695), p38 (cat. no. 8690), p-p38 (cat. no. 4511), JNK (cat. no. 9252), p-JNK (cat. no. 4668), p-PI3K (cat. no. 17366), CDK2 (cat. no. 18048) and cyclin A2 (cat. no. 67955) and the goat anti-rabbit IgG (cat. no. 7074) and anti-mouse IgG (cat. no. 7076) secondary antibodies were purchased from Cell Signaling Technology, Inc. The voltage dependent anion channel 1 (VDAC1; cat. no. ab14734), p-AKT (cat. no. ab192623), AKT (cat. no. ab179463), PI3K (cat. no. ab191606), LC3 (cat. no. ab48394) and p62 (cat. no. ab56416) antibodies were obtained from Abcam. The GAPDH antibody (cat. no. 21612) was purchased from Signalway Antibody LLC.

Cell Counting Kit 8 (CCK8) cell viability assay. Growing osteosarcoma and MC3T3-E1 cells were trypsinized, centrifuged (180 x g; 25˚C; 3 min) and resuspended in complete MEM or α-MEM to prepare cell suspensions. Cells (~$4.0x10^3$) were seeded into 96-well plates and incubated at 37˚C for 24 h. Subsequently, the osteosarcoma cells were treated with asiatic acid (0, 10, 20 and 40 μM) at 37˚C for 24 and 48 h. The culture medium was replaced with 10 μl CCK8 (Beyotime Institute of Biotechnology) in MEM and cells were incubated at 37˚C for 2-4 h. OD values were determined using a microplate reader (MK3; Thermo Fisher Scientific, Inc.) at 450 nm.

Cell cycle analysis. The osteosarcoma cells were treated with asiatic acid (0, 20 and 40 μM) at 37˚C for 24 h, then collected by centrifugation (180 x g; 4˚C; min) and fixed with 70% chilled ethanol at 4˚C for 12 h. The fixed cells were stained with PI dye (Beyotime Institute of Biotechnology) and incubated at 37˚C for 30 min. A flow cytometer (FACSCelesta; BD Biosciences) and Modfit LT software (5.0 version; Verity Software House, Inc.) were used to analyze the cell cycle phases.

Apoptosis analysis. The osteosarcoma cells (~$15.0x10^4$) were seeded into six-well plates and incubated with asiatic acid (0, 10, 20 and 40 μM) at 37˚C for 24 h. The adherent cells from each well were collected, and suspended and placed in a flow tube. To stain the cells, the cells were resuspended in 5 μl annexin V-FITC and 10 μl PI (Beyotime Institute of Biotechnology) and incubated at room temperature for 30 min. Finally, the stained cells were analyzed by flow cytometry (FACSCelesta; BD Biosciences) and BD FACSDiva Software (version 6.1.3; BD Biosciences). The apoptotic rate was calculated by the percentage of early + late apoptotic cells.

Measurement of MMP. The osteosarcoma cells were treated with asiatic acid for 24 h and then resuspended in 0.5 ml cell culture medium. JC-1 (0.5 ml; Beyotime Institute of Biotechnology) staining working solution was added to the culture medium and the cultures were placed in a cell incubator at 37˚C for 20 min. Subsequently, the cells were washed twice with staining buffer and analyzed using a flow cytometer (FACSCelesta; BD Biosciences) and BD FACSDiva Software (version 6.1.3; BD Biosciences).

Measurement of intracellular reactive oxygen species (ROS) content. The 143B and HOS human osteosarcoma cell lines were treated with asiatic acid (0 and 40 μM) at 37˚C for 24 h. Dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology) was then diluted with serum-free MEM to a final working solution concentration of 10 μM, and the cells were resuspended in the diluted DCFH-DA and incubated at 37˚C for 20 min. After washing the cells three times with serum-free MEM, intracellular ROS levels were measured using a flow cytometer (FACSCelesta; BD Biosciences) and BD FACSDiva Software (version 6.1.3; BD Biosciences).

Transmission electron microscopy. The preparation and observation of electron microscope specimens was completed in the following seven steps. In the first step, the collected cells were fixed with 3% glutaraldehyde (SPI-CHEM Inc.) for 4 h at 4˚C, and then fixed with 0.1 M sodium dimethylarsenate buffer at 4˚C for three times, with a 2 h interval between changes. In the second step, the samples were subsequently immersed with 1% osmium acid (SPI-CHEM Inc.) for 2 h at 4˚C, then rinsed with 0.1 M sodium dimethylarsenate buffer twice, each time at 4˚C for 15 min. Step three was staining with saturated uranyl acetate dye (SPI-CHEM Inc.) for 2 h at room temperature. In the fourth step, the stained samples were soaked in 50% alcohol at 4˚C for 10 min, 70% alcohol at 4˚C for 10 min, 80% alcohol at room temperature for 10 min, 90% alcohol at room temperature for 10 min, anhydrous ethanol soaks twice at room temperature for 10 min, times acetone permeations twice at room temperature for 15 min, a complete encapsulation solution (Eponate12 epoxy resin; Ted Pella, Inc.) and acetone in a 1:1 ratio at room temperature for 3 h, then changing the above ratio to a 1:2 penetration for 3 h, and then overnight penetration of the complete embedding solution at room temperature. In the fifth step, the samples soaked with the complete embedding solution were placed in a 40˚C incubator for 12 h, and then transferred to an embedding plate still soaked with the complete embedding solution and placed in a 60˚C incubator for 24 h. In the sixth step, the samples were sectioned by using an ultrathin sectioning machine (UC7; Leica Microsystems GmbH), with a section thickness of 90 nm. Finally, the samples were observed by a JEM-1400 transmission electron microscope (JEOL Ltd.) with an operating voltage of 80 kV, and image

acquisition was performed with DigitalMicrograph Software version 832 (Gatan, Inc.; Thermo Fisher Scientific, Inc.).

Western blot analysis. Osteosarcoma cells were trypsinized and collected by centrifugation (180 x g; 4˚C; 3 min) before mixing with appropriate amounts of protein lysate (PMSF; RIPA, 1:100; Beyotime Institute of Biotechnology). The cells were lysed on ice, sonicated and centrifuged at high speed (13,800 x g; 4˚C; 15 min) to collect the supernatant protein. Next, the protein concentration of each sample was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology) and each protein sample was adjusted to the same concentration. Subsequently, 5X SDS-PAGE protein loading buffer was added to the protein solutions and these were boiled for 10 min. Proteins (20 μg/lane) were separated by SDS-PAGE on 10 and 12% separation gels and then transferred (250 mA; 2.5 h) to PVDF membranes (pore size, 0.2 μm; Merck KGaA). Fresh 5% skimmed milk was used to block the membranes for 60 min. Subsequently, the membranes were cut and incubated with primary antibodies (dilution, 1:1,000) at 4˚C for 12 h and with secondary antibodies (dilution, 1:3,000) at room temperature for 2 h. The ECL reagents (Zeta-Life Inc.) and Fluorescent Imaging System (Tanon 5200; Tanon Science and Technology Co., Ltd.) were used to visualize the protein bands. In addition, grey value intensities were calculated using ImageJ software (v1.8.0; National Institutes of Health).

Statistical analysis. All data are presented as the mean ± SD of at least three independent experiments, and all data were analyzed and graphs were plotted using the Figdraw Platform (https://www.figdraw.com/), SPSS Statistics 25.0 (IBM Corp.) and GraphPad Prism 8.0 (GraphPad Software; Dotmatics) software. Unpaired Student's t-tests were used for comparisons between 2 groups. Statistical differences among ≥3 groups were determined using one-way ANOVA followed by Tukey's post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Potential targets of asiatic acid in osteosarcoma. The asiatic acid structure was obtained from the PubChem database, 341 drug targets after de-duplication were obtained from the PharmMapper, Swiss Target Prediction and Uniprot databases (Table SI), and 911 disease targets were obtained from the OMIM, TTD and Genecards databases (Table SII). A Venn diagram (Fig. 1A) identified 78 common targets, which were entered into Cytoscape to draw a network diagram of 'osteosarcoma-target-asiatic acid' interactions (Fig. 1B). The common targets of asiatic acid were entered into the STRING database and a PPI network graph with 78 nodes, 969 edges and 4 concentric circles was obtained (Fig. 1C). Node size, color and depth represent the degree values. The PPI network data were then subjected to topological analysis and 30 core targets were identified based on mean degree values. Among the core target genes, EGFR (64 edges), Caspase-3 (55 edges), ESR1 (54 edges), HSP90AA1 (54 edges), IL-6 (54 edges) and SRC (54 edges) had a high node degree, indicating their close association with the effect of asiatic acid against osteosarcoma (Fig. 1D).

GO and KEGG analysis of asiatic acid treatment-associated genes in osteosarcoma. Common targets were analyzed via GO enrichment analysis after running the R programming language to select 1,581 biological process (BP) pathways, 27 items associated with cellular component (CC) expression processes and 110 items associated with molecular function (MF) processes. Fig. 2A shows the top 10 BP, CC and MF items. KEGG analysis of the common targets revealed 134 related pathways, enriched in cancer-related signaling pathways such as 'Proteoglycans in cancer' (hsa05205), 'PI3K-AKT signaling pathway' (hsa04151), 'Rap1 signaling pathway' (hsa04015) 'MAPK signaling pathway' (hsa04010) and 'Ras signaling pathway' (hsa04014). Fig. 2B shows the top 20 results from a KEGG enrichment bubble map. The present results suggested that the PI3K-AKT and MAPK signaling pathways may be associated with the effects of asiatic acid against osteosarcoma.

Molecular docking analysis of the core targets. The PPI network analysis of asiatic acid and osteosarcoma revealed target genes with a significant degree value of association with anti-osteosarcoma effects. Therefore, the top six highest-ranking target genes were selected for molecular docking. Binding energy and hydrogen bond interactions were assessed to evaluate the affinity and binding capacity between asiatic acid and target proteins. Molecular docking visualization showed hydrogen bonds between asiatic acid and amino acids in the active sites of the target proteins. asiatic acid binds the following proteins: EGFR (binding energy, -7.01 kcal/mol) forming hydrogen bonds with LYS-721, ASP-776, CYS-773 and ASP-831 amino acids in its active site (Fig. 3A); Caspase-3 (binding energy, -6.51 kcal/mol) forming hydrogen bonds with TRP-206, GLY-122 and THR-62 amino acids in its active site (Fig. 3B); ESR1 (binding energy, -6.85 kcal/mol) forming hydrogen bonds with VAL-533, THR-347 and ASP-351 amino acids in its active site (Fig. 3C); HSP90AA1 (binding energy, -5.83 kcal/mol) forming hydrogen bonds with LYS-581, ASN-51, GLY-135 and LEU-107 amino acids in its active site (Fig. 3D); IL-6 (binding energy, -6.69 kcal/mol) forming hydrogen bonds with LYS-86, TYR-97 and ASN-63 amino acids in its active site (Fig. 3E); and SRC (binding energy, -7.02 kcal/mol) forming hydrogen bonds with GLY-346, SER-347 and ASN-393 amino acids in its active site (Fig. 3F). In summary, molecular docking revealed that asiatic acid exhibited strong affinity and binding potential with the target proteins EGFR, Caspase-3, ESR1, HSP90AA1, IL-6 and SRC.

Asiatic acid inhibits proliferation and induces cell cycle arrest of osteosarcoma cells. Asiatic acid inhibited the proliferation of 143B, MG63 and HOS human osteosarcoma cell lines in a dose- and time-dependent manner. No cytotoxicity was observed in MC3T3-E1 normal osteoblast cells treated with asiatic acid under the same conditions (Fig. 4A). Cell cycle analysis revealed that the proportion of cells in the G_0/G_1 phase was decreased and that in the G_2/M phase was increased

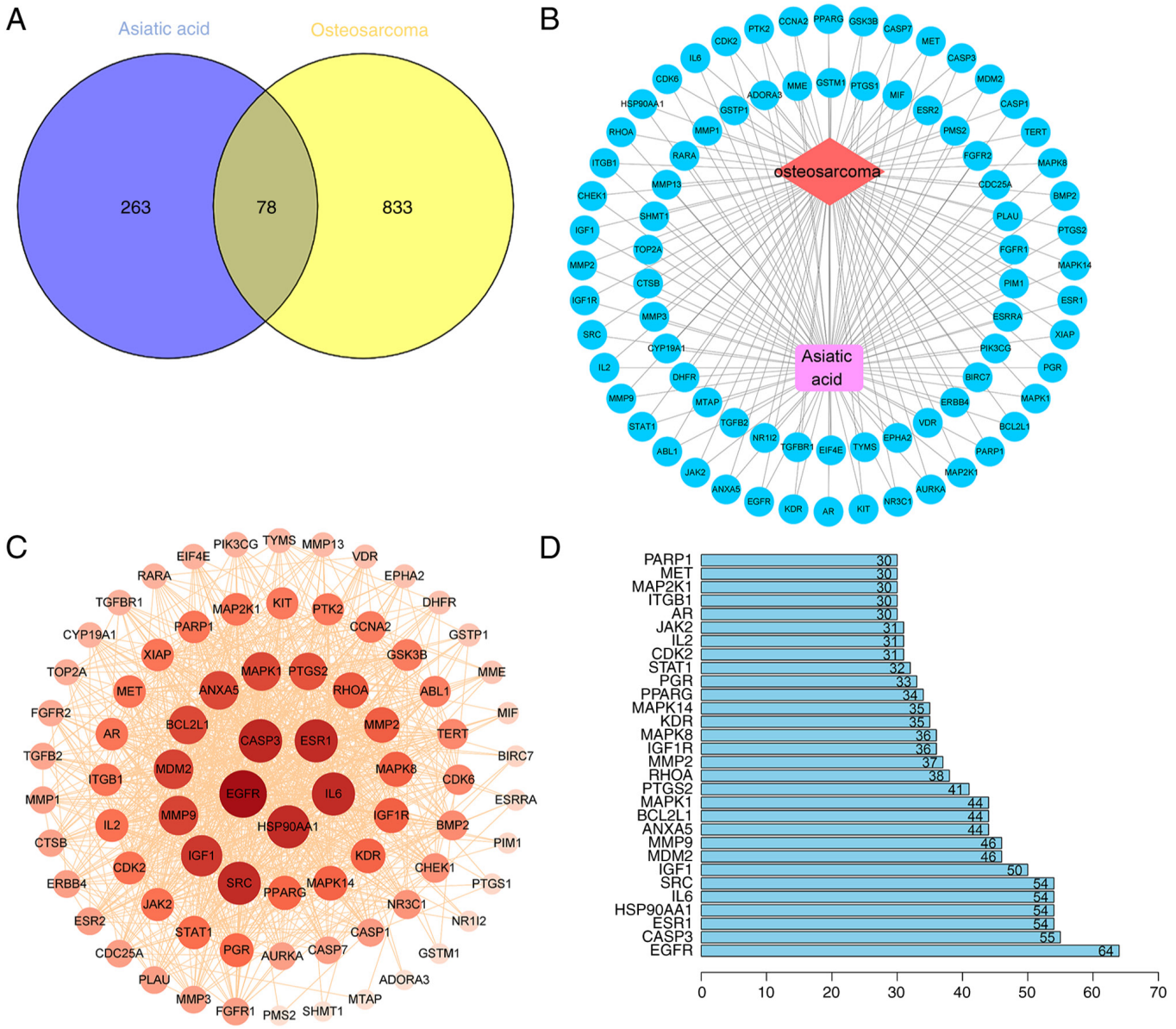

Figure 1. Potential targets of asiatic acid associated with osteosarcoma. (A) Venn diagram of asiatic acid and osteosarcoma-associated targets. (B) Network diagram of osteosarcoma-target-asiatic acid interactions. The purple lines represent asiatic acid, blue lines represent 78 common targets, and red lines represent osteosarcoma. (C) PPI network of common targets of asiatic acid and osteosarcoma. (D) Bar graph showing the top ranked 30 core targets based on PPI topological analysis.

(Fig. 4B). Western blot analysis further demonstrated that asiatic acid suppressed the expression of cyclin A2 and CDK2 in osteosarcoma cells (Fig. 4C).

Asiatic acid induces apoptosis in osteosarcoma cells. To investigate whether asiatic acid induced apoptosis in 143B, MG63 and HOS osteosarcoma cells, annexin V-FITC/PI double staining reagent was used to analyze the proportion of apoptotic cells. The present results demonstrated that the proportion of apoptotic cells increased with increasing asiatic acid concentrations (Fig. 5A). MMP level reductions are an essential feature of early apoptosis stages (41). Therefore, the MMP was examined and an increase in green fluorescence was observed in JC-1-labeled mitochondrial membranes, indicating early apoptosis with a reduced MMP (Fig. 5B). In addition, changes in apoptosis-related proteins validated these findings. The present results indicated that asiatic acid increased the expression levels of Bax and VDAC1, and decreased the expression levels of Bcl-2 (Fig. 5C). Thus, asiatic acid promoted mitochondrial dysfunction and induced apoptosis in osteosarcoma cells.

Asiatic acid triggers autophagy in osteosarcoma cells. Autophagy is closely related to apoptosis and both processes are involved in tumor cell death mechanisms (42,43). Therefore, the present study next investigated whether asiatic acid triggers autophagy in 143B, MG63 and HOS osteosarcoma cells. Transmission electron microscopy is the gold standard for examining autophagy by confirming the presence of autophagosomes at the subcellular level. Osteosarcoma cells were treated with 40 μM asiatic acid. The transmission electron microscopy images revealed

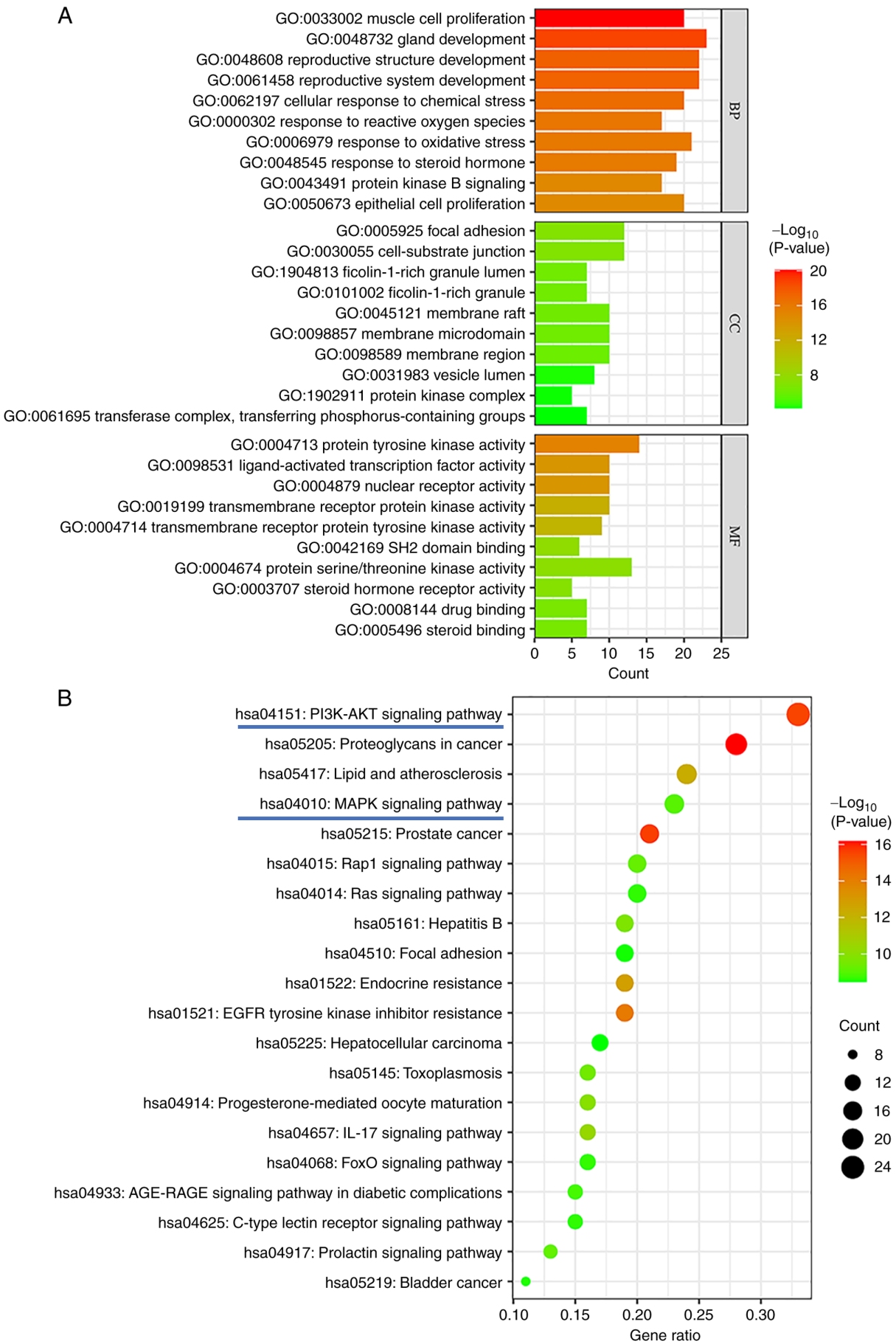

Figure 2. GO and KEGG analysis results. (A) GO enrichment analysis of target genes. (B) KEGG enrichment analysis of target genes. The bubble plot shows the top 20 enriched KEGG signaling pathways. P-values represent significant differences in enriched KEGG pathways. The red color intensity increases with the level of significance. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

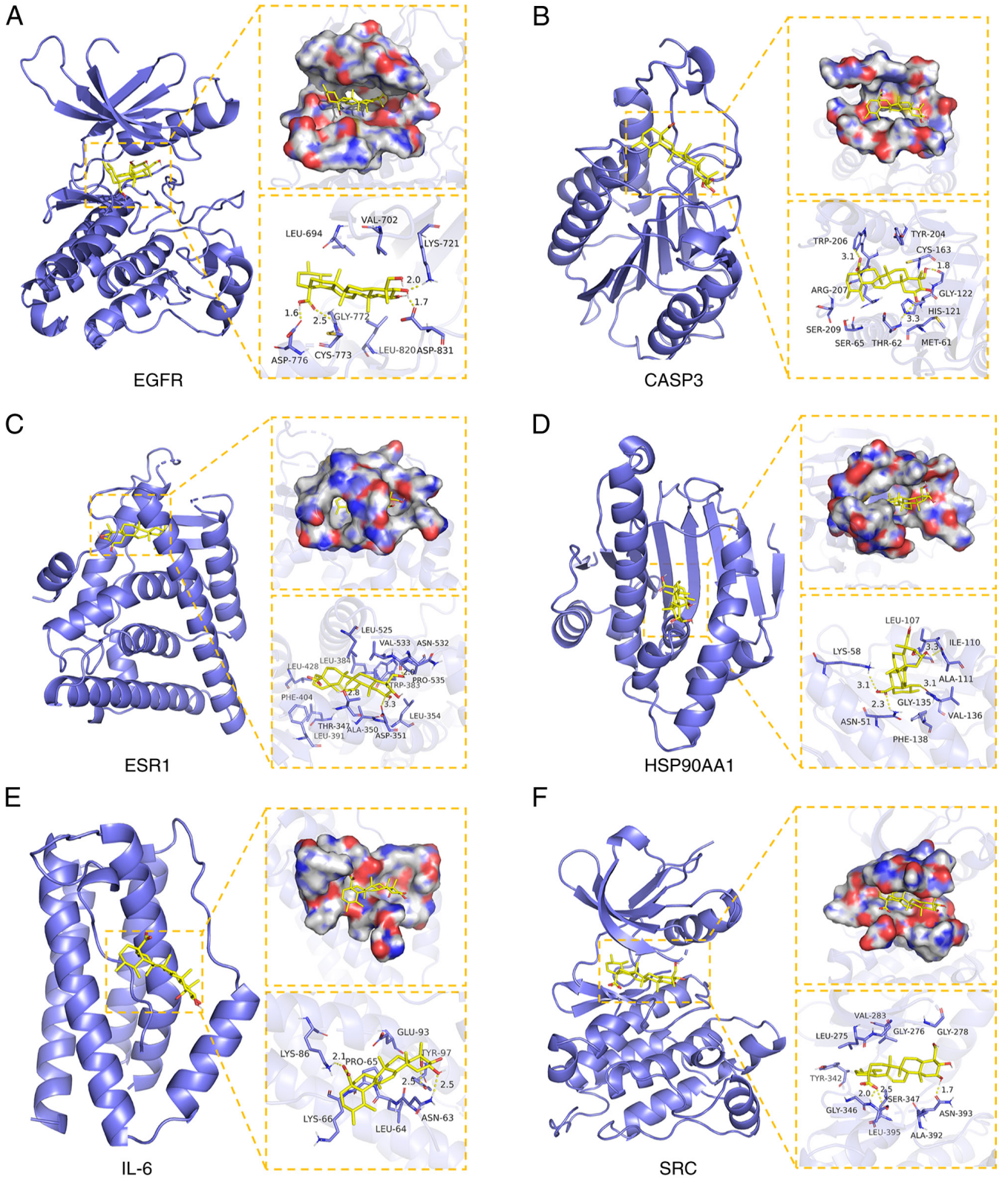

Figure 3. Molecular Docking Analysis of asiatic acid with its core target. (A) Asiatic acid binds four amino acid residues (LYS-721, ASP-776, CYS-773, and ASP-831) in EGFR protein via hydrogen bonds. (B) Asiatic acid binds three amino acid residues (TRP-206, GLY-122, and THR-62) in Caspase-3 protein via hydrogen bonds. (C) Asiatic acid binds to three amino acid residues (VAL-533, THR-341, and ASP-351) in the ESR1 protein via hydrogen bonds. (D) Asiatic acid binds four amino acid residues (LYS-581, ASN-51, GLY-135, and LEU-107) in the HSP90AA1 protein via hydrogen bonds. (E) Asiatic acid binds three amino acid residues (LYS-86, TYR-97, and ASN-63) in the IL-6 protein via hydrogen bonds. (F) Asiatic acid binds three amino acid residues (GLY-346, SER-347, and ASN-393) in the SRC protein via hydrogen bonds. Yellow dashed lines represent hydrogen bonds.

marked autophagosome increases in all the treated cells (Fig. 6A). Furthermore, the present study assessed the effects of asiatic acid on the levels of autophagy-related proteins LC3 and p62, which are marker proteins, and the western

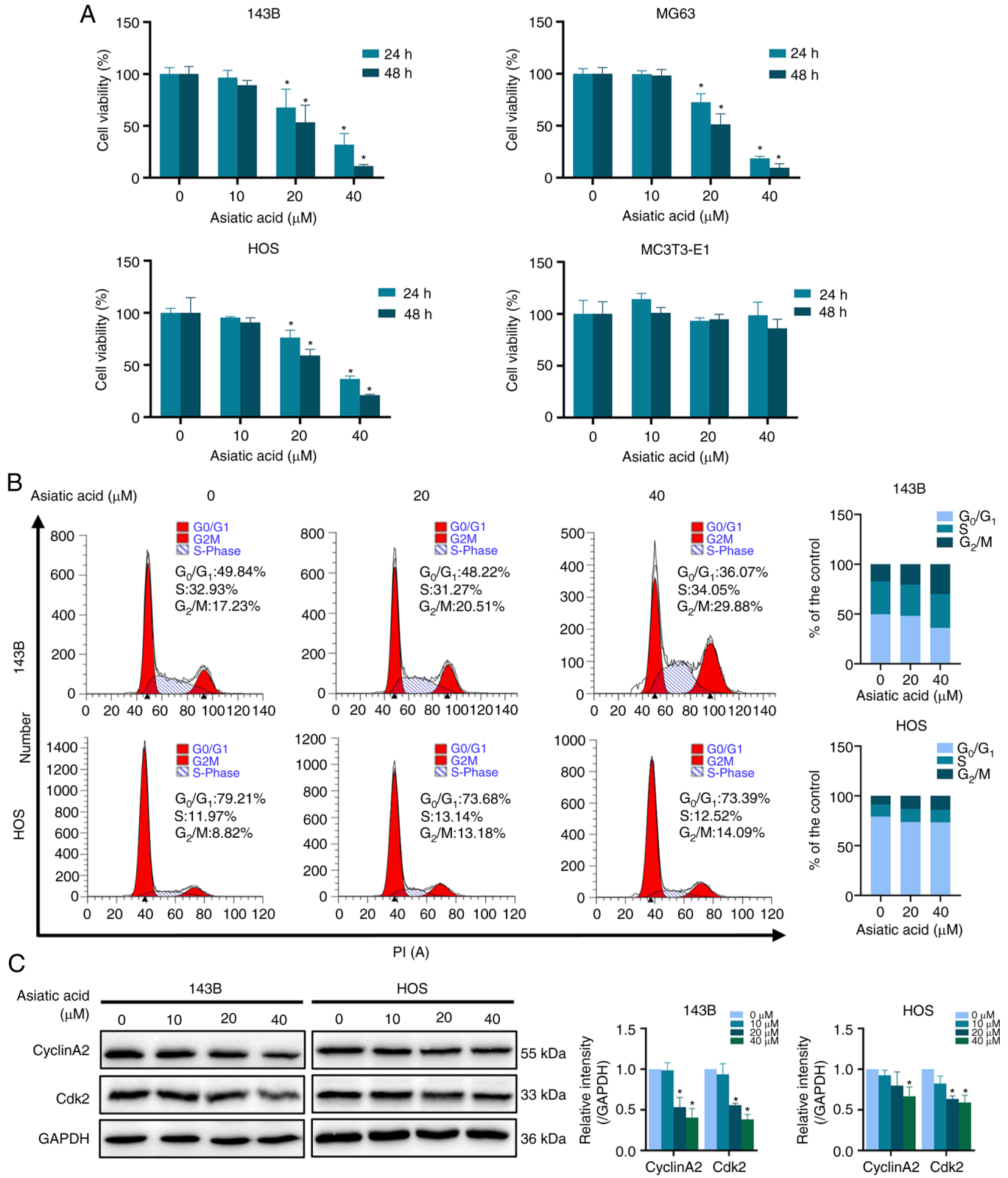

Figure 4. Asiatic acid inhibits the proliferation of osteosarcoma cells. (A) Cell viabilities of osteosarcoma (143B, MG63, and HOS) and MC3T3-E1 cells treated with asiatic acid (0, 10, 20, and 40 μM) for 24-48 h using CCK8 assays. (B) Cycle arrest of 143B and HOS cells detected via flow cytometry. (C) Expression levels of cell cycle-related CyclinA2 and Cdk2 proteins via western blot analysis. *P<0.05 vs. control group.

blot analysis results demonstrated that asiatic acid treatment markedly increased the LC3-II/I ratio and decreased the p62 levels, indicating that asiatic acid could trigger autophagy in osteosarcoma cells (Fig. 6B).

Asiatic acid inhibits the PI3K/AKT signaling pathway and activates the ROS/MAPK signaling pathway in osteosarcoma cells. The KEGG enrichment analysis results suggested that the PI3K/AKT and the MAPK signaling pathways were

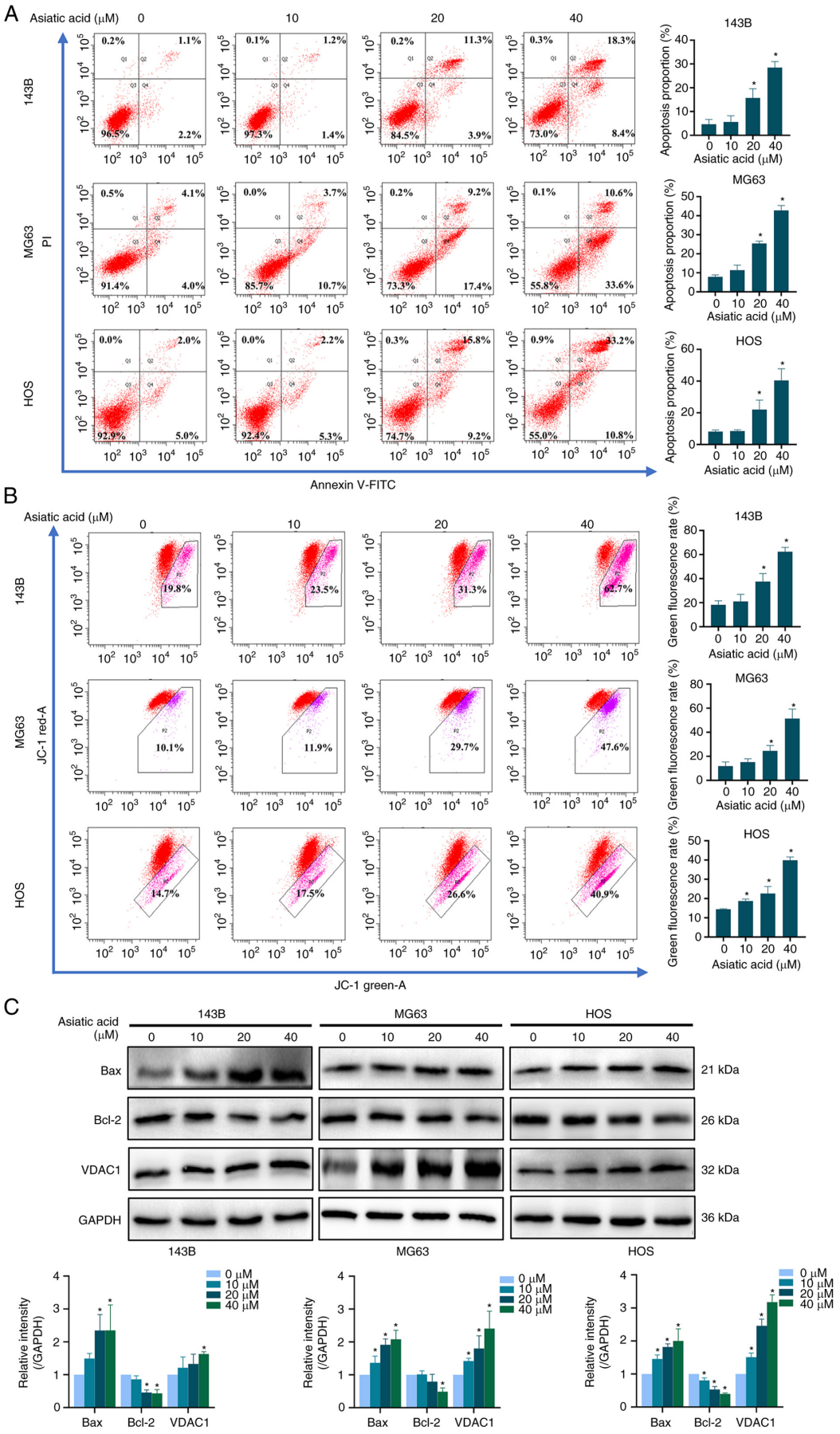

Figure 5. Asiatic acid-induced apoptosis in osteosarcoma cells. (A) 143B, MG-63 and HOS osteosarcoma cell double staining with Annexin V-FITC/PI to measure apoptosis rates. (B) Flow cytometry detection of MMP in osteosarcoma cells stained with mitochondrial probe JC-1. (C) Western blot analysis showing apoptosis-related proteins Bax, Bcl-2, and VDAC1 after being treated with asiatic acid for 24 h. *P<0.05 vs. control group. MMP, mitochondrial membrane potential.

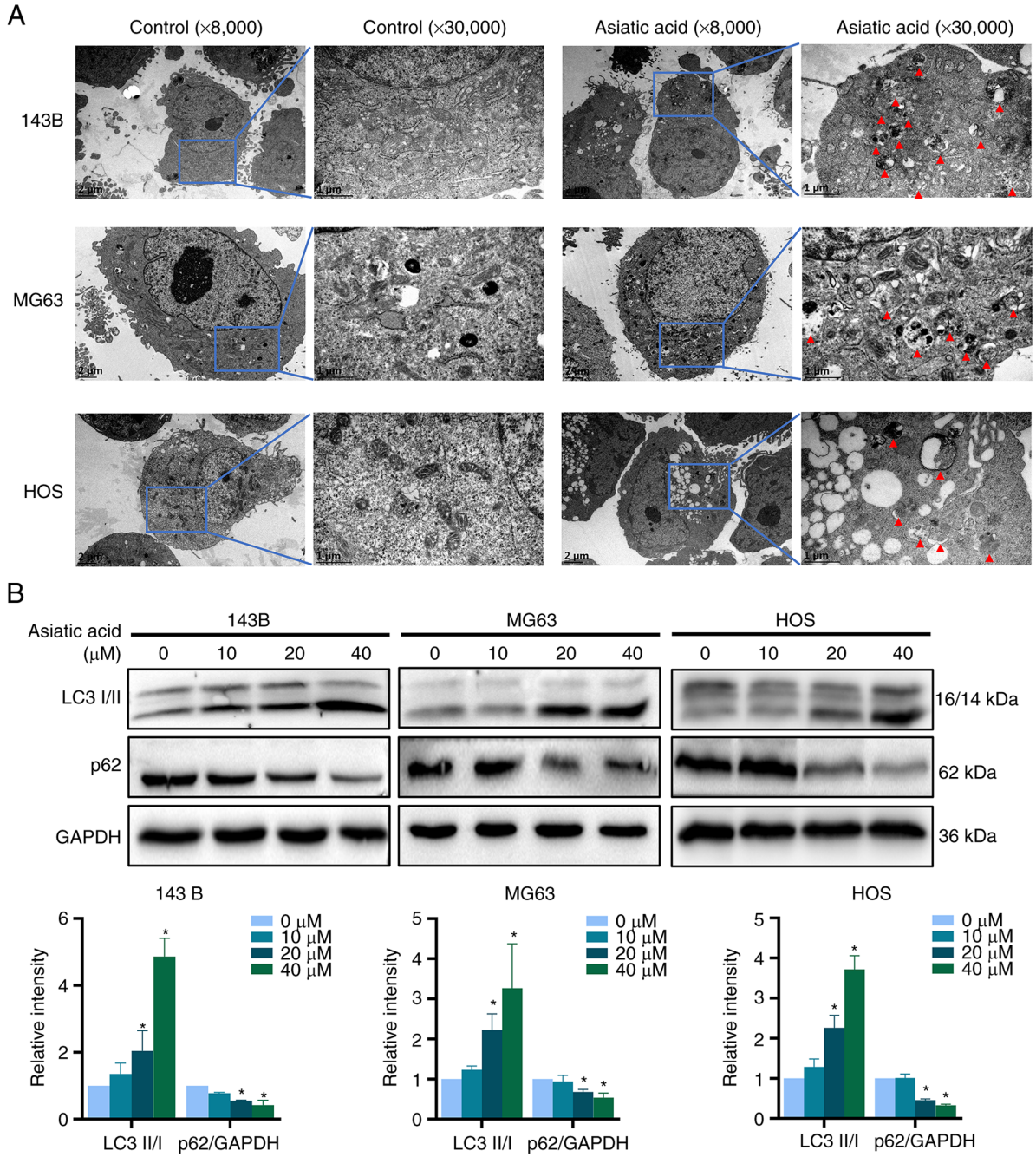

Figure 6. Asiatic acid triggers autophagy in osteosarcoma cells. (A) Autophagosome quantities in 143B, MG-63, and HOS osteosarcoma cells as observed via transmission electron microscopy. Red triangles indicate autophagosomes. Low magnification, 8,000x; High magnification, 30,000x. Scale bars, 2 and 1 μm. (B) LC3I/II and p62 protein expressions as detected by western blot analysis following asiatic acid treatment. *P<0.05 vs. control group.

associated with the anti-osteosarcoma effects of asiatic acid. Thus, the present study examined the expression levels of PI3K/AKT signaling pathway-related proteins. Fig. 7A shows that asiatic acid treatment markedly decreased the levels of p-PI3K/PI3K and p-AKT/AKT. ROS analysis using DCFH-DA staining (Fig. 7B) revealed that asiatic acid treatment markedly increased intracellular ROS content. Western blot analysis results further demonstrated that asiatic acid upregulated the protein levels of p-ERK1/2/ERK, p-p38/p38 and p-JNK/JNK (Fig. 7C). Consequently, asiatic acid may inhibit the PI3K/AKT signaling pathway and activate the ROS/MAPK signaling pathway to regulate osteosarcoma cell death mechanisms.

Discussion

Doxorubicin, cisplatin and methotrexate are common chemotherapy agents used against osteosarcoma (44); however, their cytotoxic effects and drug resistance limit their use in the clinic (45,46). Therefore, uncovering the pathogenesis of osteosarcoma and developing safe and effective therapeutic agents is important. In the present study, the potential targets and pathways of asiatic acid for osteosarcoma treatment were predicted using network pharmacology and molecular docking, and validated by *in vitro* experiments in osteosarcoma cells. The results indicated 78 common targets of asiatic acid and osteosarcoma. PPI network analysis identified EGFR,

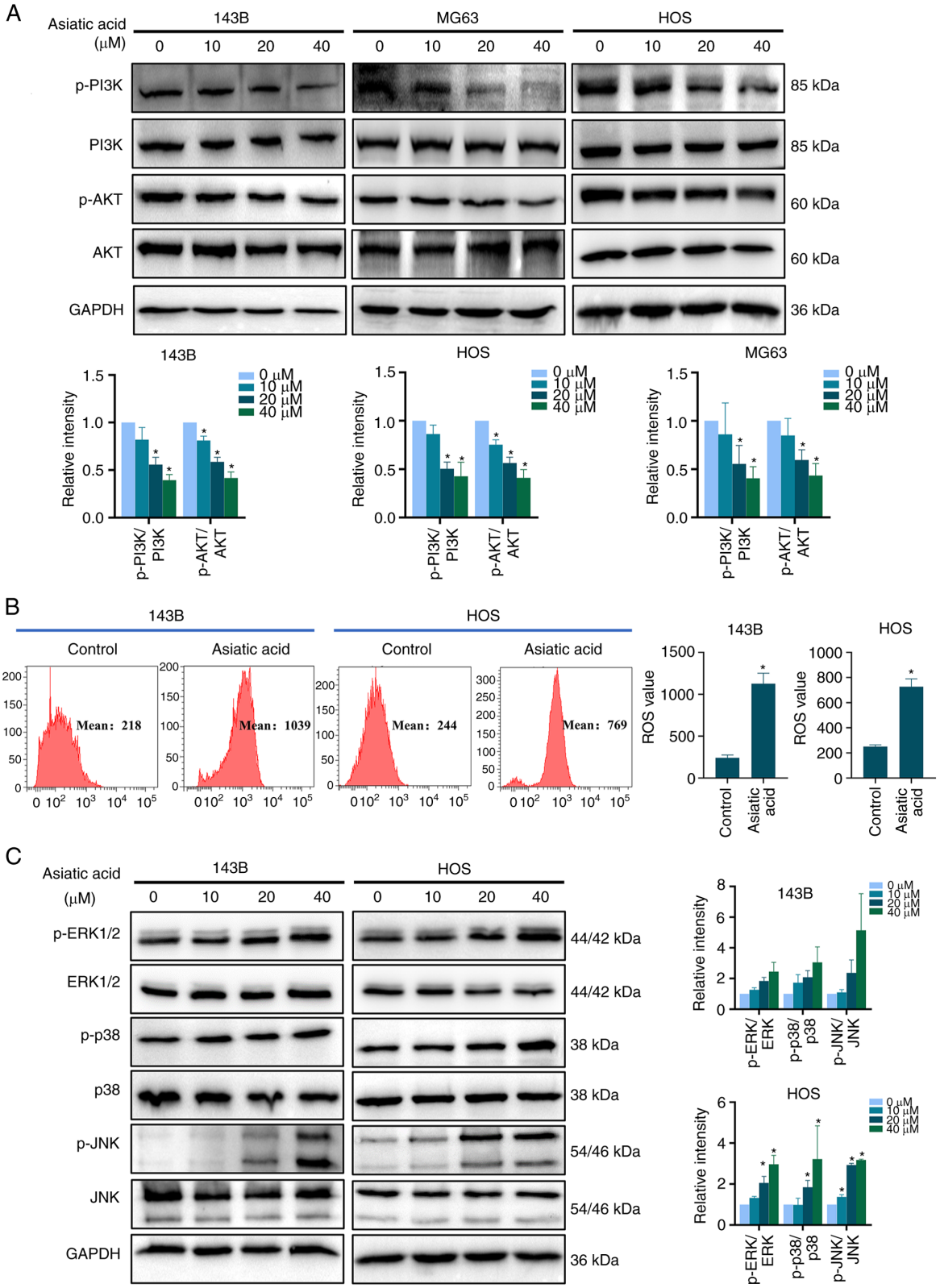

Figure 7. Asiatic acid inhibits PI3K/AKT and activates ROS/MAPK pathways in osteosarcoma cells. Osteosarcoma cells were treated with asiatic acid for 24 h. (A) Protein levels of p-PI3K, PI3K, p-AKT, and AKT as measured by western blot analyses. (B) Intracellular ROS content by flow cytometry. (C) Protein levels of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, and JNK by western blot analyses. *P<0.05 vs. control group. ROS, reactive oxygen species; p-, phosphorylated.

Caspase-3, ESR1, HSP90AA1, IL-6 and SRC as potential targets of asiatic acid in osteosarcoma. KEGG pathway analysis suggested a mechanism for asiatic acid against osteosarcoma associated with cancer-related pathways such as 'Proteoglycans in cancer' (hsa05205), 'PI3K-AKT signaling pathway' (hsa04151), 'Rap1 signaling pathway' (hsa04015) 'MAPK signaling pathway' (hsa04010) and 'Ras signaling pathway' (hsa04014).

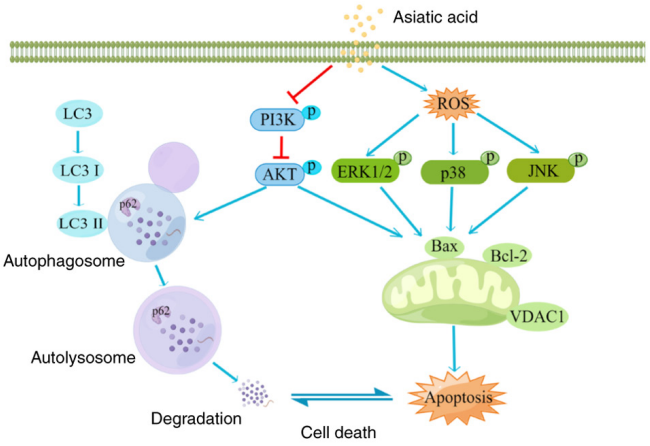

Figure 8. Potential mechanisms of asiatic acid-induced apoptosis and autophagy in osteosarcoma cells. ROS, reactive oxygen species.

Numerous types of cancer develop after genetic aberrations in EGFR facilitate growth, motility and metastasis of tumor cells (47,48). Abnormal expression and mutations of EGFR are associated with the development of osteosarcoma (49,50). Upregulation of EGFR expression is prevalent in osteosarcoma samples and EGFR expression is associated with chemotherapy-induced stress survival of tumor cells (51,52). Caspase-3 is the main terminal cleaving enzyme of apoptosis and is an essential target for cancer therapy (53). ESR1 is positive in most osteosarcoma specimens, its level is associated with the tumor volume and inhibition of the ESR1 enhances chemotherapeutic effects in P53(+) osteosarcomas (54). HSP90AA1, a heat shock protein, promotes cancer metastases and drug resistance in multiple tumors (55-57). In addition, as an essential autophagy regulator, it participates in the drug resistance of osteosarcoma cells (58). Abnormal IL-6 expression is associated with tumor angiogenesis, invasion, metastases, diagnoses and prognoses (59,60). IL-6 can improve invasion by promoting the expression of intercellular adhesion molecule-1 in osteosarcoma cells (61). It also promotes osteosarcoma stemness and carcinogenicity by upregulating the STAT3 signaling pathway (62). SRC is a member of the non-receptor protein tyrosine kinase family. Its abnormal expression can cause the development of certain tumors (63), and the protein has been associated with malignant features of osteosarcomas (64,65). The activated SRC kinase activates the MAPK and PI3K/AKT signaling pathways by phosphorylating target tyrosine residues (65,66). Based on PPI network analysis, EGFR, Caspase-3, ESR1, HSP90AA1, IL-6 and SRC proteins were selected for molecular docking. The present findings demonstrated that asiatic acid had a strong affinity for these targets, indicating its anti-osteosarcoma effect through these targets and related pathways.

Cyclins and CDKs determine cell cycle progression (67). Numerous anticancer drugs arrest cancer cell cycles (68). CDK2/cyclin E and CDK2/cyclin A promote the initiation and progression of DNA replication through the S phase (69), while CDK1/cyclin A and CDK1/cyclin B complexes activate the expression of genes essential for the mitotic process (70) during the G_2/M phase. Through *in vitro* validation, the present results indicated that asiatic acid inhibited proliferation, induced G_2/M arrest and suppressed cyclin A2 and CDK2 levels in osteosarcoma cells. The utilization of MC3T3-E1 mouse-derived normal osteoblast cells as controls in safe concentration trials for osteosarcoma has been extensively documented (71-74). Therefore, the present study purposively opted for MC3T3-E1 normal osteoblast cells as a reference acid on normal cells. However, it would be preferable to select human normal osteoblast cells, such as the hFOB 1.19 cell line. Unfortunately, the lack of toxicity assessment of asiatic acid on normal human osteoblast cells is a limitation in the present study. In addition, it was observed that the viability of MC3T3-E1 normal osteoblasts remained unaffected under the same conditions, thereby demonstrating the anti-osteosarcoma effects of asiatic acid within a safe concentration range. Thus, asiatic acid inhibited osteosarcoma cell proliferation through G_2/M cell cycle arrest.

Elevations in the Bax/Bcl-2 ratio reduce the MMP in the early apoptosis stages (75), triggering the release of cytochrome C, which further activates the caspase family and induces cell death (76,77). The channel protein VDAC1 on the outer mitochondrial membrane controls the entry and exit of substances and energy into and out of mitochondria (78), including the entry of cytochrome C into the cytoplasm. VDAC1 is also a crucial component of apoptosis (79,80) regulating related proteins to induce apoptosis (81). The present flow cytometry experiments revealed that asiatic acid treatment reduced the MMP and induced apoptosis in osteosarcoma cells, as demonstrated by increased Bax and VDAC1 levels and decreased Bcl-2 levels, indicating mitochondria-dependent apoptosis. Autophagy includes four stages: Initiation, autophagosome formation, binding of autophagosomes to lysosomes and autophagosome degradation (82,83). During autophagy, cytoplasmic LC3 becomes hydrolyzed to LC3-I, which is conjugated with phosphatidylethanolamine to form LC3-II, which is found in autophagosome membranes (84,85). During this dynamic process, p62 is selectively encapsulated into autophagosomes and later degraded by autolysosomes (86). The present results revealed an increase in the LC3-II/LC3-I ratio and a decrease in p62 protein expression in osteosarcoma cells treated with asiatic acid, indicating that autophagy had been triggered. In addition, the high autophagosome abundance observed in asiatic acid-treated cells under the electron microscope further confirmed that asiatic acid induced autophagy in osteosarcoma cells.

KEGG enrichment analysis of network pharmacology revealed that the PI3K/AKT and MAPK signaling pathways are essential for asiatic acid to combat osteosarcomas. The PI3K/AKT signaling pathway is fixed and activated in various tumors and promotes tumor development (87). It mediates the progression of osteosarcoma cell proliferation, metastasis, apoptosis and autophagy (88,89). Furthermore, studies have demonstrated that inhibiting the activity of this pathway and its related upstream and downstream molecules via small molecule inhibitors is vital for treating osteosarcomas (90,91). The ERK1/2, JNK and p38 proteins of the MAPK cascade pathway are sensitive to intracellular oxidative stress (92-94). ROS within osteosarcoma cells activate the MAPK signaling

pathway, which induces apoptosis and autophagy (95,96). Notably, asiatic acid has antioxidant properties in osteoporotic mice, RAW264.7 cells, H9c2 rat cardiomyocytes and HepG2 human hepatoma cells, mainly involving peroxisome proliferator-activated receptor γ expression and the AKT/GSK-3β/hypoxia-inducible factor-1α, sirtuin 1/FOXO1 and nuclear factor erythroid 2-related factor 2/heme oxygenase 1 (Nrf2/HO-1) pathways (97-100). However, asiatic acid also promotes ROS generation in MCF7 human breast cancer cells, A549 and H1299 human lung cancer cells, and SK-MEL-2 human melanoma cells (101-103). Antioxidant and pro-oxidant effects in different cellular biological environments, probably due to different targets and mechanisms of asiatic acid. The present findings suggested that asiatic acid decreased the phosphorylation of PI3K and AKT, increased intracellular ROS levels, and resulted in markedly higher p-ERK1/2/ERK1/2, p-p38/p38 and p-JNK/JNK levels in osteosarcoma cells. Regrettably, the absence of evaluation for transfection of target gene overexpression, mutation and inhibition experiments and *in vivo* experiments are limitations of the present study. Based on the present results, it was hypothesized that asiatic acid may induce apoptosis and autophagy in osteosarcoma cells by inhibiting PI3K/AKT and activating ROS/MAPK pathways.

In conclusion, the present study explored the multi-target and multi-signaling pathways of asiatic acid against osteosarcoma using network pharmacology and molecular docking techniques. Subsequently, the present *in vitro* experiment results demonstrated that asiatic acid mediated apoptosis and autophagy in osteosarcoma cells by inhibiting the PI3K/AKT signaling pathways and activating the ROS/MAPK signaling pathways. The excellent anti-osteosarcoma efficacy and associated mechanisms of asiatic acid (Fig. 8) make it a promising novel anti-osteosarcoma agent.

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Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

Authors' contributions

BW, LS, and HP conducted and designed the research. HP, HW, and ZZ performed the experiments, analyzed data and wrote the paper. HP, MX and TW provided technical support and purchased all the reagents and chemicals needed. ZW, HP, HW, and TW designed the figures. HP and HW contributed equally to this work and should be considered co-first authors. HP and HW confirm the authenticity of all the raw data. All authors contributed to the article and all authors read and approved the final manuscript.

Ethics approval and consent to participate

The PubChem, PharmMapper, Swiss Target Prediction, Uniprot, OMIM, TTD, Genecards, and STRING databases are publicly available and allows researchers to download and analyze. Thus, our Ethics Committee of Affiliated Hospital of Guangdong Medical University waived ethical approval for open public databases that were used in this work.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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