

Preclinical antitumor activity of Ferulasong A from *Ferula songarica* against gastric cancer: multi-omics profiling and computational assessment of a putative interaction with MDM2

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Abstract

Background

Ferulasong A (FA) is a structurally characterized sesquiterpenoid isolated from *Ferula songarica*. Although preliminary anti-gastric cancer activity has been observed in vitro, its effects on multiple malignant phenotypes and tumor growth in vivo, as well as the associated molecular changes, remain insufficiently characterized. This study evaluated the preclinical anti-gastric cancer activity of FA and explored its potential molecular basis.

Methods

FA with a purity greater than 98% was evaluated in AGS and MKN-45 gastric cancer cells using CCK-8, EdU incorporation, colony formation, flow-cytometric, wound-healing, and Transwell assays. RNA sequencing and untargeted metabolomic profiling were performed in AGS cells. Public transcriptomic datasets were analyzed to prioritize candidate genes, and quantitative real-time PCR and Western blotting were used to assess MDM2/p53-related expression changes. Molecular docking and two independent 100-ns molecular dynamics simulations were conducted to investigate a potential interaction between FA and MDM2. Antitumor activity was further evaluated in an MKN-45 xenograft model.

Results

FA reduced cell viability, DNA synthesis, clonogenic growth, migration, and invasion, altered cell-cycle distribution, and increased apoptosis. The 48-h half-maximal inhibitory concentrations were 39.38 μM in AGS cells and 20.61 μM in MKN-45 cells. Transcriptomic and metabolomic profiling revealed broad molecular alterations involving cellular signaling and multiple metabolic processes, including amino acid metabolism, the tricarboxylic acid cycle, oxidative phosphorylation, and central carbon metabolism. Public transcriptomic analyses supported the disease relevance and prognostic associations of *MDM2* and *TP53* in gastric cancer. FA decreased *MDM2* mRNA and increased *TP53* mRNA expression in both AGS and MKN-45 cells, while corresponding decreases in MDM2 protein and increases in p53 protein were demonstrated in AGS cells. Molecular docking and molecular dynamics simulations provided computational support for a potential FA–MDM2 interaction. In vivo, FA suppressed MKN-45 xenograft growth and reduced final tumor weight without marked body-weight loss. FA-treated tumors also exhibited reduced Ki-67 and MDM2 immunoreactivity and increased p53 immunoreactivity.

Conclusions

FA exhibited broad anti-gastric cancer activity in vitro and in vivo and was consistently associated with decreased MDM2 and increased p53/TP53 expression. Computational analyses further suggested a potential interaction between FA and MDM2. These findings support further investigation of FA as a natural-product-derived antitumor candidate and identify MDM2/p53-related molecular changes as a potential component of its activity. Direct FA–MDM2 binding and the functional dependence of the antitumor effects of FA on MDM2 remain to be experimentally established.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and poses a substantial threat to human health [1]. According to GLOBOCAN 2024 estimates, approximately 980,000 new cases of GC and 642,000 GC-related deaths occurred worldwide in 2024, placing GC fifth globally in terms of both cancer incidence and mortality [2]. Despite continued advances in multimodal treatment strategies, including surgery, chemotherapy, targeted therapy, and immunotherapy, patients with advanced GC continue to face limited therapeutic efficacy, drug resistance, recurrence, metastasis, and poor prognosis [1, 3]. Therefore, the development of novel candidate compounds with demonstrable antitumor activity and the capacity to modulate key molecular pathways is of considerable importance for improving therapeutic outcomes in GC [4, 5].

Natural products constitute an important source of structurally diverse scaffolds for anticancer drug discovery [6]. Bioactive constituents derived from medicinal plants can interfere with multiple cancer-associated processes, including cell proliferation, cell-cycle progression, apoptosis, and metabolic regulation [7]. Species of the genus *Ferula* produce structurally diverse natural products, including sesquiterpenoids and coumarins, and several *Ferula*-derived compounds have demonstrated antitumor activity in experimental models [8–10]. Ferulasong A (FA) is a sesquiterpenoid previously isolated and structurally characterized from the roots of *Ferula songarica* Pall. ex Schult. by our research group, with preliminary anti-gastric cancer activity reported during its initial characterization [11]. However, its effects on multiple malignant phenotypes, its antitumor activity in vivo, and the molecular changes associated with these effects have not been systematically investigated.

The MDM2/p53 regulatory axis plays a central role in tumor-cell fate by integrating DNA-damage responses, cell-cycle control, apoptosis, and cellular stress responses [12]. MDM2 is a principal negative regulator of p53 and promotes its ubiquitination and proteasomal degradation through E3 ubiquitin ligase activity, thereby restraining p53-mediated tumor-suppressive functions [12]. Accordingly, pharmacological modulation of the MDM2–p53 interaction has attracted considerable interest as an anticancer strategy [13]. Whether FA influences MDM2/p53-related molecular responses in gastric cancer, however, remains unknown.

In the present study, we systematically evaluated the preclinical anti-GC activity of FA in AGS and MKN-45 cells and in an MKN-45 xenograft model. RNA sequencing and untargeted metabolomic profiling were used to characterize FA-associated molecular alterations, and public transcriptomic analyses were

combined with molecular experiments to prioritize and evaluate candidate molecules. Molecular docking and molecular dynamics simulations were further performed to assess a putative interaction between FA and MDM2. This study therefore aimed to define the antitumor activity of FA across complementary in vitro and in vivo models and to provide molecular and computational evidence for further investigation of its relationship with MDM2/p53-related responses in gastric cancer.

Materials and Methods

Preparation and characterization of FA

The underground parts of *Ferula songarica* were collected from Jianggesi Township, Yumin County, Tacheng Prefecture, Xinjiang Uygur Autonomous Region, China. The plant material was authenticated as *F. songarica* by Guoxu Ma, a researcher at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. A voucher specimen was deposited under accession number 654225240717024LY. FA is a sesquiterpenoid natural product previously isolated from the underground parts of *F. songarica* by our research group. Briefly, the dried medicinal plant material was pulverized and extracted under reflux with 95% ethanol. The resulting extract was concentrated under reduced pressure to obtain a crude extract. After dispersion in distilled water, the crude extract was sequentially partitioned with petroleum ether, dichloromethane, and ethyl acetate to yield fractions of different polarities. The target compound was predominantly enriched in the dichloromethane fraction, which was subjected to silica gel column chromatography using petroleum ether/ethyl acetate and dichloromethane/methanol gradient elution systems, yielding seven major fractions. The third fraction was further separated by silica gel column chromatography, after which the twelfth subfraction was purified by semipreparative high-performance liquid chromatography (HPLC). A methanol–water system was used as the mobile phase at a flow rate of 2 mL/min, and the target peak eluting at approximately 17 min was collected to obtain purified FA.

The chemical structure of FA was elucidated using liquid chromatography–mass spectrometry (LC–MS) and multidimensional nuclear magnetic resonance spectroscopy, including ^1H NMR, ^{13}C attached proton test (^{13}C -APT), ^1H – ^1H correlation spectroscopy (^1H – ^1H COSY), heteronuclear single-quantum coherence spectroscopy, heteronuclear multiple-bond correlation spectroscopy, and nuclear Overhauser effect spectroscopy. The FA used in the present study had a purity of > 98%, as determined by HPLC, and was suitable for subsequent cellular and animal experiments. Before the in vitro experiments, FA was dissolved in dimethyl sulfoxide (DMSO) to prepare a 40 mM stock solution. The stock solution was aliquoted and stored at -20°C in the dark. Working solutions were freshly prepared before use, and repeated freeze–thaw cycles were avoided.

Cell lines and culture conditions

The human gastric cancer cell lines AGS and MKN-45 were used for the in vitro experiments. The identities of both cell lines were confirmed by short tandem repeat (STR) profiling, and no cross-contamination with other human cell lines was detected. Following recovery, the cells were routinely cultured under sterile conditions. Only cells in good condition and in the logarithmic growth phase were used for subsequent experiments. Information on the sources of the cell lines and the culture media is provided in Supplementary Table S1.

AGS cells were cultured in complete F-12K medium, whereas MKN-45 cells were cultured in a specialized complete medium based on RPMI-1640. The cells were maintained at 37°C in a humidified incubator containing 5% CO₂ and were routinely passaged according to their growth status. To ensure experimental consistency, all in vitro experiments were conducted using cells within a predefined passage range after recovery. Before treatment, the FA stock solution was serially diluted in the corresponding complete culture medium to achieve the required final concentrations. The final DMSO concentration was maintained below 0.1% (v/v) in all cell-based experiments, and the control groups received an equivalent volume of DMSO as the vehicle control.

Cell viability and proliferation assays

The effects of FA on the viability of AGS and MKN-45 cells were evaluated using the Cell Counting Kit-8 (CCK-8) assay, and the half-maximal inhibitory concentration (IC₅₀) values were determined. AGS and MKN-45 cells were seeded in 96-well plates at densities of 1.5×10^3 and 3.0×10^3 cells/well, respectively, in a final volume of 100 µL per well. After allowing the cells to adhere for 24 h, the cells were treated as indicated. For IC₅₀ determination, cells were treated with FA at final concentrations of 7, 14, 21, 28, 35, and 42 µM, whereas CDDP was tested at final concentrations of 0.625, 1.25, 2.5, 5, 10, and 20 µM. After 48 h of treatment, CCK-8 reagent was added, and absorbance at 450 nm was measured. Cell viability was normalized to that of the vehicle control group, and IC₅₀ values were calculated by nonlinear regression analysis using GraphPad Prism software. Information on the relevant reagents and consumables is provided in Supplementary Table S1.

Based on the results of the preliminary experiments and IC₅₀ determination, FA concentrations below or close to the respective IC₅₀ values were selected for all subsequent in vitro experiments, except for the IC₅₀ assay, to balance treatment efficacy against cytotoxicity while preserving cell viability and minimize the influence of excessive cytotoxicity on functional phenotypes and molecular measurements. For AGS cells, the low, medium, and high concentrations of FA were set at 10, 20, and 40 µM, respectively. For MKN-45 cells, the corresponding concentrations were set at 5, 10, and 20 µM, respectively. The treatment duration was determined according to the requirements of each experiment. CDDP was used as a positive control at a final concentration of 1.25 µM. To evaluate the time-dependent effects of FA on cell viability, AGS and MKN-45 cells were treated according to the standardized treatment scheme for 24, 48, or 72 h before CCK-8 analysis.

A 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed to evaluate the effect of FA on DNA synthesis in gastric cancer cells. AGS and MKN-45 cells were seeded in 24-well plates at densities of 1.0×10^4 and 1.5×10^4 cells/well, respectively, in 500 μ L of complete culture medium per well. After attachment, the cells were treated according to the standardized treatment scheme for 24 h. The cells were then incubated with EdU working solution, followed by fixation, permeabilization, click-reaction labeling, and Hoechst counterstaining. Fluorescence images were acquired using a fluorescence microscope. Five fields were randomly selected from each group for analysis, and the EdU-positive rate was calculated as the percentage of EdU-positive cells relative to the total number of Hoechst-stained cells.

A colony formation assay was performed to evaluate the long-term proliferative capacity of gastric cancer cells following FA treatment. AGS and MKN-45 cells were seeded in six-well plates at densities of 2.0×10^3 and 3.0×10^3 cells/well, respectively. After attachment, the cells were treated according to the standardized treatment scheme for 48 h. The culture medium was then replaced with fresh drug-free complete medium, and the cells were cultured for an additional 14 days. At the end of the culture period, the colonies were fixed, stained with crystal violet, and photographed. A cell cluster containing at least 50 cells was defined as a colony, and the number of colonies was quantified using ImageJ software. All experiments were conducted with three replicate wells per group and independently repeated three times.

Cell-cycle and apoptosis analyses

Flow cytometry was used to evaluate the effects of FA on cell-cycle distribution and apoptosis in AGS and MKN-45 gastric cancer cells. AGS and MKN-45 cells were seeded in six-well plates at densities of 2.0×10^5 and 3.0×10^5 cells/well, respectively, in 1.5 mL of the corresponding complete culture medium per well. After allowing the cells to adhere for 24 h, they were treated according to the standardized treatment scheme for an additional 24 h. Information on the relevant assay reagents is provided in Supplementary Table S1.

Cell-cycle distribution was analyzed using propidium iodide (PI) staining. Following treatment, cells from each group were collected, washed with phosphate-buffered saline (PBS), and fixed overnight at 4°C in prechilled 70% ethanol. The fixed cells were subsequently treated with RNase A and stained with PI for 30 min at room temperature in the dark. Cellular DNA content was measured by flow cytometry, and the proportions of cells in the G0/G1, S, and G2/M phases were determined.

Apoptosis was assessed using Annexin V-PE/7-aminoactinomycin D (7-AAD) double staining. Following treatment, floating cells in the culture supernatant and trypsinized adherent cells were collected and pooled. After washing with PBS, the cells were stained according to the manufacturer's instructions. Apoptotic cells were then analyzed by flow cytometry. The total apoptotic rate was calculated as the sum of the percentages of early and late apoptotic cells. All experiments were conducted with three replicate wells per group and independently repeated three times.

Cell migration and invasion assays

Wound-healing and Transwell assays were performed to evaluate the effects of FA on the migratory and invasive capacities of AGS and MKN-45 gastric cancer cells. Information on the relevant reagents and consumables is provided in Supplementary Table S1.

For the wound-healing assay, AGS and MKN-45 cells were seeded in six-well plates at densities of 3.0×10^5 and 5.0×10^5 cells/well, respectively, in 2 mL of the corresponding complete culture medium per well. Once the cells reached confluence, a linear scratch was created across the center of the monolayer using a sterile 200 μ L pipette tip. Detached cells were removed by washing with PBS. Low-serum medium containing 1% fetal bovine serum (FBS) was then added, and the cells were treated according to the standardized treatment scheme. Images were acquired immediately after treatment and designated as the 0-h images. The same wound areas were photographed again at 12, 24, and 48 h. Wound width was measured using ImageJ software, and the wound closure rate was calculated as follows:

$$\text{Wound closure rate (\%)} = \frac{W_0 - W_t}{W_0} \times 100\%$$

where W_0 represents the wound width at 0 h and W_t represents the wound width at the corresponding time point.

Transwell assays were used to evaluate cell migration and invasion. For the migration assay, the membrane of the upper chamber was left uncoated, whereas for the invasion assay, the membrane was precoated with diluted Matrigel. AGS and MKN-45 cells were pretreated according to the standardized treatment scheme for 24 h, harvested by trypsinization, and counted to determine the number of viable cells. For each cell line, equal numbers of viable cells from the different treatment groups were then seeded into the upper chambers, with 2.0×10^4 AGS cells and 3.0×10^4 MKN-45 cells per insert. Serum-free medium containing the corresponding treatment was added to the upper chamber, whereas complete medium containing 10% FBS was added to the lower chamber as a chemoattractant. The same concentrations of FA or CDDP, or an equivalent volume of DMSO, were also added to the lower chamber. After 24 h of incubation, cells remaining on the upper surface of the membrane were removed. Cells that had migrated or invaded through the membrane were fixed, stained with crystal violet, and photographed. Five fields were randomly selected from each insert for imaging, and the numbers of migrated or invaded cells were quantified using ImageJ software. All experiments were performed with three technical replicates per group and independently repeated three times.

Transcriptomic sequencing and metabolomic analysis

Transcriptomic sequencing and untargeted metabolomic analyses were performed using AGS gastric cancer cells. Cells were treated with FA at its IC_{50} concentration (39.38 μ M) for 24 h, whereas control cells received an equivalent volume of vehicle. Six biological replicates were included in each group.

Detailed information on the analytical platforms, databases, and software is provided in Supplementary Table S2.

For transcriptomic analysis, total RNA was extracted following treatment, and samples meeting the quality requirements were subjected to library preparation and sequencing. Raw reads were filtered using fastp, and sequencing quality was assessed using FastQC and RNA-SeQC. Clean reads were aligned to the human reference genome GRCh38.p13 (GCF_000001405.39) using HISAT2 and processed using SAMtools. Gene-level expression was quantified using HTSeq-count. Principal component analysis (PCA) and sample-to-sample distance analysis were performed to assess global variation and sample similarity based on the gene-expression profiles. Differential expression analysis was conducted using DESeq2, with differentially expressed genes defined as those with $P < 0.05$ and $|\log_2 \text{fold change}| > 1$. Functional enrichment analyses of the differentially expressed genes were performed using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and WikiPathways. Gene set enrichment analysis (GSEA) was additionally conducted to assess coordinated pathway-level changes across the transcriptome.

For untargeted metabolomic analysis, intracellular metabolites were extracted using a precooled methanol–water system containing mixed internal standards. Quality-control samples were prepared by pooling equal aliquots of all experimental extracts to monitor analytical reproducibility and instrument stability. Metabolomic profiling was performed by liquid chromatography–mass spectrometry in both positive- and negative-ion modes. Raw data were subjected to peak detection, alignment, retention-time correction, peak-area integration, and normalization. Metabolites were annotated on the basis of accurate mass, retention time, isotopic distribution, and tandem mass spectrometric fragmentation patterns. Differential metabolites were defined using $P < 0.05$ and a fold change ≥ 1.2 or $\leq 1/1.2$. PCA was used to characterize global metabolic variation, and orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to further evaluate group separation. Model robustness was assessed by seven-fold cross-validation and 200-response permutation testing. KEGG pathway enrichment analysis was subsequently performed for the differential metabolites.

Analysis of public transcriptomic datasets

To evaluate the expression patterns and prognostic relevance of candidate genes in gastric cancer, transcriptomic data from The Cancer Genome Atlas Stomach Adenocarcinoma cohort (TCGA-STAD) and the Gene Expression Omnibus dataset GSE146996 were analyzed. TCGA-STAD was used for differential expression and overall survival analyses, whereas GSE146996 served as an independent dataset for validation of differential gene expression. Detailed information on the data sources, platforms, and sample characteristics is provided in Supplementary Table S3.

For TCGA-STAD, raw RNA-sequencing count data were used for differential expression analysis, whereas normalized expression data were used for gene-expression visualization and survival analysis. For GSE146996, probe identifiers were mapped to gene symbols according to the corresponding platform

annotation, and the expression matrix was $\log_2$ -transformed and normalized before analysis. Samples were classified as gastric tumor or normal gastric tissues according to the accompanying annotation information, and samples with incomplete group assignments or missing expression data were excluded. Differential expression between tumor and normal tissues was assessed using DESeq2 for TCGA-STAD and limma for GSE146996. Genes with $P < 0.05$ and $|\log_2 \text{ fold change}| > 0.5$ were considered differentially expressed. Volcano plots were generated to visualize genome-wide differential expression patterns. Differentially expressed genes from TCGA-STAD and GSE146996 were subsequently compared with the FA-related candidate genes identified from the transcriptomic analysis of FA-treated AGS cells to prioritize candidate genes showing consistent relevance across experimental and public datasets.

Expression levels of the prioritized candidate genes were further compared between tumor and normal tissues in both datasets and visualized using violin plots. For TCGA-STAD cases with available survival information, associations between candidate gene expression and overall survival were evaluated using Kaplan–Meier analysis. Patients were stratified into high- and low-expression groups using gene-specific optimal expression cutoffs, and survival distributions were compared using the log-rank test. Statistical analyses and data visualization were performed in R using the corresponding packages.

qRT-PCR analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the transcriptional responses of MDM2 and TP53 to FA treatment in AGS and MKN-45 cells. Cells were treated for 48 h according to the treatment scheme described in Section 2.3 and subsequently harvested for RNA extraction. Information on the reagents and primer sequences is provided in Supplementary Tables S1 and S4.

Total RNA was extracted using TRIzol reagent, and RNA concentration and purity were assessed before reverse transcription. For each sample, 1,000 ng of total RNA was reverse-transcribed into complementary DNA (cDNA). qRT-PCR was performed in a total reaction volume of 20 μL containing 2 $\times$ qPCR Master Mix, gene-specific forward and reverse primers, cDNA template, and RNase-free water. The amplification protocol consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. β -Actin was used as the reference gene, and relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Three independent biological replicates were analyzed, with three technical replicates performed for each sample.

Western blot analysis

Western blotting was performed to assess MDM2 and p53 protein expression in FA-treated AGS cells. Cells were treated for 48 h according to the treatment scheme described in Section 2.3 and subsequently harvested. After washing with ice-cold phosphate-buffered saline (PBS), total cellular protein was extracted using RIPA lysis buffer supplemented with protease inhibitors. Protein

concentrations were determined using the bicinchoninic acid assay. Information on the antibodies and major reagents is provided in Supplementary Table S1.

Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. Following transfer, membranes were sectioned according to the molecular-weight regions of the target proteins. After blocking, membrane regions were incubated with the corresponding primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence. MDM2 and p53 were detected on membrane regions originating from the same original PVDF membrane and the same sample lanes. Following target-protein detection, the relevant membrane regions were stripped and reprobed for β -actin. Thus, the target proteins and the β -actin loading control used for normalization were derived from the same original membrane and corresponding sample lanes. Densitometric analysis was performed using ImageJ, and MDM2 and p53 protein levels were normalized to β -actin. Three independent biological replicates were analyzed.

Molecular docking and molecular dynamics simulations

Molecular docking and molecular dynamics (MD) simulations were performed to investigate the potential interaction between FA and MDM2. The crystal structure of MDM2 was obtained from the RCSB Protein Data Bank (PDB ID: 5C5A). Prior to docking, water molecules, the co-crystallized ligand, and nonessential heteroatoms were removed. Because a suitable three-dimensional structure of FA was unavailable in public databases, its structure was constructed according to the structural characterization reported in our previous study and subjected to energy minimization before docking. Molecular docking was performed using AutoDock Vina within the predefined ligand-binding region of MDM2. The pose with the lowest predicted binding energy was selected for subsequent structural analysis and MD simulations. Protein–ligand interactions, including hydrogen bonds, hydrophobic contacts, and π interactions, were examined using PyMOL and Discovery Studio Visualizer.

Two independent 100-ns MD simulations were performed using the selected docking pose as the initial structure. Simulations were conducted using GROMACS, with MDM2 and FA parameterized using the AMBER14SB force field and the General AMBER Force Field, respectively. The complex was solvated using the TIP3P water model, and counterions were added to neutralize the system. Following energy minimization and equilibration under the NVT and NPT ensembles, production simulations were conducted at 300 K and 1 bar. Trajectory analyses included root-mean-square deviation (RMSD), intermolecular hydrogen-bond occupancy, and time-dependent distances between selected ligand atoms and key MDM2 residues. Distances associated with the predicted interactions involving HIS96 and VAL93 were specifically monitored to assess the persistence of these contacts during the simulations.

Binding free-energy components of the predicted FA–MDM2 complex were estimated using gmx_MMPBSA based on the molecular mechanics/generalized Born surface area (MM/GBSA) approach. Van der Waals, electrostatic, polar solvation, and nonpolar solvation contributions were

evaluated together with the total estimated binding free energy. Detailed docking and MD simulation parameters are provided in Supplementary Table S5.

Nude mouse xenograft experiment

Specific pathogen-free (SPF) male BALB/c nude mice, aged 42–62 days and weighing 20–24 g at baseline, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; production license No. SCXK [Jing] 2021-0006). Mice were acclimatized for 7 days before experimental procedures and maintained under SPF barrier conditions at approximately 26°C and 40–60% relative humidity, with 10–15 air changes per hour and a 10-h light/14-h dark cycle. Five mice were housed per cage on wood-shaving bedding, and cages were cleaned every 5 days and autoclaved before reuse. Standard feed formulated for highly immunodeficient mice and acidified drinking water were provided *ad libitum*. Drinking water was sterilized before use, acidified with hydrochloric acid to pH 2.5–2.8, supplemented with compound vitamins, and replaced every other day. Water bottles and drinking tubes were cleaned and disinfected between uses. No additional environmental enrichment was provided. The animal facility held an experimental animal use license (SYXK [Xin] 2024-0004).

All animal procedures were approved by the Laboratory Animal Welfare and Ethics Committee of the General Hospital of the Xinjiang Military Command of the Chinese People's Liberation Army (ethics application No. 2025042906; approval No. DWLL2025043018) and were conducted in accordance with institutional guidelines for laboratory animal welfare and ethics. Information on the animals, tumor-modeling reagents, and dosing vehicles is provided in Supplementary Table S1.

A preliminary dose-ranging study was conducted before the formal efficacy experiment to assess FA tolerability and establish the dose range. FA was administered at 5, 15, or 45 mg/kg, with three mice per dose group. Based on the preliminary observations, FA doses of 5, 20, and 60 mg/kg were selected for the formal experiment. Animals used in the preliminary study were not included in the subsequent efficacy analysis. The cisplatin (CDDP) dose was selected with reference to previously reported gastric cancer xenograft protocols.

MKN-45 cells were used to establish the subcutaneous xenograft model. Cells in the logarithmic growth phase were harvested, resuspended in sterile PBS, and mixed with Matrigel at a 1:1 ratio. Approximately 1×10^7 cells in a total volume of 200 μ L were injected subcutaneously into the right axillary region of each mouse. Treatment was initiated when individual tumor volumes reached approximately 50–100 mm³. Tumor-bearing mice were allocated to the vehicle-control, low-dose FA (FA-L), medium-dose FA (FA-M), high-dose FA (FA-H), and CDDP groups, with allocation balanced according to baseline tumor volume and body weight. Five eligible mice were included in each group, yielding a total of 25 mice for the formal efficacy analysis.

FA was administered by oral gavage once daily for 14 consecutive days at 5, 20, or 60 mg/kg in the FA-L, FA-M, and FA-H groups, respectively. The FA formulation consisted of 10% DMSO, 40% PEG300, 5% Tween-80, and 45% normal saline. The vehicle-control group received an equivalent volume of blank

vehicle by oral gavage. The CDDP group received CDDP at 3 mg/kg by intraperitoneal injection on treatment days 0 and 7 and received an equivalent volume of normal saline by oral gavage once daily.

Body weight, tumor dimensions, general activity, food and water intake, skin condition, tumor ulceration, and other signs of pain or distress were monitored every 2 days throughout the 14-day treatment period. The longest tumor diameter (length) and the perpendicular shortest diameter (width) were measured using calipers, and tumor volume was calculated as: $V = 0.5 \times \text{length} \times \text{width}^2$. Relative tumor volume (RTV) was calculated as follows: $RTV = V_t/V_0$, where V_0 represents the tumor volume on the first day of treatment and V_t represents the tumor volume at each observation time point. Humane endpoints were predefined before initiation of the experiment. Mice were to be euthanized before the scheduled endpoint if the maximum tumor dimension reached 20 mm or if tumor ulceration, infection, or necrosis occurred. Additional humane endpoints included loss of > 20% of baseline body weight, inability to eat or drink, severe lethargy or markedly reduced activity, or unresponsiveness. Animals meeting any predefined endpoint were to be euthanized promptly.

At the end of the 14-day treatment period, the remaining mice were euthanized by gradual-fill CO₂ inhalation. CO₂ was introduced gradually into the euthanasia chamber containing ambient air at a displacement rate of approximately 30% of the chamber volume per minute, followed by cervical dislocation as a secondary physical method to confirm death. Tumors were excised immediately after euthanasia, photographed, measured, and weighed. Tumor tissues required for subsequent histological and immunohistochemical analyses were collected and processed accordingly.

Immunohistochemical analysis

Xenograft tumor tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Following deparaffinization and rehydration, antigen retrieval was performed, and endogenous peroxidase activity was blocked with 3% H₂O₂. Sections were subsequently blocked with bovine serum albumin and incubated with the corresponding primary antibodies overnight at 4°C. The immunohistochemical markers evaluated were Ki-67, MDM2, and p53, consistent with the proliferative and MDM2/p53-related endpoints investigated in the present study. Antibody information and dilution ratios are provided in Supplementary Table S1.

After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactivity was visualized using 3,3'-diaminobenzidine, followed by hematoxylin counterstaining, dehydration, clearing, and mounting. Sections were examined under a light microscope, and representative images were acquired at 100× and 400× magnification.

Immunohistochemical staining was evaluated using a semiquantitative immunoreactive score (IRS) based on the proportion of positively stained cells and staining intensity. The proportion of positive cells was scored as follows: 0, no positive cells; 1, < 10%; 2, 10–50%; 3, 51–80%; and 4, > 80%. Staining intensity was scored as 0, absent; 1, weak; 2, moderate; and 3, strong. The IRS was calculated as the product of the positive-cell proportion score and staining-intensity score, yielding a possible range of 0–

12. Representative tumor regions were evaluated using the same scoring criteria across treatment groups, and the resulting IRS values were used for statistical analysis.

Statistical analysis

All experimental data were analyzed and plotted using GraphPad Prism version 10.6 and R software. Image quantification was performed using ImageJ software. Continuous variables are presented as the mean $\pm$ standard deviation (mean $\pm$ SD) or the median and interquartile range [M (P25–P75)]. Comparisons between two groups were performed using a two-tailed Student's *t*-test or the Mann–Whitney *U* test, depending on the data distribution. Comparisons among multiple groups were performed using one-way analysis of variance or the Kruskal–Wallis test, followed by the corresponding post hoc multiple-comparison tests.

Longitudinal data, including tumor volume, relative tumor volume, and body weight, were analyzed using two-way repeated-measures analysis of variance. Semiquantitative immunohistochemical IRS values are presented as M (P25–P75), and comparisons among multiple groups were performed using the Kruskal–Wallis test. Differences in candidate gene expression between gastric cancer and normal tissues in public databases were assessed using appropriate parametric or nonparametric tests according to the data distribution. Survival curves were generated using the Kaplan–Meier method, and survival differences between expression groups were compared using the log-rank test. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. Statistical significance in the figures was denoted as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Results

FA suppresses gastric cancer cell viability and proliferative capacity

The chemical structure of FA is shown in Fig. 1A. CCK-8 dose–response curves demonstrated that FA exerted marked growth-inhibitory effects on both gastric cancer cell lines. After 48 h of treatment, the IC_{50} values were 39.38 μ M for AGS cells and 20.61 μ M for MKN-45 cells (Fig. 1B, C), suggesting that MKN-45 cells may be more sensitive to FA treatment. Time-course CCK-8 assays further showed that FA suppressed the increase in OD_{450} values in both cell lines compared with the control group and that this inhibitory effect became more pronounced with increasing FA concentrations and treatment duration (Fig. 1D, E). In AGS cells, medium- and high-dose FA markedly suppressed the increase in OD_{450} values, whereas MKN-45 cells showed marked reductions in cell viability following treatment with low, medium, and high doses of FA.

To further assess the effects of FA on gastric cancer cell proliferation, EdU incorporation assays were performed to evaluate DNA synthesis. Compared with the control group, FA treatment markedly reduced the proportion of EdU-positive cells in both AGS and MKN-45 cells (Fig. 1F, G). In AGS cells, the reduction in the EdU-positive rate was more pronounced in the medium- and high-dose FA groups, whereas all

three FA doses significantly reduced the EdU-positive rate in MKN-45 cells. These findings were consistent with the CCK-8 results, indicating that FA impaired DNA synthesis and the proliferative capacity of gastric cancer cells. Colony formation assays further showed that FA reduced colony numbers in both AGS and MKN-45 cells, with an overall dose-dependent trend (Fig. 1H). In AGS cells, the number of colonies decreased from 325.33 ± 14.01 in the control group to 282.33 ± 13.01 , 103.33 ± 7.51 , and 45.67 ± 2.52 in the low-, medium-, and high-dose FA groups, respectively. In MKN-45 cells, the number of colonies decreased from 351.33 ± 15.63 in the control group to 271.00 ± 12.12 , 203.00 ± 8.00 , and 65.00 ± 7.55 in the corresponding FA groups. Collectively, these results indicated that FA not only suppressed short-term cell viability and DNA synthesis but also impaired the long-term clonogenic growth of gastric cancer cells.

FA alters cell-cycle distribution and promotes apoptosis in gastric cancer cells

Given the inhibitory effects of FA on gastric cancer cell viability and proliferation, flow cytometry was used to examine its effects on cell-cycle distribution and apoptosis. Cell-cycle analysis showed that FA markedly altered the cell-cycle distribution of AGS cells (Fig. 2A, C–E). Compared with the control group, high-dose FA reduced the proportion of cells in the G0/G1 phase and increased the proportions of cells in the S and G2/M phases. The effects of low- and medium-dose FA on the distribution of AGS cells across different cell-cycle phases did not follow a strictly progressive dose-dependent pattern. This finding suggested that the effect of FA was not confined to arrest at a single cell-cycle phase but may instead have involved broader cell-cycle redistribution or disruption of cell-cycle progression. FA-induced cell-cycle redistribution was more pronounced in MKN-45 cells (Fig. 2B, F–H). As the FA concentration increased, the proportion of cells in the G0/G1 phase gradually decreased, whereas the proportions of cells in the S and G2/M phases increased. Following high-dose FA treatment, the proportion of MKN-45 cells in the G0/G1 phase decreased from 57.17% in the control group to 32.10%, whereas the proportions of cells in the S and G2/M phases increased from 19.40% to 31.43% and from 22.83% to 34.30%, respectively. These findings indicated that FA markedly altered cell-cycle progression in gastric cancer cells and promoted the accumulation of MKN-45 cells in the S and G2/M phases.

Annexin V-PE/7-AAD double staining was used to evaluate the effect of FA on gastric cancer cell apoptosis. FA treatment significantly increased the proportion of apoptotic cells in both AGS and MKN-45 cell populations (Fig. 2I–L). In AGS cells, the apoptotic rate increased from 1.64% in the control group to 9.35%, 20.73%, and 34.59% in the low-, medium-, and high-dose FA groups, respectively (Fig. 2I, J). In MKN-45 cells, the apoptotic rate increased from 3.18% in the control group to 11.10%, 18.87%, and 37.53% in the corresponding FA groups (Fig. 2K, L). Notably, the apoptosis-inducing effect of high-dose FA was comparable to that of the CDDP positive-control group. Taken together, these results showed that FA altered cell-cycle distribution and increased the proportion of apoptotic gastric cancer cells, suggesting that disruption of cell-cycle progression and induction of apoptosis may jointly contribute to the growth-inhibitory effects of FA.

FA suppresses the migratory and invasive capacities of gastric cancer cells

Given the close association of tumor cell migration and invasion with gastric cancer progression, the effects of FA on the motility of AGS and MKN-45 cells were evaluated. Wound-healing assays showed progressive wound closure over time in the control group, whereas FA treatment slowed wound closure in both gastric cancer cell lines (Fig. 3A–D). In AGS cells, this reduction was more pronounced following medium- and high-dose FA treatment. FA also reduced the migratory capacity of MKN-45 cells, although the responses across the different dose groups did not follow a fully consistent dose-dependent pattern. These findings indicated that FA impaired the two-dimensional migration of gastric cancer cells.

Transwell migration assays showed that FA treatment reduced the numbers of AGS and MKN-45 cells migrating through the membrane compared with the control group (Fig. 3E, F). This reduction was more pronounced in the medium- and high-dose FA groups in both cell lines. Transwell invasion assays further demonstrated that FA reduced the numbers of AGS and MKN-45 cells invading through the Matrigel-coated membrane (Fig. 3E, F). In AGS cells, the numbers of invading cells were markedly reduced in the medium- and high-dose FA groups, whereas the inhibitory effect of low-dose FA was relatively modest. In MKN-45 cells, the number of invading cells decreased in a dose-dependent manner following FA treatment. Collectively, these results showed that FA suppressed the migratory and invasive capacities of AGS and MKN-45 gastric cancer cells, suggesting that it may attenuate the invasive phenotype of gastric cancer cells.

Transcriptomic and metabolomic profiling reveals FA-induced molecular reprogramming in AGS cells

To characterize the molecular alterations associated with FA treatment, transcriptomic profiling was performed in AGS cells. PCA showed separation between FA-treated and control samples, with PC1 and PC2 accounting for 52.51% and 13.75% of the total variance, respectively (Fig. 4A). Using the predefined criteria of $P < 0.05$ and $|\log_2 \text{fold change}| > 1$, 68 differentially expressed genes were identified, including 22 upregulated and 46 downregulated genes (Fig. 4B). The volcano plot and hierarchical clustering heatmap further illustrated distinct transcriptional changes between the two groups (Fig. 4C, D). Consistently, sample-to-sample distance analysis showed group-dependent differences in global gene-expression profiles (Fig. 4E). KEGG enrichment analysis of the differentially expressed genes identified multiple pathways associated with cellular signaling and metabolism, including ABC transporters, TGF- β signaling, lipid and atherosclerosis, retinol metabolism, and arachidonic acid metabolism (Fig. 4F). These results indicate that FA treatment induces broad transcriptional alterations in AGS cells.

Untargeted LC–MS-based metabolomic profiling was subsequently performed to characterize FA-associated metabolic alterations. PCA demonstrated distinct overall metabolic profiles between FA-treated and control samples, with PC1 and PC2 explaining 33.1% and 17.1% of the variance, respectively

(Fig. 4G). Consistent with the PCA results, OPLS-DA showed clear separation between the two groups (Fig. 4H). In the 200-permutation test, the R^2 and Q^2 intercepts were 0.982 and -0.144 , respectively (Fig. 4I). Based on the predefined criteria, 288 differential metabolites were identified, comprising 115 increased and 173 decreased metabolites (Fig. 4J). Hierarchical clustering of representative differential metabolites further demonstrated distinct metabolic patterns between FA-treated and control cells (Fig. 4K). KEGG enrichment analysis identified several metabolic pathways, including alanine, aspartate and glutamate metabolism, the citrate cycle, oxidative phosphorylation, central carbon metabolism in cancer, nicotinate and nicotinamide metabolism, taurine and hypotaurine metabolism, and pyruvate metabolism (Fig. 4L).

Collectively, the transcriptomic and metabolomic analyses demonstrate that FA treatment is associated with broad transcriptional and metabolic reprogramming in AGS cells, providing a molecular basis for subsequent candidate-gene prioritization and mechanistic investigation.

Public transcriptomic analyses support the expression and prognostic relevance of MDM2 and TP53 in gastric cancer

To prioritize FA-responsive candidate genes with relevance to gastric cancer, seven candidate genes derived from the RNA-seq analysis were compared with differentially expressed genes identified in the TCGA-STAD and GSE146996 datasets. Four genes were shared across all three gene sets (Fig. 5A). Among these overlapping candidates, MDM2 and TP53 were prioritized for further investigation. Differential expression analyses of TCGA-STAD and GSE146996 revealed widespread transcriptional differences between gastric tumor and normal tissues, with MDM2 and TP53 highlighted among the differentially expressed genes in both datasets (Fig. 5B, C).

Expression analysis further showed that MDM2 and TP53 were significantly upregulated in gastric tumor tissues compared with normal tissues in TCGA-STAD (MDM2, $P = 2.1517 \times 10^{-23}$; TP53, $P = 6.2270 \times 10^{-10}$; Fig. 5D). Consistent expression patterns were observed in the independent GSE146996 dataset, in which both MDM2 ($P = 0.0004$) and TP53 ($P = 8.7430 \times 10^{-5}$) were significantly elevated in tumor tissues (Fig. 5E). These results demonstrate consistent tumor-associated expression patterns of MDM2 and TP53 across two independent gastric cancer transcriptomic cohorts.

The prognostic associations of MDM2 and TP53 expression were subsequently evaluated in the TCGA-STAD cohort. Kaplan–Meier analysis showed significant differences in overall survival between the high- and low-expression groups for both MDM2 (log-rank $P = 0.016$) and TP53 (log-rank $P = 0.0017$; Fig. 5F). In both analyses, higher expression was associated with more favorable overall survival. Collectively, these public transcriptomic analyses support the differential expression and prognostic relevance of MDM2 and TP53 in gastric cancer and provide a rationale for their subsequent experimental evaluation following FA treatment.

FA modulates MDM2/p53 expression in gastric cancer cells

Based on the candidate-gene prioritization from the transcriptomic and public-dataset analyses, the effects of FA on MDM2/p53-related expression were further examined in MKN-45 and AGS cells. In MKN-45 cells, qRT-PCR analysis showed that FA treatment significantly decreased *MDM2* mRNA expression and increased *TP53* mRNA expression compared with the control group (Fig. 6A, B). Similar transcriptional changes were observed in AGS cells, with reduced *MDM2* and increased *TP53* mRNA expression following FA treatment (Fig. 6C, D).

Protein-level changes were further examined in AGS cells by Western blotting. FA treatment reduced MDM2 protein abundance, with progressively lower levels observed across the FA-treated groups (Fig. 6E, F). In contrast, p53 protein abundance increased, with significant elevations observed at the medium and high FA concentrations (Fig. 6E, G). Thus, the protein-level changes in AGS cells were directionally consistent with the corresponding transcriptional responses.

Collectively, these findings show that FA treatment is associated with decreased MDM2 expression and increased p53/TP53 expression in gastric cancer cells, with transcriptional changes observed in both MKN-45 and AGS cells and corresponding protein-level changes demonstrated in AGS cells. These results provide experimental support for further investigation of MDM2/p53-related signaling in the response of gastric cancer cells to FA.

Molecular docking and molecular dynamics simulations suggest a potential interaction between FA and MDM2

The above findings showed that FA treatment was associated with altered MDM2/p53 expression. To explore whether FA could potentially interact with MDM2, molecular docking and molecular dynamics (MD) simulations were performed. Molecular docking predicted that FA could occupy a putative ligand-binding pocket of MDM2 and form multiple noncovalent interactions with surrounding residues (Fig. 7A–C). In the selected docking pose, FA formed a predicted hydrogen-bond interaction with HIS96, together with hydrophobic interactions involving LEU54, ILE61, and TYR67. The lowest-energy docking pose yielded a predicted docking score of -6.655 kcal/mol, supporting the feasibility of a potential FA–MDM2 interaction.

Two independent 100-ns MD simulations were subsequently performed to examine the dynamic behavior and reproducibility of the predicted complex. RMSD analysis of the trajectory shown in Fig. 7D indicated that the protein, ligand, and complex remained relatively stable over the simulation period, without evident large-scale structural deviation. Intermolecular hydrogen bonds between FA and MDM2 were dynamically formed and disrupted throughout the trajectory (Fig. 7E).

To further assess the persistence of specific ligand–protein contacts, time-dependent distances between selected FA atoms and key MDM2 residues were analyzed across the two independent simulations. The HIS96-related and VAL93-related distances exhibited temporal fluctuations, with recurrent periods in which the interacting atoms remained in close proximity (Fig. 7F, G). These observations provided additional support for the persistence of selected contacts within the predicted FA–MDM2 binding mode.

MM/GBSA analysis was further used to estimate the binding free energy of the predicted FA–MDM2 complex. The estimated ΔTOTAL was -22.16 ± 3.53 kcal/mol. Energy decomposition showed a favorable van der Waals contribution ($\Delta\text{VDWAALS} = -36.69 \pm 3.56$ kcal/mol) and favorable total gas-phase energy ($\Delta\text{GGAS} = -40.48 \pm 4.72$ kcal/mol), whereas the polar solvation component ($\Delta\text{EGB} = 22.83 \pm 3.37$ kcal/mol) opposed binding (Fig. 7H).

Collectively, the docking, MD, interaction-distance, and MM/GBSA analyses provided computational support for a potential interaction between FA and MDM2. These findings offer a structural basis for further investigating the relationship between FA and MDM2/p53-related responses; however, direct FA–MDM2 binding remains to be experimentally established.

FA suppresses MKN-45 xenograft growth and alters Ki-67, MDM2, and p53 immunoreactivity in vivo

To evaluate the *in vivo* antitumor activity of FA, a subcutaneous MKN-45 xenograft model was established in nude mice. At the end of the 14-day treatment period, representative images of tumor-bearing mice and excised tumors showed smaller tumors in the FA-treated groups than in the vehicle-control group, particularly at the medium and high doses (Fig. 8A, B). Endpoint tumor weights were significantly reduced in the medium- and high-dose FA groups, with a comparable inhibitory effect observed in the CDDP group (Fig. 8D). Longitudinal measurements further showed slower tumor growth following FA treatment, with more pronounced effects at the medium and high doses (Fig. 8E). Relative tumor-volume changes were consistent with the absolute tumor-volume measurements (Fig. 8F). Body weights remained relatively stable in the FA-treated groups throughout the treatment period, whereas a more evident decrease was observed in the CDDP group (Fig. 8C). Thus, no marked body-weight reduction was observed with FA at the tested doses.

Immunohistochemical analysis was subsequently performed to assess tumor-cell proliferation and MDM2/p53-related changes in xenograft tissues. Compared with the control group, Ki-67 immunoreactivity was reduced following FA treatment, with significant reductions observed in the medium- and high-dose groups (Fig. 8G, J). MDM2 staining showed a similar decrease, reaching statistical significance in the medium- and high-dose FA groups (Fig. 8H, K). In contrast, p53 immunoreactivity increased following FA treatment, with significant increases observed at the medium and high doses (Fig. 8I, L).

Collectively, these findings demonstrate that FA suppresses MKN-45 xenograft growth in vivo without causing marked body-weight loss under the present experimental conditions. The accompanying reduction in Ki-67 and MDM2 immunoreactivity and increase in p53 immunoreactivity are consistent with reduced proliferative activity and alterations in the MDM2/p53-related molecular profile of xenograft tumors.

Discussion

Gastric cancer remains a major gastrointestinal malignancy with a substantial global disease burden. Although patients with early-stage gastric cancer may achieve long-term survival following standardized treatment, those with advanced disease continue to face recurrence, metastasis, therapeutic resistance, and poor prognosis [3]. Therefore, the identification of natural-product-derived compounds with antitumor activity and the potential to modulate cancer-associated molecular processes remains of considerable interest. Building on the previous isolation and structural characterization of FA by our research group, the present study systematically evaluated its antitumor activity in gastric cancer and explored the associated molecular changes through transcriptomic and metabolomic profiling, public transcriptomic analyses, molecular experiments, and computational simulations. FA inhibited cell viability, proliferation, colony formation, migration, and invasion, altered cell-cycle distribution, increased apoptosis, and suppressed MKN-45 xenograft growth in vivo. At the molecular level, FA treatment was consistently associated with decreased MDM2 and increased p53/TP53 expression, while computational analyses suggested a potential interaction between FA and MDM2. Together, these findings identify MDM2/p53-related molecular changes as a potential component of the antitumor response to FA, although the causal relationship and direct FA–MDM2 interaction require further experimental validation.

Natural products have long served as an important source of anticancer agents because of their structural diversity and broad biological activities [16]. Gao et al. [17] identified a core combination of bioactive constituents with synergistic antitumor activity from a traditional Chinese medicine formula, further illustrating the potential of natural products as sources of anticancer lead compounds. Plants of the genus *Ferula* contain diverse bioactive constituents, including sesquiterpenoids, coumarins, and sulfur-containing compounds, several of which have demonstrated antiproliferative, pro-apoptotic, and anti-migratory effects in tumor models [9]. For example, the *Ferula*-derived compound umbelliprenin has been reported to inhibit gastric cancer cell growth and migration through modulation of Wnt/ β -catenin-related signaling [18]. In this context, the present study extends the investigation of FA beyond its previous isolation and structural characterization by providing a systematic evaluation of its anti-gastric cancer activity in two gastric cancer cell lines and an MKN-45 xenograft model, together with molecular and computational analyses aimed at identifying potential mechanisms underlying its effects.

In the in vitro functional assays, FA exhibited marked antiproliferative activity against both AGS and MKN-45 cells. CCK-8, EdU incorporation, and colony formation assays consistently demonstrated reductions in cell viability, DNA synthesis, and clonogenic growth following FA treatment. MKN-45 cells

showed greater sensitivity to FA than AGS cells, suggesting that the response to FA may vary among gastric cancer cells with different molecular backgrounds. Flow-cytometric analysis further showed that FA altered cell-cycle distribution and increased apoptosis in both cell lines. Notably, the changes in cell-cycle distribution did not indicate a uniform accumulation in a single phase across the two cell models, suggesting that FA may exert broader effects on cell-cycle progression rather than inducing a strictly phase-specific arrest. In addition, wound-healing and Transwell assays demonstrated that FA impaired migratory and invasive phenotypes. Taken together, these findings indicate that the antitumor activity of FA extends beyond growth inhibition and encompasses multiple malignant phenotypes, including proliferation, survival, migration, and invasion.

Transcriptomic and metabolomic profiling provided further insight into the molecular changes associated with FA treatment. RNA-seq revealed broad transcriptional alterations in AGS cells, with enrichment of pathways related to cellular signaling and metabolism, including ABC transporters, lipid-associated processes, retinol metabolism, and arachidonic acid metabolism. Metabolic reprogramming is a well-recognized feature of cancer cells and supports membrane synthesis, energy production, biosynthetic demands, and adaptation to microenvironmental stress [19]. Consistent with this concept, Wang et al. [20] demonstrated that a natural product could suppress gastric cancer progression through modulation of glycolytic and metabolic–epigenetic programs. In the present study, untargeted metabolomic profiling identified extensive changes in metabolite abundance and enrichment of pathways involving amino acid metabolism, the tricarboxylic acid cycle, oxidative phosphorylation, central carbon metabolism in cancer, nicotinate and nicotinamide metabolism, taurine and hypotaurine metabolism, and pyruvate metabolism. Although the transcriptomic and metabolomic datasets were not used to establish a single causal metabolic mechanism, taken together, these findings indicate that FA is associated with broad molecular remodeling at both the transcriptional and metabolic levels. These molecular changes provided a context for subsequent prioritization of candidate genes potentially associated with the antitumor effects of FA.

Among the prioritized candidates, the MDM2/p53 axis was of particular interest because of its central role in the regulation of tumor-cell fate. MDM2 is a major negative regulator of p53 and promotes its ubiquitination and proteasomal degradation through E3 ubiquitin ligase activity, whereas restoration of p53 activity can influence cell-cycle control, apoptosis, and cellular stress responses [21]. Previous studies have also implicated the MDM2/p53 axis in gastric cancer biology and have shown that therapeutic strategies targeting MDM2 together with cell-cycle checkpoints may enhance antitumor responses in gastric cancer models [22]. In the present study, FA treatment consistently decreased *MDM2* expression and increased *TP53* expression at the mRNA level in both AGS and MKN-45 cells, while corresponding decreases in MDM2 protein and increases in p53 protein were further demonstrated in AGS cells. Public transcriptomic analyses also showed that *MDM2* and *TP53* were differentially expressed between gastric tumor and normal tissues in both TCGA-STAD and GSE146996 and were associated with overall survival in TCGA-STAD. These observations support the biological relevance of MDM2 and TP53 in gastric cancer and demonstrate their associations with patient prognosis, providing a rationale for examining their relationship with FA. However, the present data establish an association

between FA treatment and altered MDM2/p53 expression rather than a causal MDM2-dependent mechanism.

The molecular docking and molecular dynamics simulations provided computational support for a potential interaction between FA and MDM2. FA was predicted to occupy a putative ligand-binding pocket of MDM2 and to form noncovalent contacts involving residues including HIS96, LEU54, ILE61, TYR67, and VAL93. MD-derived structural metrics, together with the analysis of selected intermolecular distances across independent simulations, supported the persistence of several predicted ligand–protein contacts over time. MM/GBSA analysis further yielded a favorable estimated binding free energy, with van der Waals interactions contributing substantially to the predicted interaction. These findings provide a structural hypothesis for a possible FA–MDM2 interaction but do not constitute direct evidence of physical binding or target engagement. Future studies should therefore combine biophysical binding assays, such as surface plasmon resonance, with cellular target-engagement approaches such as cellular thermal shift or drug-affinity-responsive target-stability assays. Genetic perturbation and rescue experiments will also be required to determine whether the biological effects of FA are functionally dependent on MDM2.

The *in vivo* experiments further supported the antitumor activity of FA. In the MKN-45 xenograft model, FA treatment reduced endpoint tumor weight and slowed tumor growth, particularly at the medium and high doses. Body weight remained relatively stable in the FA-treated groups, suggesting that FA did not cause marked body-weight loss under the present experimental conditions. Immunohistochemical analysis showed reduced Ki-67 and MDM2 immunoreactivity and increased p53 immunoreactivity following FA treatment, with more pronounced changes at the medium and high doses. The reduction in Ki-67 was consistent with the observed inhibition of xenograft growth, whereas the accompanying changes in MDM2 and p53 were directionally consistent with the molecular responses observed *in vitro*. Nevertheless, immunohistochemical staining provides semiquantitative evidence of protein-associated changes and does not establish that MDM2/p53 signaling is causally required for the *in vivo* antitumor effect of FA. Further studies incorporating MDM2-directed genetic manipulation, pharmacological rescue, and direct target-engagement assays will therefore be necessary to define the mechanistic role of MDM2 *in vivo*.

Several limitations of this study should be acknowledged. First, the potential involvement of MDM2 in the response to FA is currently supported by changes in MDM2/p53 expression together with molecular docking and molecular dynamics simulations. Direct FA–MDM2 binding and target engagement have not yet been experimentally established, and genetic perturbation or rescue experiments are required to determine whether the antitumor effects of FA are functionally dependent on MDM2. Second, although transcriptional changes in *MDM2* and *TP53* were observed in both AGS and MKN-45 cells, protein-level validation was performed only in AGS cells. Moreover, the *in vivo* efficacy of FA was evaluated using a single MKN-45 xenograft model. Additional gastric cancer models with distinct molecular backgrounds and TP53 status, as well as organoid and patient-derived models, will be required to assess the generalizability of these findings. Third, the transcriptomic and metabolomic analyses were performed in

AGS cells at a single treatment condition and primarily provide associative molecular information rather than evidence of causal pathway regulation. Further functional studies are therefore needed to define the biological significance of the molecular changes identified by these omics analyses. Finally, the pharmacokinetic properties, tissue distribution, comprehensive safety profile, and drug-like characteristics of FA remain to be established. Future studies should address these translational parameters and evaluate whether optimization of formulation, delivery, or combination strategies can improve the therapeutic potential of FA.

Conclusions

Overall, the present study demonstrates that FA exerts anti-gastric cancer activity by inhibiting cell viability, proliferation, migration, and invasion, altering cell-cycle distribution, promoting apoptosis, and suppressing xenograft growth in vivo. FA treatment was consistently associated with decreased MDM2 and increased p53/TP53 expression, while molecular docking and molecular dynamics simulations suggested a potential interaction between FA and MDM2. These findings identify MDM2/p53-related molecular changes as a potential component of the antitumor response to FA and provide a basis for further investigation of FA as a natural-product-derived candidate for gastric cancer therapy. Direct FA–MDM2 binding and the functional dependence of FA-induced antitumor effects on MDM2 remain to be experimentally established.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics approval

All animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of the General Hospital of the Xinjiang Military Command of the Chinese People's Liberation Army (ethics application no. 2025042906; approval no. DWLL2025043018) and were conducted in accordance with the relevant institutional and national guidelines for laboratory animal welfare and ethics.

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Author Contribution

DP performed experiments, analyzed the data, prepared the data visualizations, and drafted the original manuscript. YH conducted the animal experiments, collected the data, and contributed to manuscript revision. LY provided methodological guidance, participated in data analysis, and reviewed the manuscript. CJ conducted the literature search, organized the relevant literature, reviewed the manuscript, and contributed to language editing. ZW performed cell culture, cell-based functional assays, and molecular biology experiments. NL conceived and designed the study, supervised and administered the project, provided experimental guidance, and reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

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Data Availability

The publicly available transcriptomic datasets analyzed in this study were obtained from The Cancer Genome Atlas Stomach Adenocarcinoma (TCGA-STAD) cohort and the Gene Expression Omnibus (GEO) database (accession no. GSE146996). The datasets generated during the current study are available from the corresponding author on reasonable request.

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Figures

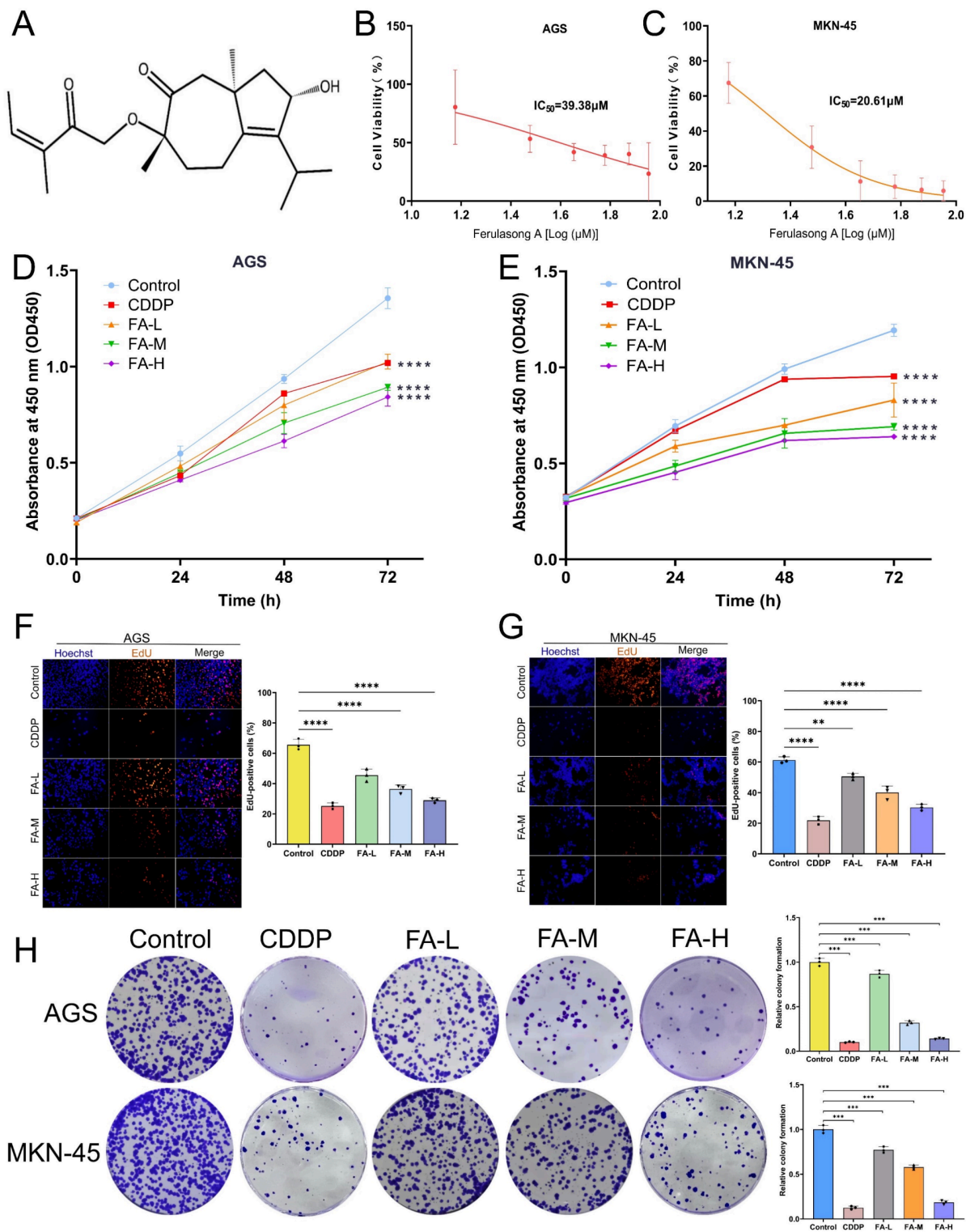


Figure 1

FA suppresses gastric cancer cell viability and proliferation in vitro. **A** Chemical structure of FA. **B**, **C** Forty-eight-hour concentration–response curves of FA in AGS and MKN-45 cells, respectively. Cell viability was normalized to the vehicle-control group, and the IC_{50} values were estimated by nonlinear regression. **D**, **E** ECK-8-derived absorbance values at 450 nm in AGS and MKN-45 cells after treatment with FA or cisplatin (CDDP, 1.25 μM) for the indicated durations. FA-L, FA-M, and FA-H correspond to 10,

20, and 40 μ M FA in AGS cells and 5, 10, and 20 μ M FA in MKN-45 cells, respectively. **F, G** Representative fluorescence images and quantitative analysis of EdU incorporation in AGS and MKN-45 cells after 24 h of treatment. Nuclei were stained with Hoechst and are shown in blue, whereas EdU-positive cells are shown in red. Scale bars, 100 μ m. **H** Representative images and quantitative analysis of colony formation in AGS and MKN-45 cells. Cells were treated with the indicated agents for 48 h and subsequently cultured in drug-free medium for 14 days.

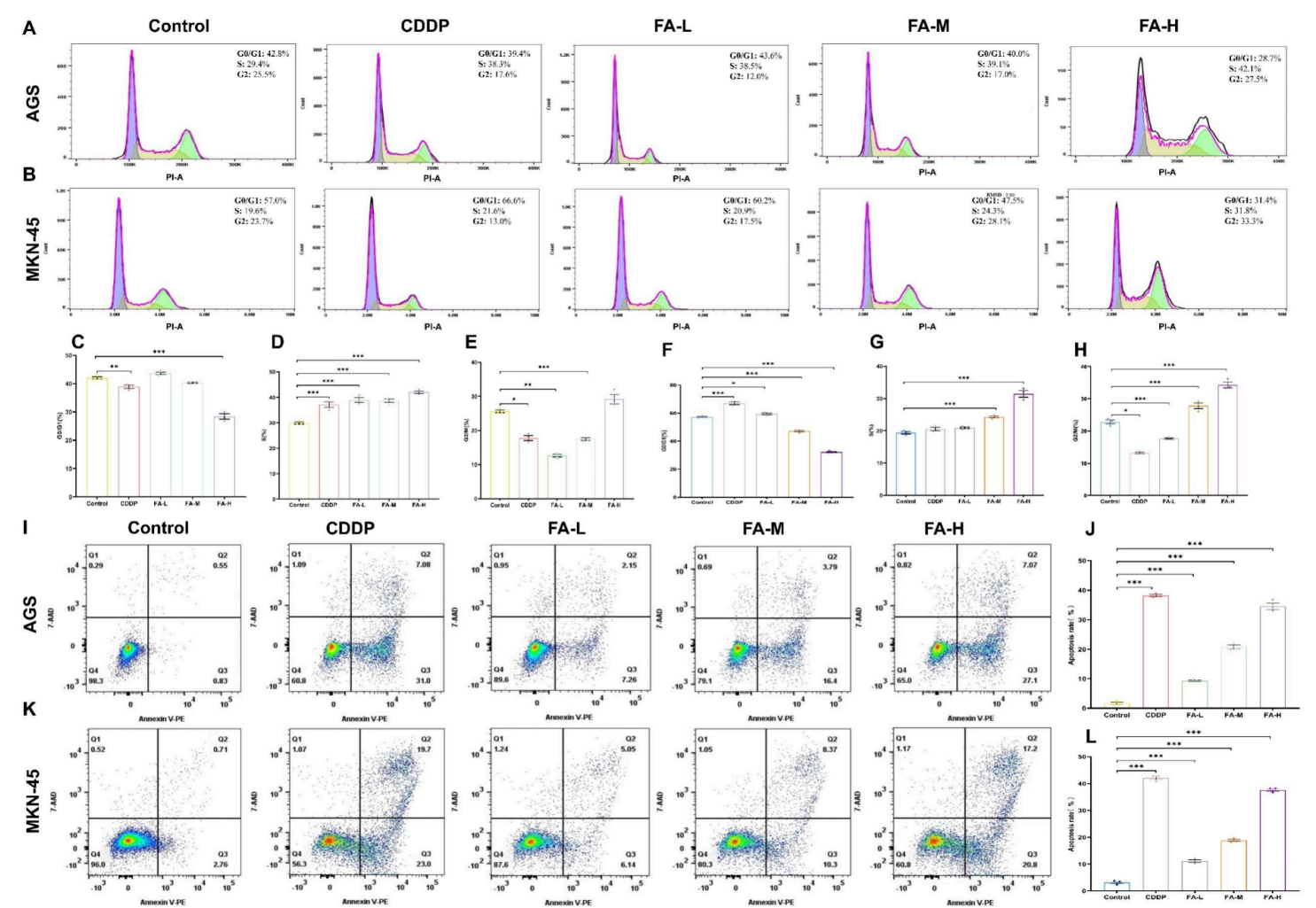


Figure 2

FA alters cell-cycle distribution and promotes apoptosis in gastric cancer cells. **A, B** Representative flow-cytometric histograms of propidium iodide-stained AGS and MKN-45 cells after treatment with FA or CDDP for 24 h. **C–E** Quantitative analysis of the percentages of AGS cells in the G0/G1, S, and G2/M phases, respectively. **F–H** Quantitative analysis of the percentages of MKN-45 cells in the G0/G1, S, and G2/M phases, respectively. **I, K** Representative Annexin V-PE/7-AAD flow-cytometric plots of apoptosis in AGS and MKN-45 cells after 24 h of treatment. The lower-right quadrant represents early apoptotic cells, and the upper-right quadrant represents late apoptotic cells. **J, L** Quantitative analysis of the total apoptotic rates in AGS and MKN-45 cells, calculated as the sum of early and late apoptotic cells.

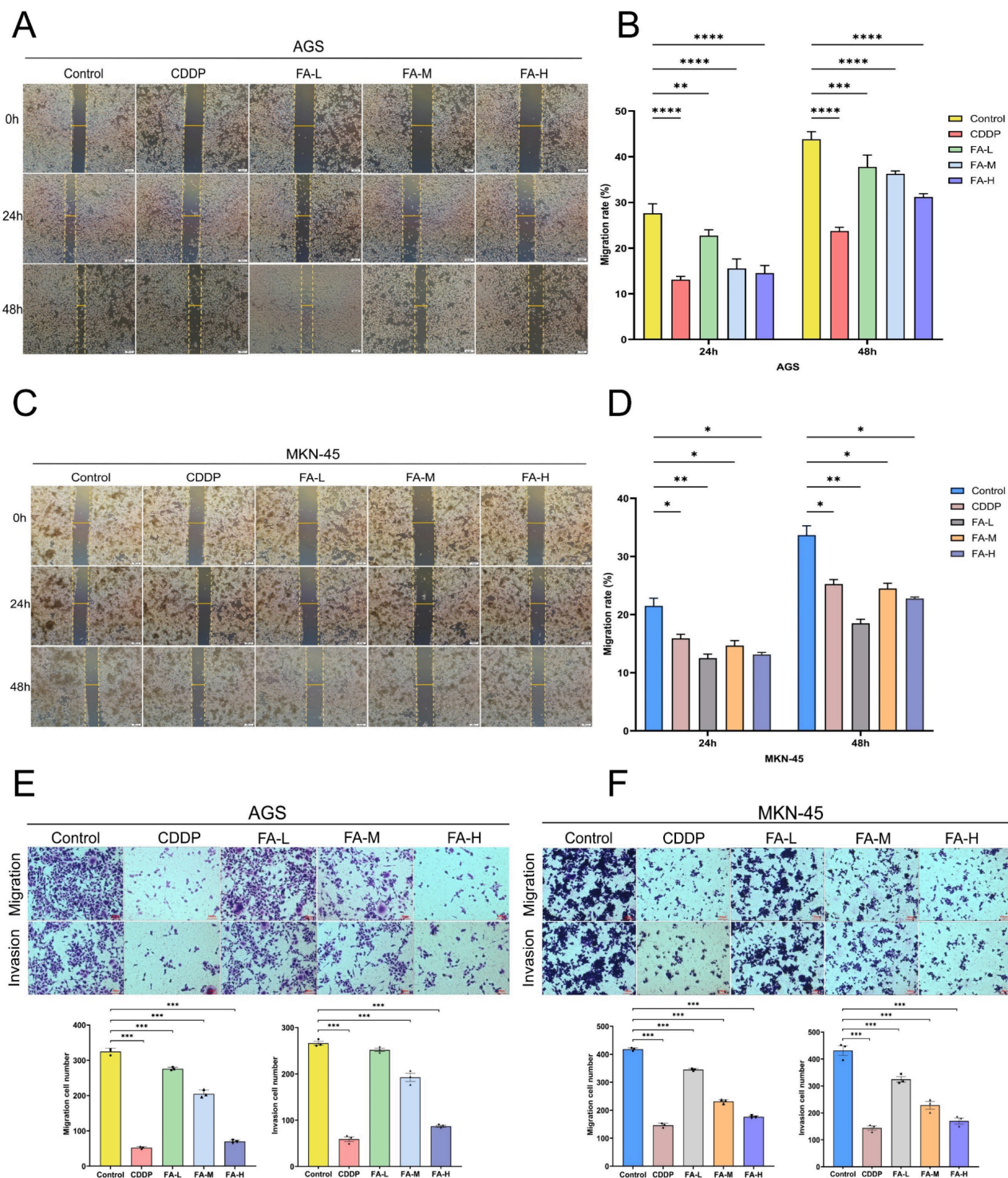


Figure 3

FA suppresses the migration and invasion of gastric cancer cells in vitro. **A, C** Representative wound-healing images of AGS and MKN-45 cells treated with FA or CDDP at the indicated time points. **B, D** Quantitative analysis of wound closure in AGS and MKN-45 cells at 24 and 48 h. **E, F** Representative images and quantitative analysis of Transwell migration and Matrigel-coated Transwell invasion assays in AGS and MKN-45 cells. Cells were pretreated with the indicated agents for 24 h, followed by migration

or invasion for an additional 24 h. Cells that migrated through the membrane or invaded through the Matrigel-coated membrane were stained with crystal violet and counted. Scale bars, 200 μ m in A and C, and 100 μ m in E and F.

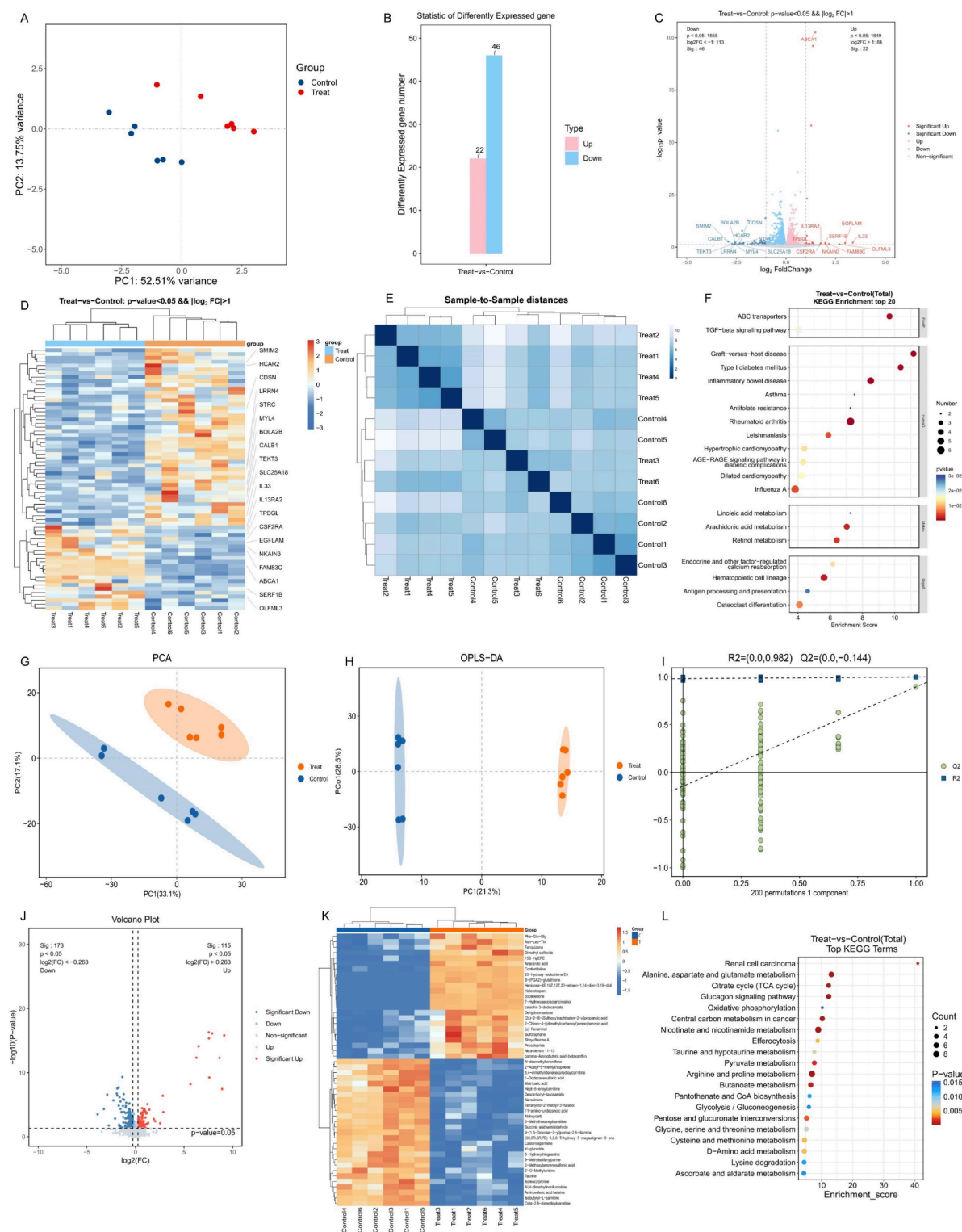


Figure 4

Integrated transcriptomic and metabolomic profiling reveals FA-associated molecular reprogramming in AGS cells. AGS cells were treated with vehicle or FA at 39.38 μ M for 24 h, with six biological replicates per group. **A–F**, Transcriptomic analysis. **A** Principal component analysis (PCA) of transcriptomic profiles in FA-treated and vehicle-control cells. **B** Numbers of differentially expressed genes (DEGs), comprising 22 upregulated and 46 downregulated genes. **C** Volcano plot showing the distribution of DEGs between the two groups. DEGs were identified using the criteria of $P < 0.05$ and $|\log_2 \text{fold change}| > 1$. **D** Hierarchical clustering heatmap of the DEGs. **E** Sample-to-sample distance heatmap showing transcriptomic similarity among individual samples. **F** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs, showing the top 20 enriched pathways. **G–L**, Untargeted metabolomic analysis. **G** PCA of the metabolic profiles in FA-treated and vehicle-control cells. **H** Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the two groups. **I** A 200-response permutation test of the OPLS-DA model; the R^2 and Q^2 intercepts were 0.982 and -0.144 , respectively. **J** Volcano plot showing differentially abundant metabolites, comprising 115 increased and 173 decreased metabolites. Differential metabolites were identified using the criteria of $P < 0.05$ and fold change ≥ 1.2 or $\leq 1/1.2$. **K** Hierarchical clustering heatmap of representative differential metabolites. **L** KEGG enrichment analysis of the differential metabolites, showing the top enriched pathways. DEG, differentially expressed gene; KEGG, Kyoto Encyclopedia of Genes and Genomes; OPLS-DA, orthogonal partial least squares-discriminant analysis; PCA, principal component analysis.

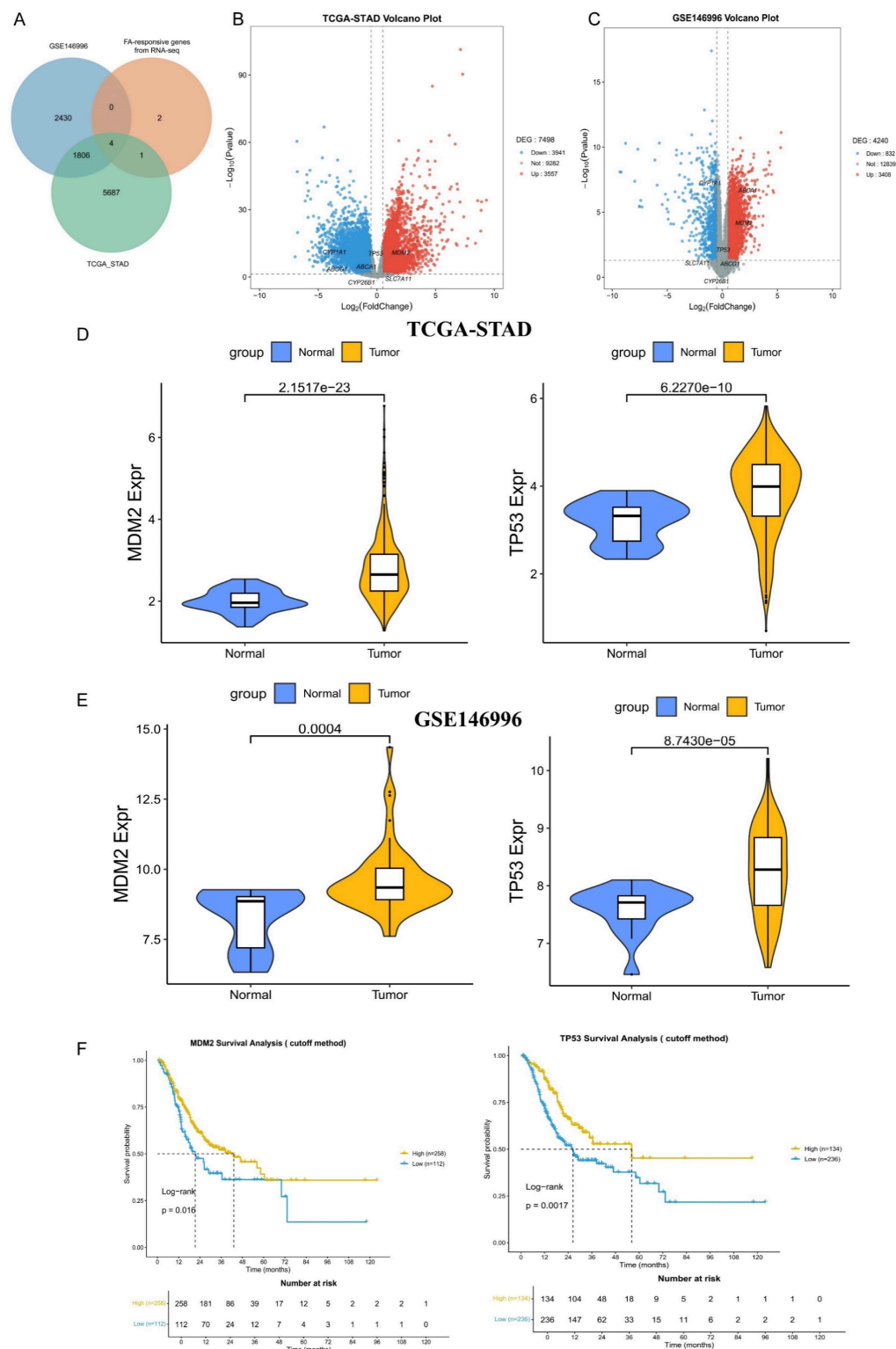


Figure 5

Public transcriptomic analyses prioritize MDM2 and TP53 as FA-associated candidate genes in gastric cancer. **A** Venn diagram showing the overlap between seven RNA-seq-derived FA-responsive candidate genes and differentially expressed genes identified in the TCGA-STAD and GSE146996 datasets. **B, C** Volcano plots showing differentially expressed genes between gastric tumor and normal tissues in TCGA-STAD and GSE146996, respectively; selected candidate genes are labeled. **D, E** Violin plots

comparing *MDM2* and *TP53* expression between gastric tumor and normal tissues in TCGA-STAD and GSE146996, respectively. **F** Kaplan–Meier curves showing overall survival according to *MDM2* and *TP53* expression in the TCGA-STAD cohort. Patients were stratified into high- and low-expression groups using gene-specific optimal expression cutoffs, and the numbers at risk are shown below the survival curves. DEG, differentially expressed gene; GEO, Gene Expression Omnibus; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.

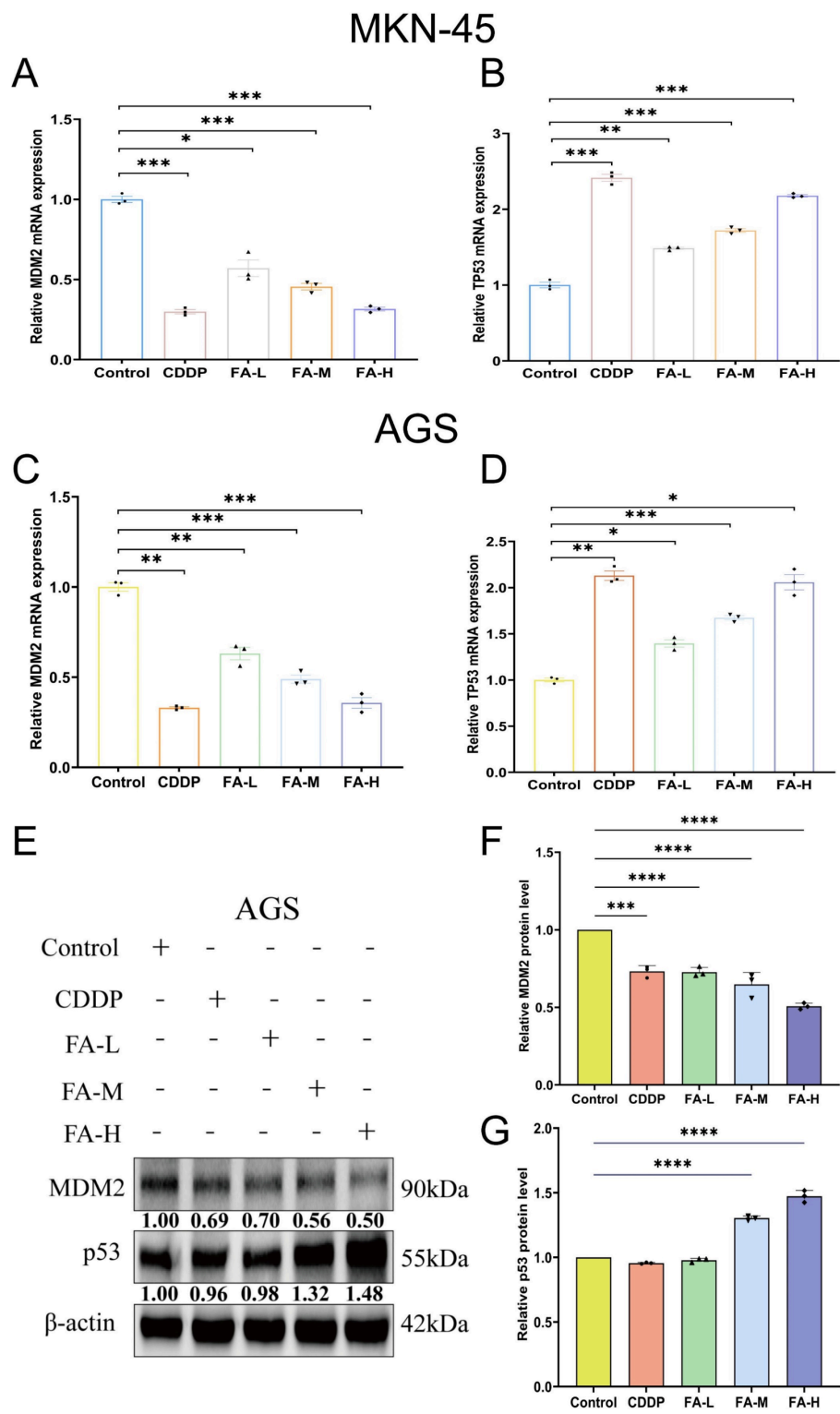


Figure 6

FA modulates MDM2/p53 expression in gastric cancer cells. **A, B** Relative mRNA expression levels of *MDM2* and *TP53*, respectively, in MKN-45 cells after treatment with control, CDDP, or the indicated concentrations of FA for 48 h, as determined by qRT-PCR. **C, D** Relative mRNA expression levels of *MDM2* and *TP53*, respectively, in AGS cells after the indicated treatments. **E** Representative Western blot images of MDM2, p53, and β -actin in AGS cells after treatment for 48 h. **F, G** Densitometric quantification of MDM2 and p53 protein levels, respectively, in AGS cells. Protein levels were normalized to β -actin and expressed relative to the control group. qRT-PCR data were normalized to β -actin and expressed relative to the corresponding control group. Data are presented as mean $\pm$ SD from three independent biological replicates. $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ versus the control group.

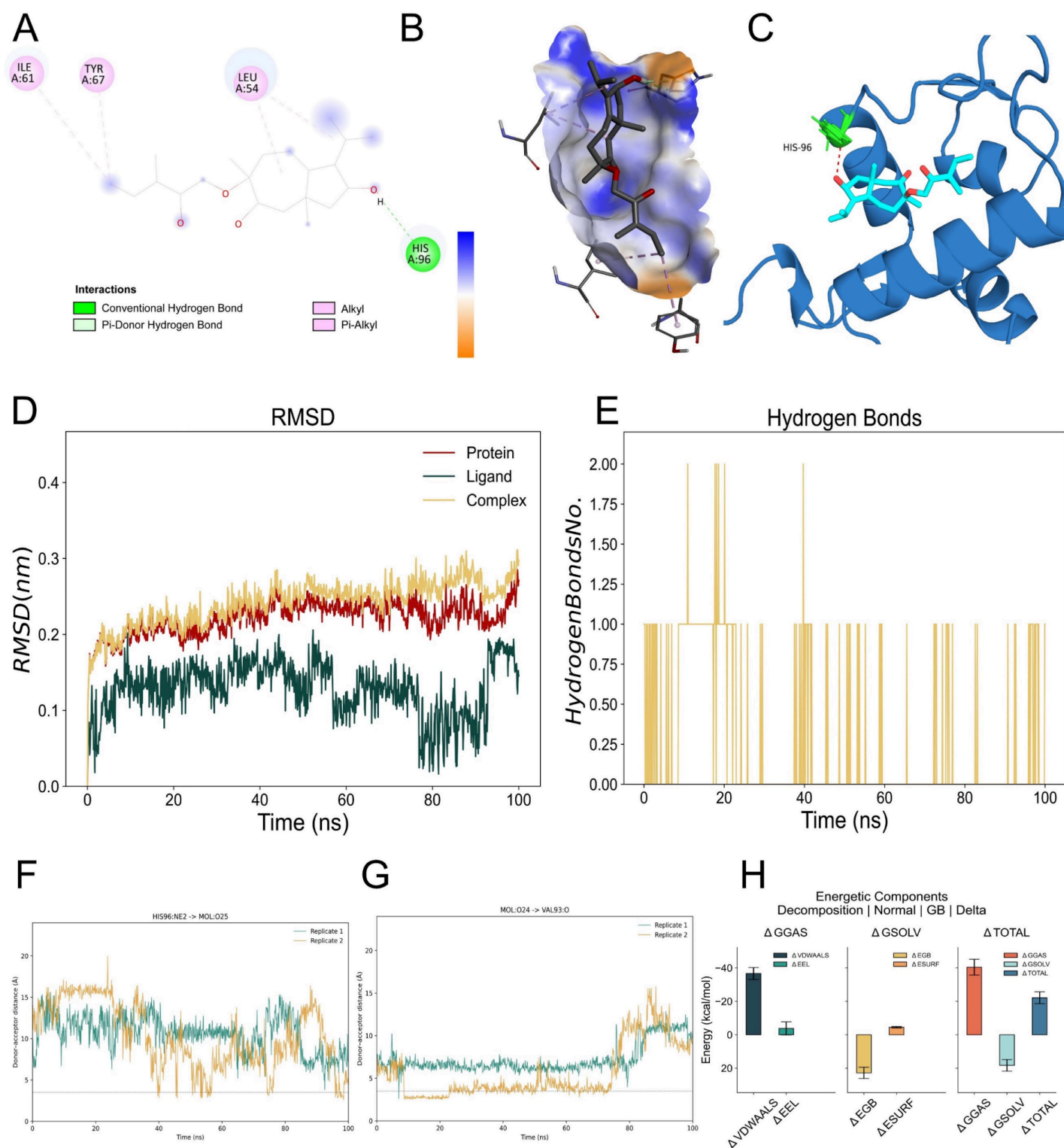


Figure 7

Molecular docking and molecular dynamics simulations suggest a potential interaction between FA and MDM2. **A** Two-dimensional interaction diagram showing the predicted binding mode of FA within the MDM2 binding pocket and the corresponding hydrogen-bond and hydrophobic interactions with surrounding residues. **B** Molecular-surface representation of FA within the predicted ligand-binding cavity of MDM2. **C** Three-dimensional representation of the predicted FA-MDM2 complex, highlighting

the predicted interaction involving HIS96. **D** Root-mean-square deviation (RMSD) profiles of MDM2, FA, and the FA-MDM2 complex during a 100-ns molecular dynamics simulation. **E** Time-dependent variation in the number of intermolecular hydrogen bonds between FA and MDM2 during the simulation. **F** Time-dependent distance between HIS96:NE2 and the O25 atom of FA during two independent 100-ns molecular dynamics simulations. **G** Time-dependent distance between the O24 atom of FA and VAL93:O during two independent 100-ns molecular dynamics simulations. **H** Molecular mechanics/generalized Born surface area (MM/GBSA) binding free-energy decomposition of the predicted FA-MDM2 complex, including van der Waals, electrostatic, polar solvation, nonpolar solvation, gas-phase, solvation, and total energy components. MM/GBSA, molecular mechanics/generalized Born surface area; RMSD, root-mean-square deviation.

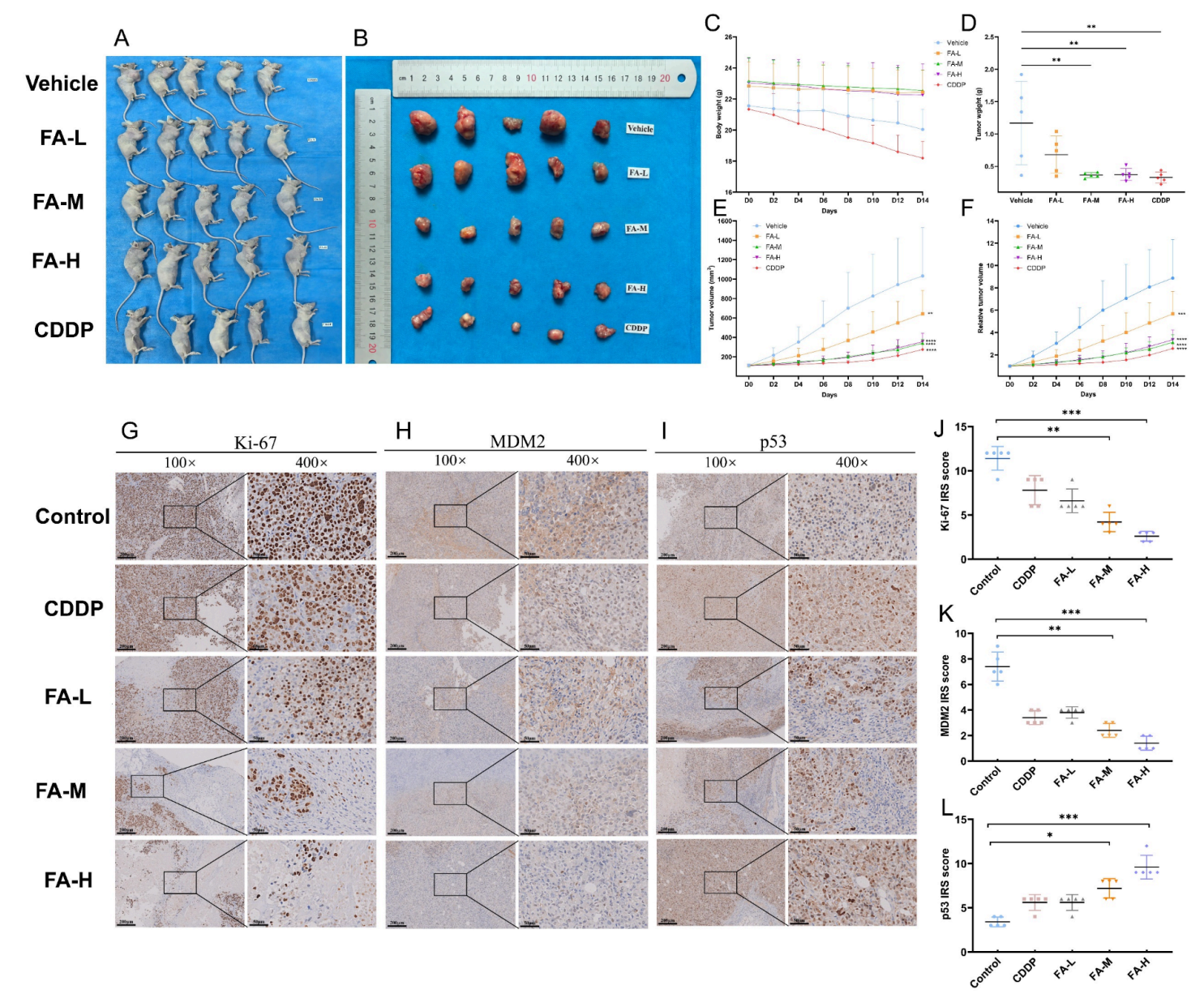


Figure 8

FA suppresses MKN-45 xenograft growth and alters Ki-67, MDM2, and p53 immunoreactivity in vivo. **A** Representative images of tumor-bearing nude mice from the vehicle-control, FA-treated, and CDDP-treated groups at the end of the 14-day treatment period. **B** Representative images of excised xenograft tumors. **C** Changes in body weight during treatment. **D** Final xenograft tumor weights. **E, F** Changes in absolute tumor volume and relative tumor volume, respectively, during the treatment period. **G–I** Representative immunohistochemical images of Ki-67, MDM2, and p53 staining, respectively, in MKN-45 xenograft tissues at 100× and 400× magnification. **J–L** IRS analyses of Ki-67, MDM2, and p53 staining, respectively. FA-L, FA-M, and FA-H correspond to FA doses of 5, 20, and 60 mg/kg, respectively, administered by oral gavage once daily for 14 consecutive days. CDDP was administered intraperitoneally at 3 mg/kg on treatment days 0 and 7. Five mice were included in each group. Scale bars, 200 µm at 100× magnification and 50 µm at 400× magnification.

Supplementary Files

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