Tomatidine improves liver fibrosis by promoting autophagy in hepatic stellate cells through the ERK/MAPK-mTOR-ULK1 pathway

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Abstract

Liver fibrosis is caused by various liver diseases and eventually develops into liver cancer. Tomatidine (TD), an aglycone of α-tomatidine, is a major glycoalkaloid found in immature tomato fruits, leaves, and stems. In this study, we investigated that tomatidine may promote autophagy in hepatic stellate cells through the ERK/MAPK-mTOR-ULK1 signaling pathway to improve liver fibrosis and combined experimental validation and molecular docking to reveal the underlying mechanism. First, we found that tomatidine could inhibit the proliferation of hepatic stellate cells and the expression of fibrosis-related proteins α-smooth muscle actin (α-SMA) and collagen type I 1 gene (COL1A1) in LX2 cells. Tomatidine was found to promote the expression of autophagy-related proteins. Next, we performed a network pharmacology screen and found that among the targets of tomatidine and the common targets of tomatidine and liver fibrosis, no target related to autophagy was found in the current database. However, pharmacological studies of tomatidine have confirmed the existence of pro-autophagy pharmacological effects. Therefore, we used molecular docking to verify that tomatidine had good binding and affinity with autophagy-related targets. Further network pharmacological analysis showed that the MAPK signaling pathway may be involved in the biological process of tomatidine against hepatic stellate cells. We further verified the ERK/MAPK-mTOR-ULK1 pathway and found that tomatidine could promote autophagy in LX-2 cells by inhibiting the expression of P-ERK and P-mTOR and activating the expression of P-ULK1. Finally, molecular docking was performed to reveal the binding of tomatidine to the active sites of ERK, MAPK, mTOR, and ULK1.

Introduction

Liver fibrosis is an intermediate process in the transformation of various liver diseases into hepatocellular carcinoma[1]. It is mainly due to the excessive accumulation of extracellular matrix (ECM) generated by activated hepatic stellate cells (HSCs) and the formation of fibrous scars[2]. This leads to structural damage to the liver[3], hepatocyte dysfunction, and eventually liver failure[4]. HSCs are highly activated after liver injury and promote the excessive proliferation and expression of ECM[5], which leads to the development of various liver diseases, liver fibrosis, and eventually liver cancer. While early liver fibrosis is reversible, as long as it is not in the late stage, there is still the possibility of reversal[6]. Therefore, early prevention and treatment of liver fibrosis are of great significance for patients with chronic liver disease. However, there is no specific drug for the treatment of liver fibrosis in the world[7]. Therefore, new pharmacological molecules or strategies for the treatment of liver fibrosis are urgently needed to improve patient survival.

Tomato is a common, nutritious, and inexpensive vegetable that contains many bioactive compounds[8]. Tomatidine (TD), a metabolite of α-tomatidine, is abundant in immature green tomatoes and exhibits a variety of activities in cells[9]. Studies have shown that tomatidine has a variety of health effects, such as anti-inflammatory, anti-atherosclerotic, and antibacterial[10]. Tomatidine has been reported to inhibit cancer cell growth[11], and tomatidine can induce autophagy in damaged cells[12]. A number of studies have

shown that tomatidine, an abundant steroidal alkaloid in Solanaceae, has pro-autophagy biological activity in vitro and in vivo for a variety of species. For example, tomatidine induces mitophagy through the SKN-1/Nrf2 pathway[13]. Tomatidine can prevent ischemic injury by enhancing the autophagic flux in neuro-2a (N2a) cells so as to achieve neuroprotection[14]. Tomatidine has the biological function of promoting autophagy, but whether it can improve liver fibrosis and the underlying molecular mechanism has not been found.

Autophagy is a process in which autophagosomes fuse with lysosomes containing a series of acid hydrolases to form autolysosomes and degrade damaged organelles or misfolded proteins in the cytoplasm. It is an essential metabolic pathway to maintain cellular homeostasis[15, 16]. ERK (mitogen-activated protein kinase) signal transduction plays an important role in cell biology. ERK (mitogen-activated protein kinase) signal transduction is a central pathway to regulate cell proliferation, differentiation, and survival[17, 18]. mTOR, a serine/threonine kinase, is a major inducer of autophagy. Autophagy can be inhibited by the mammalian target of rapamycin (mTOR), a central cell growth regulator that integrates growth factors and nutrient signals[19, 20]. ULK1 is a mammalian autophagy-initiating kinase, and Ulk1 is a homolog of yeast ATG1[21]. In this study, we revealed that ERK/MAPK promoted Ulk1 Ser 757 phosphorylation to activate autophagy by inhibiting mTOR activation. At present, it has been shown that tomatidine can exert biological functions through the MAPK/ERK signaling pathway. For example, tomatidine can inhibit neutrophil infiltration and reduce the secretion of inflammatory mediators by inhibiting the MAPK pathway and reducing the expression of ICAM-1 in mice[22]. Tomatidine could also inhibit the invasion and migration of human osteosarcoma U1OS and HOS cells by inhibiting the c-Raf-MEK-ERK pathway[23]. However, whether tomatidine exerts its biological process through the ERK/MAPK-mTOR-ULK1 signaling pathway has not been reported.

Since there are few reports on tomatidine alkaloid, we further verified it by network pharmacology and molecular docking. Network pharmacology is an effective method to find drug disease targets and predict whether there are common targets between the two, as well as the potential biological mechanisms of the common targets[24]. For example, the pharmacological mechanism of Shuanghuanglian against T-cell acute lymphoblastic leukemia was studied through network pharmacology[25]. To explore the mechanism of Danshen decoction in the treatment of ischemic heart disease through network pharmacology[26]. However, there is no report on the network pharmacology of tomatidine. In this study, we combined experimental validation, network pharmacology, and molecular docking to identify the key and potential targets of tomatidine for the treatment of liver fibrosis and predict the possible mechanisms.

Results

Figure 1

Tomatidine can inhibit the proliferation of LX-2 cells and the expression of fibrosis-related proteins α-SMA and COL1A1.

To investigate the anti-proliferative effect of tomatidine on LX-2 cells, CCK-8 was used to measure cell viability. Compared with the control group, the viability of LX-2 cells was significantly inhibited with the increase in drug concentration in a dose- and time-dependent manner. Therefore, we set up the low-medium-high concentration group in the subsequent experiments. (Figure1A). Platelet-derived growth factor BB is a drug that promotes the division and differentiation of fibroblasts[27]. PDGF-BB was used to induce stimulation of LX-2 cells, and a colony formation assay confirmed that the number of colonies in the TD group was significantly reduced in a dose-dependent manner compared with the model group under the combined action of PDGF-BB and TD. ($p < 0.05$) (Figure1 B). Western blot, real-time pcr, and immunofluorescence were used to detect the expression of the fibrosis-related proteins α-SMA and COL1A1. The model group could promote the expression of fibrosis-related proteins, while the TD group had significant reductions in the fibrosis markers α-SMA and COL1A1 at the gene, protein, and cellular levels. ($p < 0.05$) (Figure1 C-E).

Figure 2

Tomatidine can promote autophagy in LX-2 cells.

Autophagy is a self-degradation process that wraps damaged organelles and misfolded proteins into autophagosomes, which are then sent to lysosomes for disassembly[28, 29]. Chloroquine (CQ) is a lysosomal agent that is commonly used to inhibit lysosomal degradation and autophagy[30]. To confirm whether tomatidine could induce autophagy, cells were infected with mRFP-GFP-LC3 autophagy double-labeled lentivirus, and the changes of autophagosomes (green highlights) and autolysosomes (red highlights) were observed under a fluorescence microscope after adding different treatment factors according to the experimental group. We found that, compared with the model group, the number of autophagosomes in the TD group decreased in a concentration-dependent manner (quenching of green fluorescence); The number of red fluorescence and yellow fluorescence spots showed a positive correlation with the increase in TD concentration.($p < 0.05$) (Figure2 A). Western blot and real-time PCR were used to detect the expression of autophagy-related proteins and genes in LX-2 cells treated with tomatidine. We found that lycopene reduced P62 expression and increased LC3-II and Beclin-1 protein expression in LX-2 cells at the protein level. The results of real-time PCR were consistent with those of a Western blot. ($p < 0.05$)(Figure2 B-C). The results of the P62 immunofluorescence assay showed that the expression of P62 in LX-2 cells after tomatidine treatment was significantly lower than that in the CQ group in a dose-dependent manner. ($p < 0.05$)(Figure2 D). These data suggest that tomatidine is able to induce autophagy in LX-2 cells.

Figure 3

To elucidate the mechanism of tomatidine on liver fibrosis by network pharmacology

Because there are few studies on tomatidine at home and abroad, in order to strengthen the accuracy of our experimental results, we used network pharmacology to further prove whether tomatidine plays a pharmacological role in liver fibrosis. The standard string 'tomatidine' of tomatidine was input into the

Swiss Target Prediction database, Pubchem, HERB, and CTD databases to identify the targets of tomatidine, and a total of 28 potential drug targets were found. The GeneCards and DisGeNET databases have a total of 1398 potential targets for liver fibrosis. As shown in Figure 3A, the Venn diagram of tomatidine-liver fibrosis common targets contains 18 common targets. These 18 intersection targets were imported into the STRING database to explore the relationships between these targets.(Figure3 A). Based on the highest confidence screening score of 0.900, a PPI network of 18 common targets between tomatidine and liver fibrosis was constructed, and MAPK3, RELA, MAPK1, NFKB1, MAPK14, JAK1, MAPK8, AKT1, JAK2, and ESR1 were identified as the core targets.(Figure3 B). Subsequently, GO and KEGG analyses were performed, and MAPK3, RELA, MAPK1, and other core targets were input into the DAVID database to clarify the possible mechanism of tomatidine affecting liver fibrosis. GO analysis of BP (Biological Process) terms revealed that the 10 core targets were mainly related to biological processes such as response to tumor necrosis factor, intracellular signal transduction, stress-activated MAPK cascade, and positive regulation of cyclase activity. (Figure3 C). GO analysis of the CC (cellular component) term revealed that 10 core targets were mainly related to cellular components such as cytoplasm, nucleoplasm, the nucleus, and mitochondria. (Figure3 D). GO analysis of the MF (Molecular Function) term revealed that 10 core targets were mainly related to molecular functions such as MAP kinase activity, enzyme binding, ATP binding, and protein serine/threonine kinase activity. (Figure3 E). KEGG analysis showed that 10 core targets were related to the prolactin signaling pathway, toxoplasmosis, hepatitis B, and tuberculosis.(Figure3 F). Figure 3G shows the tomatidine-liver fibrosis-core target KEGG network.

We found that the targets of tomatidine and the common targets of tomatidine and liver fibrosis were not screened out in the current database. However, it has been proven that tomatidine has the pharmacological effect of promoting autophagy. Therefore, we hypothesized that there was a potential target of tomatidine for liver fibrosis related to autophagy. To further verify the reliability of our experimental results, molecular docking was performed to verify the binding of the relevant protein to the molecular structure of tomatidine in the basic experiment.

Figure 4

Molecular docking verification of tomatidine binding to autophagy-related targets.

To further strengthen the accuracy of our experimental data, we verified the potential binding mode and interaction of tomatidine with the fibrosis-related protein COL1A1, the autophagy-related proteins P62, Beclin-1, and LC3 by molecular docking. (Figure4 A-D). For the COL1A1 protein, residues involved in hydrogen bond formation after the binding of tomatidine to it include LYS-212. (Figure4 A). According to the docking results of P62 and tomatidine, the key residues involved in hydrogen bonding are ASP-7, ALA-6, and GLY-1.(Figure4 B). For Beclin-1, the key residue after docking with tomatidine is GLU-340.(Figure4 C). After the binding of LC3 protein to tomatidine, the key residue involved in hydrogen bond binding was SER-87.(Figure4 D). These results demonstrated that there are potential binding modes and interactions between tomatidine and the above targets, which further verified the reliability of our basic experimental

data. Moreover, we speculated that there are potential autophagy-related targets of tomatidine on liver fibrosis that have not been verified and have not been recorded in relevant databases.

Figure 5

Tomatidine promotes autophagy in LX-2 cells through the ERK/MAPK-mTOR-ULK1 signaling pathway.

In the network pharmacology database, we found that there was a MAPK cascade pathway in the common target of tomatidine and liver fibrosis. Therefore, we validated the MAPK-related pathway. We grouped the cells according to the experiments in Figure2 and detected the protein expression of ERK/MAPK, mTOR, and ULK1 in the cells after treatment with tomatidine. Western blotting showed that the expression of p-ULK1/ULK1 was significantly increased, while the expression of p-ERK1/2/ERK1/2 and p-mTOR/mTOR was significantly decreased. (p 0.05)(Figures5 A). We then performed molecular docking to verify the potential binding mode and interaction of tomatidine with ERK/MAPK-mTOR-ULK1. (Figure5 B-E). The key residue of ERK1/2 binding to tomatidine to form a hydrogen bond was GLU-104. (Figure5 B). The amino acid residue formed after MAPK binding to tomatidine is ASP-100.(Figure5 C). The key residue involved in hydrogen bond binding between mTOR protein and tomatidine was HIS-1806. (Figure5 D). For ULK1, the residue that forms a hydrogen bond after binding to tomatidine is GLN-227. (Figure5 E). These data indicate that tomatidine can interact with ERK/MAPK, mTOR, and ULK1 and may promote autophagy in LX-2 cells through the ERK/MAPK-MTOR-ULK1 signaling pathway.

Discussion

The pathological process of liver fibrosis results from various chronic liver injuries[31]. Liver fibrosis and its end-stage cirrhosis have been recognized as the major risk factors for hepatocellular carcinoma (HCC)[32]. Liver cancer has become the leading cause of death in the world. Approximately 90% of patients with liver cancer are associated with liver fibrosis[33, 34]. Studies have shown that it is possible to reverse liver fibrosis with appropriate treatment of non-advanced liver fibrosis[35]. At present, clinical treatment of liver fibrosis faces many challenges, and there is a lack of effective drugs for the treatment of liver fibrosis[36]. Studies have shown that traditional Chinese medicine extracts can exert pharmacological effects through multiple targets and pathways and have the characteristics of high safety, low side effects, and significant efficacy[37, 38].

TD is abundant in immature green tomatoes and exhibits a variety of activities in cells[9]. Although tomatidine is mainly derived from immature tomatoes, several studies have demonstrated its non-toxicity and safety for human treatment[39]. Studies have shown that lycopene can inhibit the growth of a variety of cancer cells through apoptosis. For example, lycopene can inhibit the cell activity of breast cancer, gastric cancer, and prostate cancer, and can also inhibit the growth of HL-60 human myeloid leukemia cells and induce cell apoptosis.[40, 41] However, the effect of tomatidine on autophagy in liver fibrosis has not been studied. Whether tomatidine has an improvement effect on liver fibrosis and the underlying molecular mechanism has not been found. Therefore, we combined experimental validation and

molecular docking through network pharmacological analysis, to investigate the potential mechanism of tomatidine in improving liver fibrosis.

In our previous experiments, it was demonstrated that tomatidine could inhibit the proliferation of LX-2 cells and reduce the expression of fibrosis-related proteins α-smooth muscle actin (α-SMA) and collagen type I 1 gene (COL1A1). ERK/MAPK-mTOR-ULK1 signaling pathways may induce autophagy in LX-2 cells. Because there are few studies on tomatidine and even fewer studies on autophagy. Therefore, network pharmacology and molecular docking were used to further verify our experimental results. Among the potential targets of tomatidine and liver fibrosis and the common targets of both, although MAPK3, RELA, MAPK1, NFKB1, MAPK14, JAK1, MAPK8, AKT1, JAK2 and ESR1 were important targets related to autophagy in the PPI network. In the process of GO analysis, it was not found that they have autophagy-related effects, but some studies have proved that tomatidine has autophagic effects, so we chose to use autophagy-related targets p62, Beclin-1, LC3, mTOR, ULK1, ERK, and tomatidine for further exploration. p62, Beclin-1, and LC3 were the key targets of autophagy, and mTOR, ULK1, and ERK were the targets related to the autophagy pathway. Through molecular docking, we found that tomatidine had good binding sites with autophagy-related proteins P62, Beclin-1, LC3, and signaling pathway-related proteins ERK, mTOR, and ULK1. ASP-7, ALA-6, and GLY-1 are the key residues produced by the binding of P62 to tomatidine; GLU-340 is the key residue produced by the binding of Beclin-1 to tomatidine; and for LC3, SER-87 is the key residue. HIS-1806 is a key residue produced by mTOR upon binding to tomatidine. GLN-227 is a key residue formed by ULK1 upon binding to tomatidine. GLU-104 is a key residue produced after ERK binds to tomatidine. At the same time, we carried out experiments to verify the expression of ERK/MAPK, mTOR, and ULK1 at the protein level. We found that the expression of P-ERK/ERK and P-ULK1/ULK1 was significantly increased, while the expression of P-mTOR/mTOR was significantly decreased. These data suggest that tomatidine may promote autophagy in LX-2 cells through the ERK/MAPK-mTOR-ULK1 signaling pathway, and these targets are potential targets that have not been identified so far. Our results suggested that tomatidine may induce autophagy in LX-2 cells through the ERK/MAPK-mTOR-ULK1 signaling pathway. Most importantly, through network pharmacology, we found that there were no autophagy-related targets reported by tomatidine in the current database, but ERK targets in the ERK/MAPK-mTOR-ULK1 signaling pathway have been reported so far. Therefore, in order to ensure the accuracy of our experiment, we performed molecular docking verification on the fibrosis-related proteins COL1A1, autophagy-related proteins P62, Beclin-1, LC3, and signal pathway-related proteins ERK, MAPK, mTOR, and ULK1, and found that tomatidine had good affinity for the above targets. Taken together, these results suggest that tomatidine may induce autophagy in LX-2 cells through the ERK/MAPK-mTOR-ULK1 signaling pathway to improve liver fibrosis. However, this study did not verify the intervention effect of the drug in animals, so there are some shortcomings.

Methods

Reagent

Tomatidine TD and Chloroquine (CQ) was purchased from MedChemExpress CQ as an autophagy inhibitor Platelet-derived growth factor-BB (PDGF-BB)was purchased from Novus PDGF-BB, as the activator of HSCs, mimics the liver fibrosis microenvironment and maintains the activation state of HSCs

Culture and treatment of hepatic stellate cells

The human hepatic stellate cell line LX-2 (from Wuhan Prosay Co.) was cultured in Dulbecco's modified Eagle's medium-high (BI, Kibbutz Beit Haemek, Israel) with 10% fetal bovine serum, and treated with different concentrations of TD (0, 40, 80, 120, 160 μM) and 40 ng/mL of PDGF-BB.

Cell viability assay

Cell viability was measured according to the Cell Counting Kit-8 (CCK-8) specification protocol (Bryotime,Shanghai,China). Cells were seeded at a density of 1×103 in 96-well plates and incubated with various concentrations of tomatidine for 24 to 72 hours. Subsequently, the absorbance at 450nm wavelength was measured using a microplate reader (Thermo, MA, USA).

Colony formation assay

Colony formation assays were performed to assess the antiproliferative activity of tomatidine in liver fibrosis. Cells were seeded in six-well plates at a density of 1000 cells per well, culture with PDGF-BB and different concentrations of tomatidine for about 10-14 days until cells grow to visible colonies. Colonies consisting of >50 cells are stained with 0.1% crystal violet and counted.

Western blot

Cells were seeded in six-well plates at a density of 5×105, after 24h of tomatidine treatment, the cells were lysed with RIPA containing a mixture of protease and phosphatase inhibitors. Protein concentrations were quantified using the BCA protein assay (#PC0021, Solarbio, Beijing, China) according to the manufacturer's instructions. Proteins were separated using SDS-PAGE gels (8 to 12%Tris-SDS gels) at 120 V for 0.5 to 2.0 hours, and wet transferred to PVDF membranes at 250 mA. After blocking the membranes with 5% skim milk for one hour at room temperature, the membranes were incubated with primary antibodies overnight at 4°C The antibodies used in the Western blot assay were all sourced from Cell Signaling Technology, USA . Then probed with horseradish peroxidase labeled anti-rabbit (1:2000) or anti-mouse (1:2000) for 1 h at room temperature. The bound immune complexes were assayed using ChemiDoc XRS (BioRad, USA).

Immunofluorescence (IF)

Using the method used to measure immunofluorescence, the samples were purified with P62(1:100; Cell Signaling Technology (Beverly, MA, USA) and a-smooth muscle actin (a-SMA; 1:200; Proteintech, Chicago,

IL, USA) antibodies. The cell slides were then placed under an OLYMPUS BX63 fluorescence microscope for observation.

mRFP-GFP-LC3

The cells were infected with the autophagy double labeled chronic disease mRFP-GFP-LC3, and the specific experimental procedures were performed according to the instructions of Shanghai Hanhang Bio-Technology Co.LTD. After successful infection, stable cell lines were obtained, passaged, and cryopreserved. After adding different treatment factors according to the experimental group, the changes in autophagosomes (green highlights) and autolysosomes (red highlights) in LX-2 cells were observed under a fluorescence microscope (OLYMPUS BX63) and photographed.

Quantitative RT-PCR

Total RNA was extracted using a total RNA extraction kit (Solarbio, Beijing, China), and cDNA was synthesized from total RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).Gene primer sequences were synthesized by QinKe (ChengDu,China), and qPCR was performed using SYBR Green Supermix (Bio-Rad). GAPDH was used as the internal control to normalize for differences in the amount of total RNA in each sample. Gene primer sequences were obtained from QinKe Gene Biology Corporation Table1

Table1 Gene primer sequences

Gene	Sequence (5’-3’)
ACTA2	Fw CTATCCAGGCGGTGCTGTCTCT
	Rv GCCACGCTCAGTCAGGATCTTC
COL1A1	Fw TTTGGATGGTGCCAAGGGAG
	Rv CACCATCATTTCCACGAGCA
SQSTM1	Fw CTGGGACTGAGAAGGCTCAC
	Rv GCAGCTGATGGTTTGGAAAT
BECN1	Fw GGGCTCCCGAGGGATGG
	Rv GCTGTTGGCACTTTCTGTGG
LC3	Fw GATGTCCGACTTATTCGAGAGC
	Rv TTGAGCTGTAAGCGCCTTCTA
GAPDH	Fw GGAGCGAGATCCCTCCAAAAT
	Rv GGCTGTTGTCATACTTCTCATGG

Network pharmacology

The tomatidine base English name 'tomatidine' input into the Swiss drug related databases Target Prediction (http://swisstargetprediction.ch/), Pubchem (https://pubchem.ncbi.nlm.nih.gov/), HERB (http://herb.ac.cn/), CTD (ctdbase.org) - the drug targets prediction analysis, potential targets for tomatidine base. Then in GeneCards (https://www.genecards.org) and DisGeNET (https://www.disgenet.org/),We searched for the English name 'liver fibrosis' in and mined the corresponding targets of liver fibrosis. Then enter Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) and input tomatidine base and liver fibrosis as separate corresponding targets to the corresponding list, The overlapping targets of tomatidine and liver fibrosis were identified, and a Venn diagram was drawn. The overlapping targets of tomatidine and liver fibrosis were input into the STRING database (https://string-db.org) for PPI network analysis, and multiple proteins were selected for analysis. The organism is selected as' homo sapiens', and the 'minimum required interaction score' is set to be greater than 0.900. The PPI network was further visualized and analyzed by Cytoscape 3.9.1, and the important targets were identified by color and shape analysis. The important targets identified in the previous step were imported into UniProt (https://www.uniprot.org/) and converted into the corresponding gene names of the targets. Will convert gene name input into DAVID Bioinformatics Resources (https://david.ncifcrf.gov/), the GO (Gene Ontology), and KEGG enrichment analysis. Items to identify molecular function (MF), biological process (BP), and cellular component (CC) enriched in the target, R 4.1.3 was used to visualize the enrichment of the top 10 pathways. Finally, the results were summarized and imported into Cytoscape 3.9.1 to construct the tomatidine -liver fibrosis- core target KEGG network. In the network, edges represent interactions between nodes, which represent drugs, diseases, core targets, and pathways.

Molecular docking

Beclin-1 (PDB code: 5HHE), COL1A1 (PDB code: 2LLP), LC3 (PDB code: 1UGM), MAPK (PDB code: 4FMQ), the 3D crystal structures of mTOR (PDB code: 3JBZ), p62 (PDB code: 1Q02), and ULK1 (PDB code: 4WNO). The three-dimensional crystal structure of ERK (ERK-AF-Q3I5G4-F1-Model_V4) was obtained from UniProt. The 2D structure of lycopene was downloaded from the Pubchem database. PyMOL (Molecular Graphics System) 2.4.0 software was used to remove all other ligands and atoms unrelated to the protein to prepare a pure protein structure. AutoDockTools 1.5.7 software was used for docking of protein-small molecule drug interactions. The key residues involved in the formation of a stable hydrogen bond after the binding of the two were determined. PyMOL 2.4.0 was used to visualize the analysis results of AutoDockTools 2.4.0, and the 3D structure diagram and pocket structure diagram were drawn.

Statistical analysis

Statistical evaluation and graphing using the GraphPad Prism 8 software package (GraphPad Software Inc, San Diego, CA, USA). All data are expressed as standard deviation (SD) ± mean. P 0.05 was considered statistically significant.

Declarations

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Author contributions statement

M.D. (Fengmei Deng) and B.T.conceived the experiment, X.H. and T.S.(Haotian Shen) conducted the experiment, X.H. and T.S.(Haotian Shen) analysed the results. All authors reviewed the manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Data Availability

The data presented in this study are available in this article (summarized in figures and tables).

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Figures

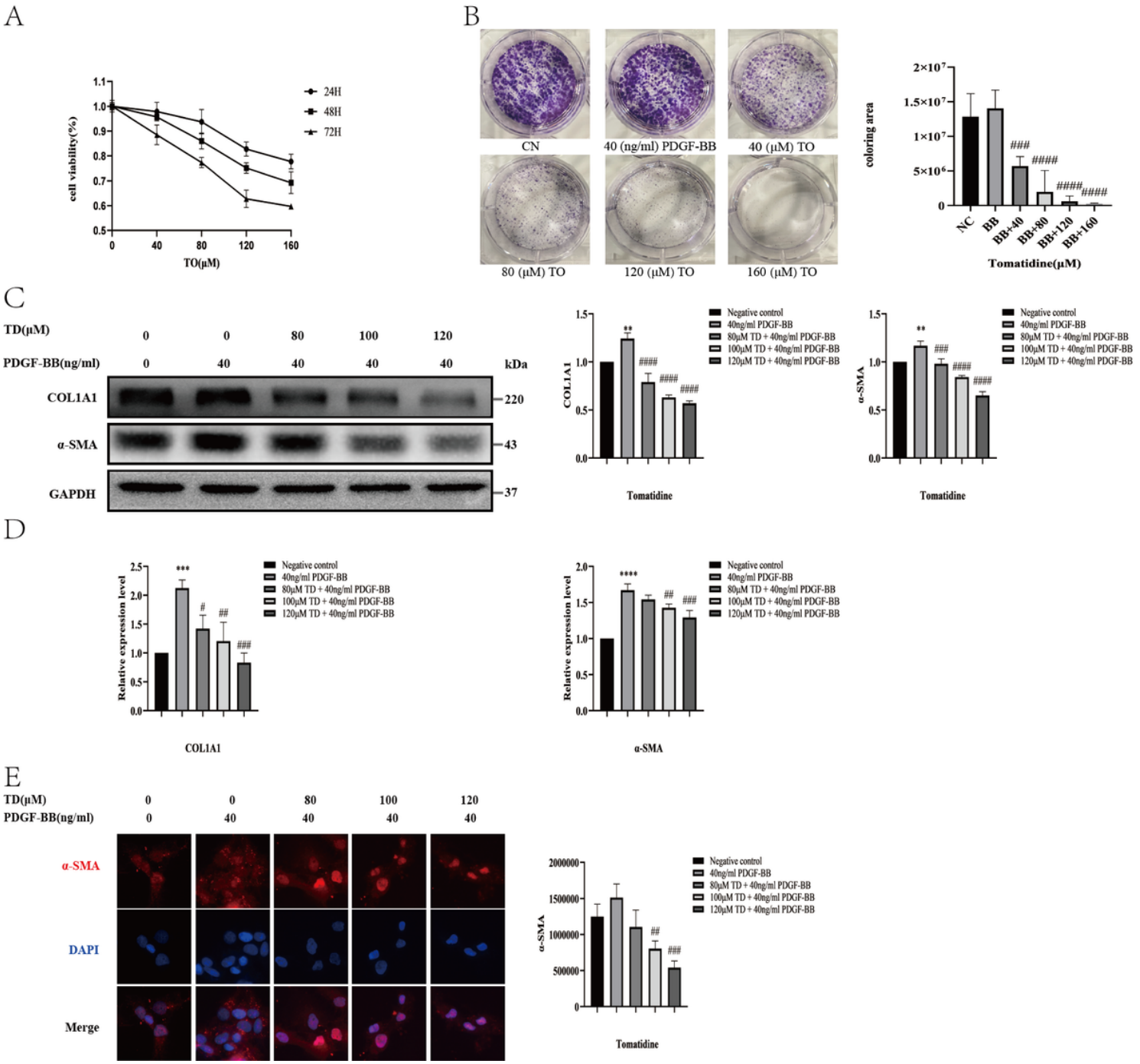

Figure 1

Tomatidine can inhibit the proliferation of LX-2 cells and the expression of fibrosis-related proteins α-SMA and COL1A1. (A)The effect of TD on the viability of LX-2 cells was determined by CCK-8 assay. (B) A colony formation assay was used to determine the anti-proliferative effect of tomatidine on LX-2. (C) Western blotting was used to detect the expression levels of the liver fibrosis phenotypes COL1A1 and α-SMA. For full-length gels see Figure S1. (D) QPCR was used to detect COL1A1 and α-SMA gene expression levels.(E)The expression level of α-SMA in cells was detected by immunofluorescence. Data are presented as mean ± SEM (n=3), compared with the control group, * $p < 0.05$, * * $p < 0.01$, * * * $p <$

0.001; compared with the PDGF-BB stimulation group, #$p < 0.05$, # #$p < 0.01$, # # #$p < 0.001$, # # # #$p < 0.0001$.

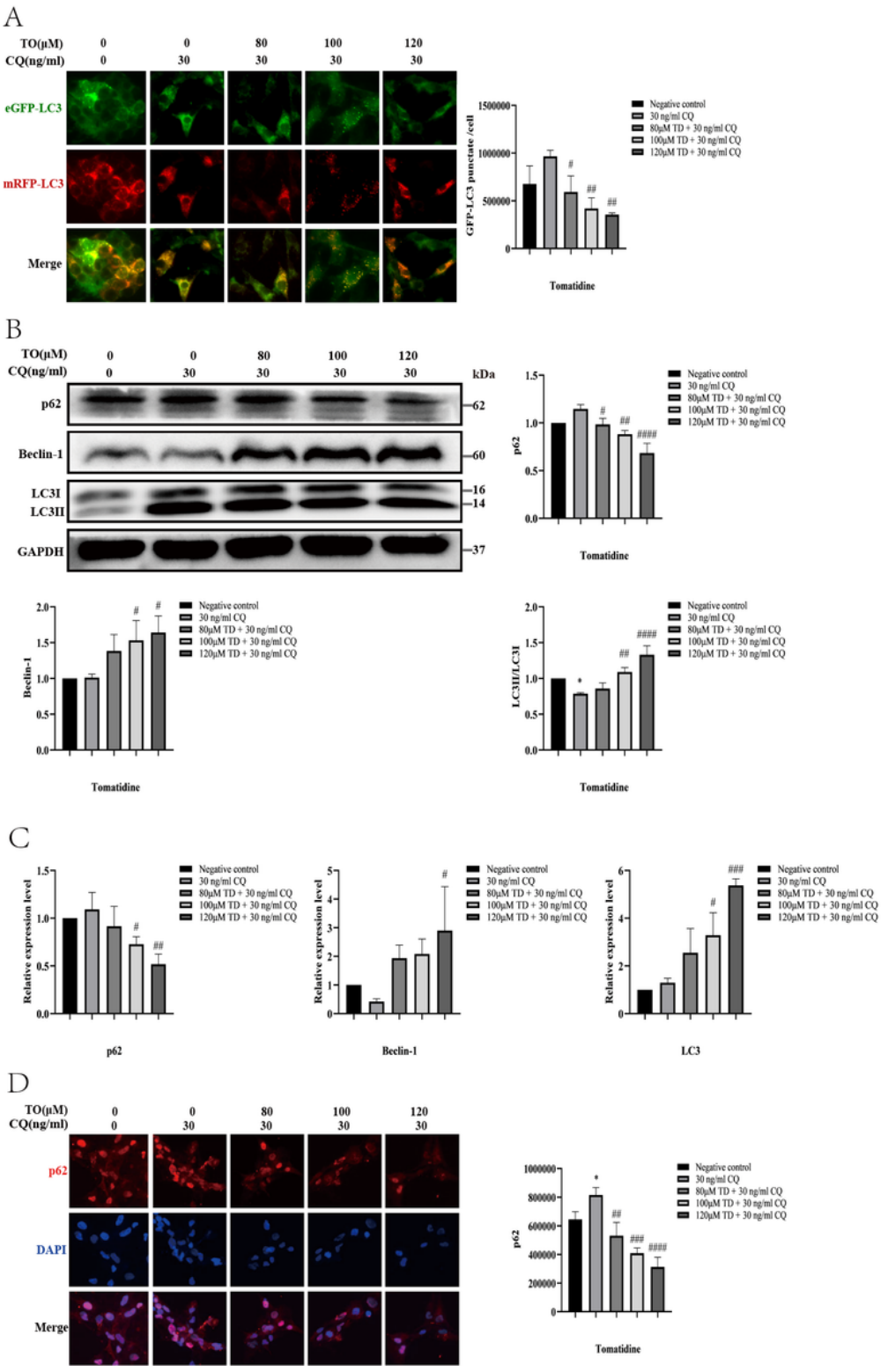

Figure 2

Tomatidine can promote autophagy in LX-2 cells. (A) is a typical image of the LC3 fluorescent bright spot to detect the amount of green fluorescence (autophagosome) and red fluorescence (autolysosome) in LX-

2 cells after tomatidine treatment. (B) After LX-2 cells were treated with tomatidine, the expression levels of autophagy-related proteins p62, Beclin-1, and LC3 were detected by Western blotting. For full-length gels see Figure S2. (C) QPCR was used to detect the gene expression levels of p62, Beclin-1, and LC3. (D) Immunofluorescence was used to detect the expression level of p62 in cells. Data are presented as mean ± SEM (n=3), compared with the control group, * p < 0.05, * * p < 0.01, * * * p < 0.001; compared with the CQ group, #p < 0.05, # #p < 0.01, # # #p < 0.001, # # # #p < 0.0001.

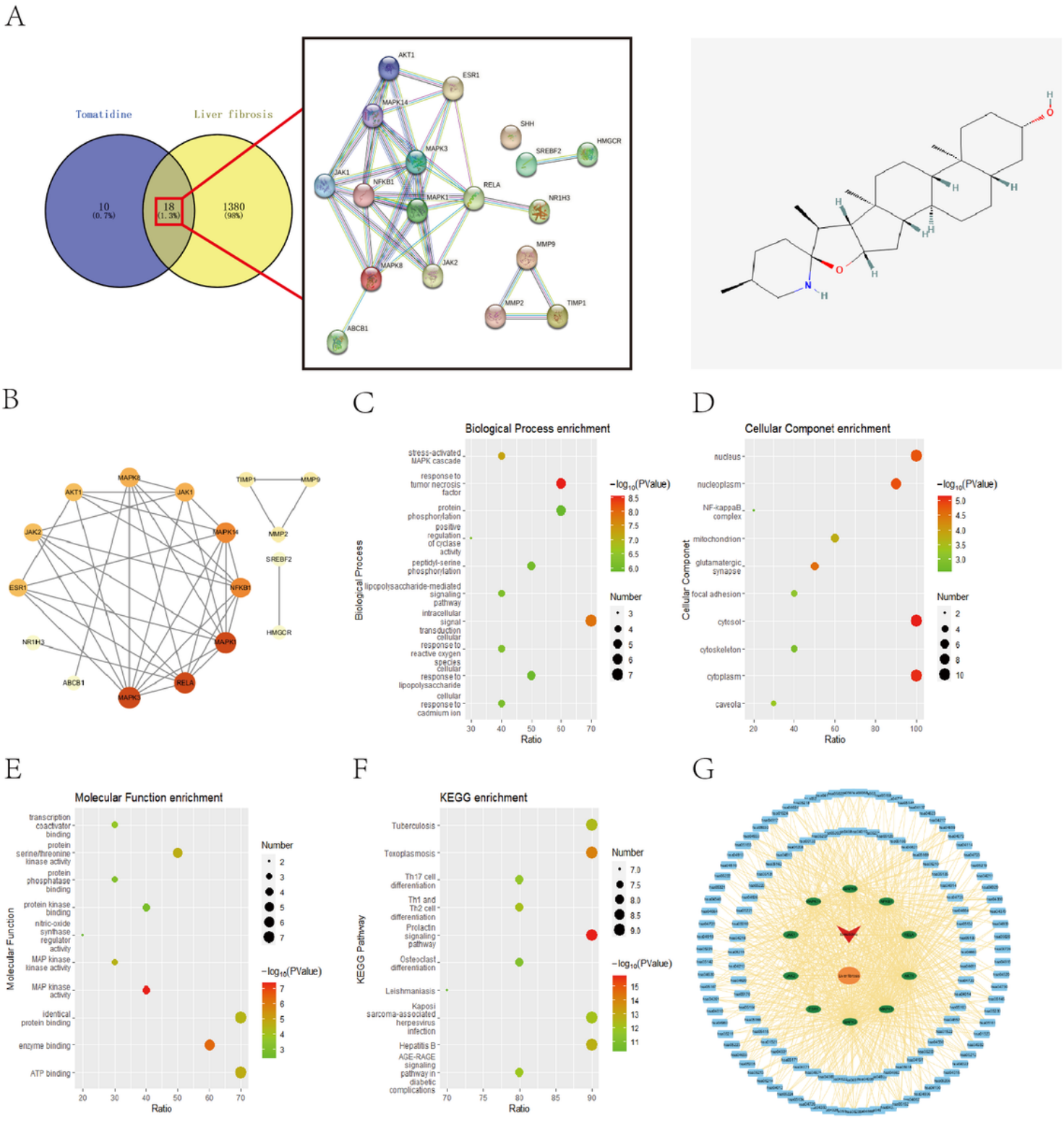

Figure 3

To elucidate the mechanism of tomatidine on liver fibrosis by network pharmacology. (A) All targets of tomatidine and liver fibrosis and 18 cross targets of tomatidine and liver fibrosis were identified by Venn diagram and PPI network. (B) The protein-protein interaction (PPI) network was constructed by Cytoscape v3.9.1 software, arranged according to the number of nodes and edges of the targets, and the core targets were screened out. (C-E) GO analysis of the core targets showed that the top 10 items related to tomatidine, an anti-liver fibrosis compound. (F) A KEGG pathway analysis was performed on the core targets, and KEGG showed that the top 10 items related to tomatidine's anti-liver fibrosis properties. (G) The network constructed by bioinformatics analysis highlighted the interaction between tomatidine, liver fibrosis, core target, and the KEGG network. Tomatidine is shown as a red V-shape, liver fibrosis is shown as an orange oval, core targets are shown as a green oval, and KEGG pathways are shown as a blue rectangle.

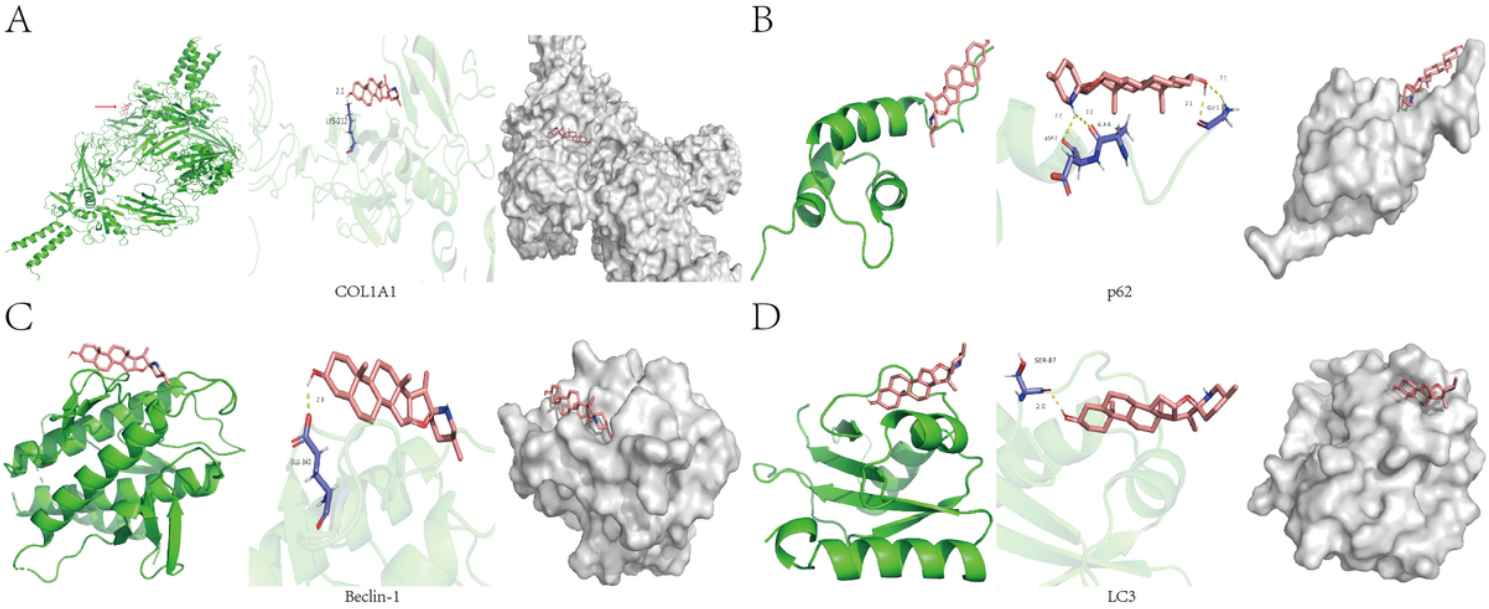

Figure 4

Molecular docking verification of tomatidine binding to autophagy-related targets. (A-D) The 3D docking of core targets COL1A1, p62, Beclin-1, and LC3 with tomatidine, the binding sites and the names of their binding residues with tomatidine, and the 3D docking of pocket structures of each target with tomatidine are shown. The tomatidine structure is shown as a red bar, the structure of the target is shown as a green band, the interacting residues are shown as a purple bar, the connected hydrogen bonds are shown as a yellow short-bar target pocket structure, and the target Mosaic model is shown in gray.

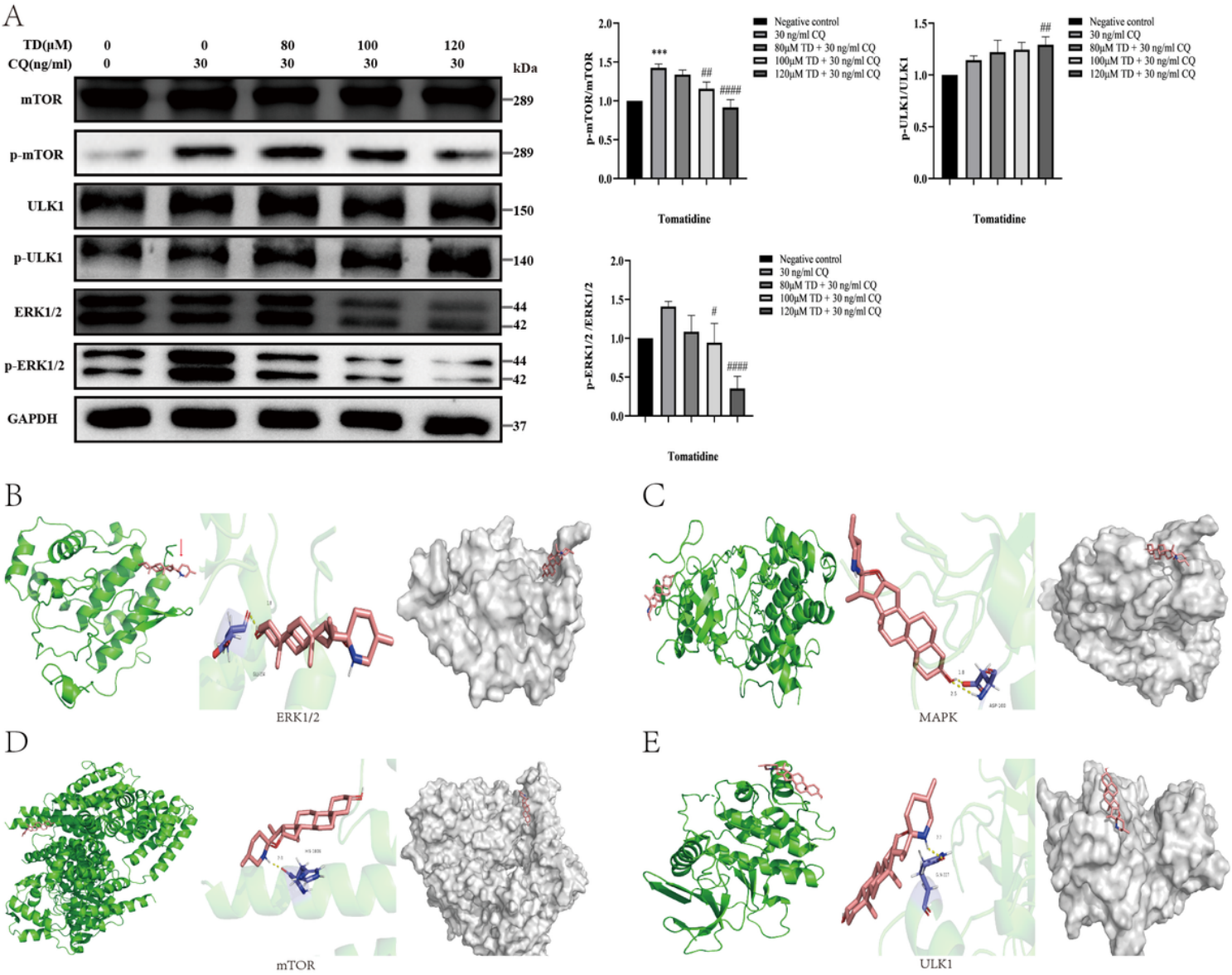

Figure 5

Tomatidine promotes autophagy in LX-2 cells through the ERK/MAPK-mTOR-ULK1 signaling pathway. (A)Western blotting was used to detect the expression levels of mTOR, ULK1 and ERK1/2 proteins in the autophagy pathway. For full-length gels see Figure S5. (B-E) The 3D docking of the proteins in the pathway, mTOR, ULK1, ERK1/2, MAPK, and tomatidine, as well as the binding sites with tomatidine and the names of their binding residues, are shown, and the 3D docking of the pocket structure of each target with tomatidine is shown. The tomatidine structure is shown as a red bar, the structure of the target is shown as a green band, the interacting residues are shown as a purple bar, the connected hydrogen bonds are shown as a yellow short-bar target pocket structure, and the target Mosaic model is shown in gray.

Supplementary Files

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