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L-741626 inhibits hepatocellular carcinoma progression by targeting Ref-1 to suppress MAPK/ERK signalling pathway activity

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Abstract

Hepatocellular carcinoma (HCC) is a common and challenging malignancy of the digestive tract. Unfortunately, patients with advanced HCC frequently experience limited long-term benefits from current treatments, highlighting the critical need for innovative therapeutic agents. The discovery and development of new small-molecule compounds that target tumours have become crucial aspects of cancer research. In this study, we report on L-741626, a compound that has significant inhibitory effects on HCC. Both in vivo and in vitro experiments confirmed that L-741626 inhibited the growth of HCC by suppressing the MAPK/ERK signalling pathway. Molecular docking simulations and drug affinity responsive target stability assays further identified redox Factor 1 (Ref-1) as a target of L-741626. Ref-1 is overexpressed in HCC and is correlated with poor prognosis and high stage. Further studies demonstrated that Ref-1 interacts with CRAF, a crucial component of the MAPK/ERK signalling pathway. Knockdown of Ref-1 in HCC cells led to inhibition of the MAPK/ERK pathway. Sorafenib is a well-established targeted therapy for the treatment of HCC, with its primary antitumor mechanism being the inhibition of the MAPK/ERK signalling pathway. However, the presence of tumor stem cells is a key factor contributing to resistance to sorafenib. Our study demonstrates that L-741626 can suppress tumor stemness in HCC. The combination of L-741626 and sorafenib significantly enhances the sensitivity of HCC, resulting in increased tumoricidal effects. Our findings reveal a novel pharmacological effect of L-741626, which inhibits MAPK/ERK signalling activity in HCC by targeting Ref-1. Furthermore, L-741626 exhibits a synergistic effect when combined with sorafenib, suggesting a new potential approach for HCC treatment.

Keywords Hepatocellular carcinoma, L-741626, Redox factor 1, MAPK/ERK, Sorafenib

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignant cancer globally and the fourth leading cause of cancer-related death [1, 2]. Early-stage HCC can be curative through ablation and surgical resection. However, most HCC patients are diagnosed at an advanced stage, which significantly reduces their chances of a cure, resulting in a 5 year survival rate of less than 15% [3–5]. Compared to other malignancies, advanced HCC is generally considered a chemotherapy-resistant tumor, characterized by the overexpression of multiple chemotherapy resistance-associated proteins, such as MDR1 and DPD [6–8]. Targeted therapy is considered a promising treatment approach for HCC. Sorafenib, as the first-ever multitarget inhibitor approved for the treatment of advanced HCC, exerts its antitumor effects by inhibiting multiple kinases, including the RAF family, C-Kit, FLT-3, VEGFR, and PDGFR [9–11]. However, sorafenib monotherapy demonstrated a low objective response rate (ORR) of only 2.2% in the SHARP and ORIENTAL trials, and overall survival was extended by less than 3 months compared to placebo [12]. Hence, it is urgent to explore new antitumour regimen to enhance the OS of HCC.

Recently, targeting tumor cells with small-molecule compounds has become a critical focus in cancer drug development [13]. Over the past decade, an average of four small-molecule drugs have been approved annually by the FDA for the treatment of solid tumors [14]. Due to their smaller molecular weight, small-molecule compounds can bind to multiple intracellular and extracellular targets to inhibit cancer growth [15]. Compared to traditional therapies, they offer greater selectivity, reduced toxicity, and lower production costs [16]. In this study, we identified L-741626 as a potent small-molecule inhibitor of HCC proliferation. L-741626 is a selective antagonist of dopamine D2 receptors, including DRD2, DRD3, and DRD4, and is commonly used in research focusing on the nervous system [17]. Research has demonstrated that L-741626 can inhibit the growth of pancreatic ductal adenocarcinoma by targeting the DRD2 signalling pathway, suggesting its potential antitumor activity [18]. However, HCC does not express dopamine D2 receptor proteins, indicating that L-741626 does not inhibit HCC proliferation via the DRD2 signalling pathway. The molecular mechanism by which L-741626 suppresses HCC progression remains unclear and requires further investigation to confirm.

In this study, we found that L-741626 can specifically target and bind to Redox Factor 1 (Ref-1). Ref-1, also known as human apurinic/apyrimidinic endonuclease 1 (APE1), is a multifunctional enzyme that is highly expressed in various malignancies, including pancreatic cancer, breast cancer, prostate cancer, and gliomas. Its overexpression is associated with poor prognosis, making it a potential target for antitumor therapy [19–22]. In addition to its role in base repair, Ref-1 regulates multiple transcription factors, including STAT3, NF-κB, and HIF-1α, through its redox activity, thereby contributing to the activation of various signalling pathways [23–25]. The MAPK/ERK signalling pathway is considered one of the most crucial pathways in the tumorigenesis and progression of HCC. Investigating the molecular mechanisms of the MAPK/ERK pathway and developing targeted interventions against this pathway have been longstanding priorities in clinical drug development [26]. Research on the relationship between Ref-1 and the MAPK/ERK signalling pathway is limited. One study demonstrated that inhibiting Ref-1 in B cells significantly impairs IL-21-induced ERK activation, though the precise molecular mechanism remains unclear [27]. Interestingly, RNA-seq pathway enrichment analysis of HCC cells treated with L-741626 revealed a marked inhibition of the MAPK/ERK signalling pathway. Based on these findings, we hypothesized that Ref-1 positively regulates the MAPK/ERK signalling pathway in HCC. Subsequent experiments confirmed this hypothesis, demonstrating that Ref-1 exerts this regulatory effect through its interaction with CRAF, a critical signalling protein in the MAPK/ERK pathway. Inhibition of the MAPK/ERK signalling pathway is also a key mechanism by which sorafenib suppresses HCC [28]. However, the presence of tumor stem cells is a major cause of resistance to sorafenib [29]. Our further experiments demonstrated that L-741626 effectively suppresses tumor stemness in HCC cells. Encouragingly, the combination of L-741626 and sorafenib exhibited synergistic antitumor effects, achieving a higher tumor inhibition rate. Collectively, these findings reveal a novel pharmacological action of

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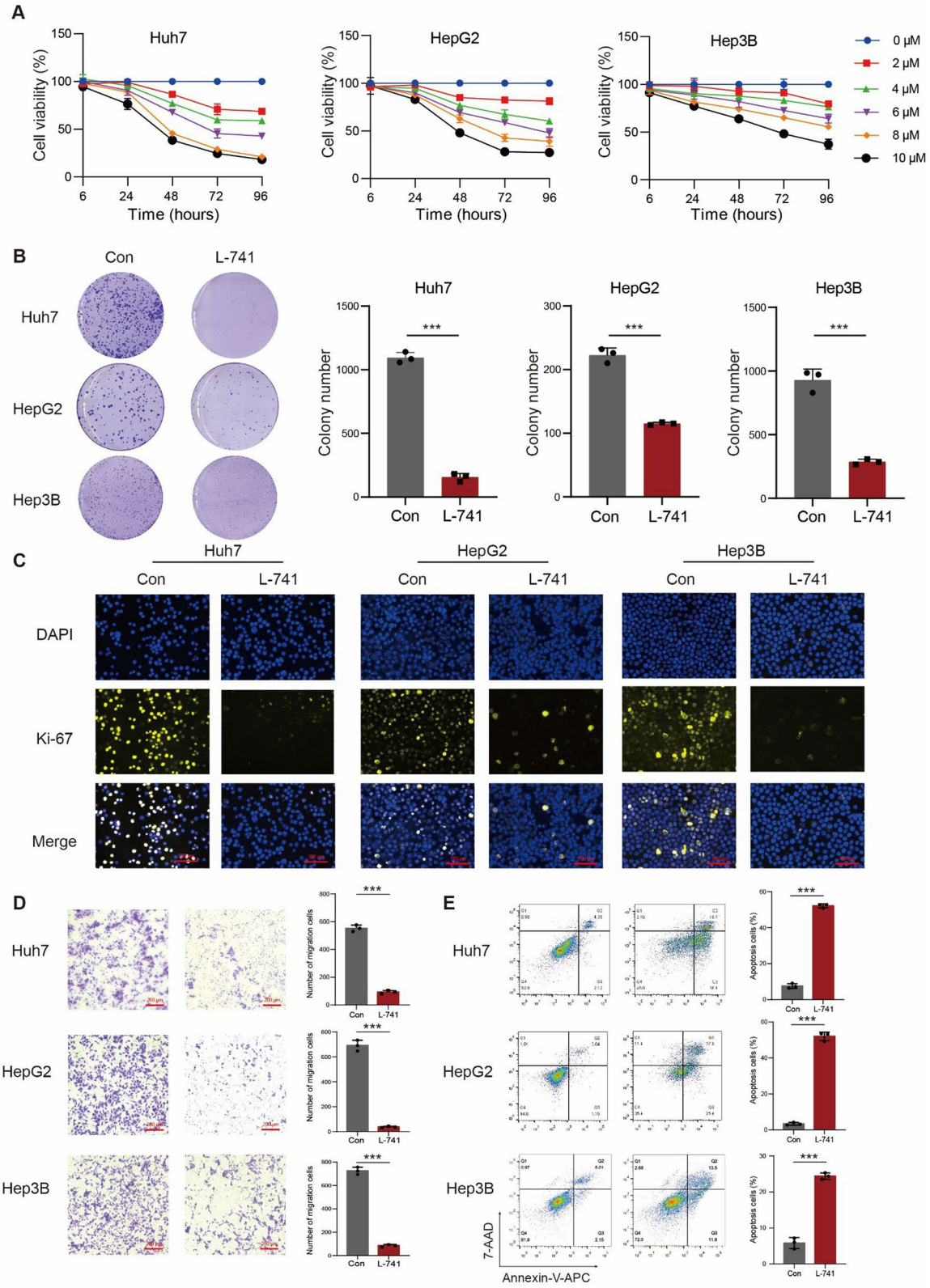
Fig. 1 **A** Huh7, HepG2, and Hep3B cells were treated with different concentrations of L-741626 and cell proliferation was measured using a CCK8 assay. **B** Huh7, HepG2, and Hep3B cells were seeded in six-well plates and treated with DMSO (control group) or L-741626 (5 μM) for 10 days, after which the number of colonies was counted. **C** Huh7, HepG2, and Hep3B cells were treated with DMSO or L-741626 (10 μM) for 48 h followed by Ki-67 immunofluorescence staining (scale bar: 100 μm). **D** Transwell assays were performed to analyse the migration ability of Huh7, HepG2, and Hep3B cells with or without L-741626 treatment (scale bar: 200 μm). **E** The apoptosis of Huh7, HepG2 and Hep3B cells treated with L-741626 (10 μM) was analysed using flow cytometry. The data are presented as means ± SDs; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



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L-741626 and provide new therapeutic possibilities for the treatment of HCC.

Materials and methods

Cell line culture and shRNAs

In this study, the human hepatocellular carcinoma cell lines Huh7, HepG2, and Hep3B were purchased from the American Type Culture Collection (ATCC). The cells were cultured in DMEM containing 10% foetal bovine serum (FBS) under conditions of 5% CO_2 at 37 °C, with all cells passaged with trypsin. shRNA oligos (shRef-1 #1, GCCGGGTGATTGTGGCTGAAT; shRef-1 #2, TGCCTGGATTAAGAAGAAAGGAT) were cloned and inserted into the pSicoR-mCherry-empty vector (Addgene, #21,907). The pSicoR-mCherry-empty or pSicoR-mCherry-Ref-1 vectors were transduced into the Huh7 and HepG2 cell lines to construct Ref-1-knockdown hepatocellular carcinoma cell lines.

Reagents and antibodies

L-741626, sorafenib, ERK1/2 antibody, phospho-ERK1/2 (Tyr204/Tyr187) antibody, and CD133 antibody were purchased from MedChemExpress. Ref-1 antibody was purchased from Proteintech. CRAF antibody was purchased from CUSABIO.

Cell viability assay and colony formation

Cell viability was assessed using a CCK-8 kit according to the manufacturer's instructions. Hepatocellular carcinoma cells were cultured in 96-well plates at a density of 2000 cells per well. The cells were treated with different concentrations of L-741626, and after the corresponding treatment time, 10 μL of CCK-8 reagent (Selleck Chemicals) was added and incubated for 1 h. The absorbance was measured at 450 nm using a microplate reader.

After hepatocellular carcinoma cells were treated with L-741626 for 2 weeks, colonies were fixed. The colonies were stained with crystal violet and counted.

Apoptosis detection

Apoptosis levels were detected using a Annexin V-APC/7-AAD apoptosis detection kit (Procell, P-CA-208). Briefly, the cells were collected and washed with 100 μL of PBS, followed by staining with 2.5 μL of Annexin V-APC and 2.5 μL of 7-AAD. The cells were incubated in the dark for 15 min, 100 μL of binding buffer was added, and the samples were analysed using flow cytometry.

Western blotting

Protein samples were loaded onto a 10% SDS–polyacrylamide gel, separated by electrophoresis, then transferred onto a PVDF membrane. After blocking, the membranes were incubated with primary antibodies at 4 °C overnight. After washing, the membranes were incubated with the appropriate secondary antibodies. Finally, the protein bands were visualized using enhanced chemiluminescence (ECL) reagents and a Western blot imaging system.

Transwell assay

A total of 1.0×10^4 hepatocellular carcinoma cells were suspended in 200 μL of serum-free DMEM and added to the upper chamber of the Transwell insert. The lower chamber was filled with 600 μL of DMEM containing 20% serum. After 48 h, the cells in the upper chamber were fixed and stained with crystal violet and the nonmigrated cells were carefully removed. The migrated cells were photographed and counted under a microscope.

Immunohistochemistry

The tumour samples were fixed in 4% paraformaldehyde and then embedded in paraffin. The paraffin-embedded samples were sectioned, deparaffinized, rehydrated, and subjected to antigen retrieval. The sections were incubated with Ref-1 primary antibody at 4 °C overnight, followed by incubation with secondary antibody for 1 h. After staining with DAB and haematoxylin, IHC images were acquired with a slide scanner (Olympus VS200).

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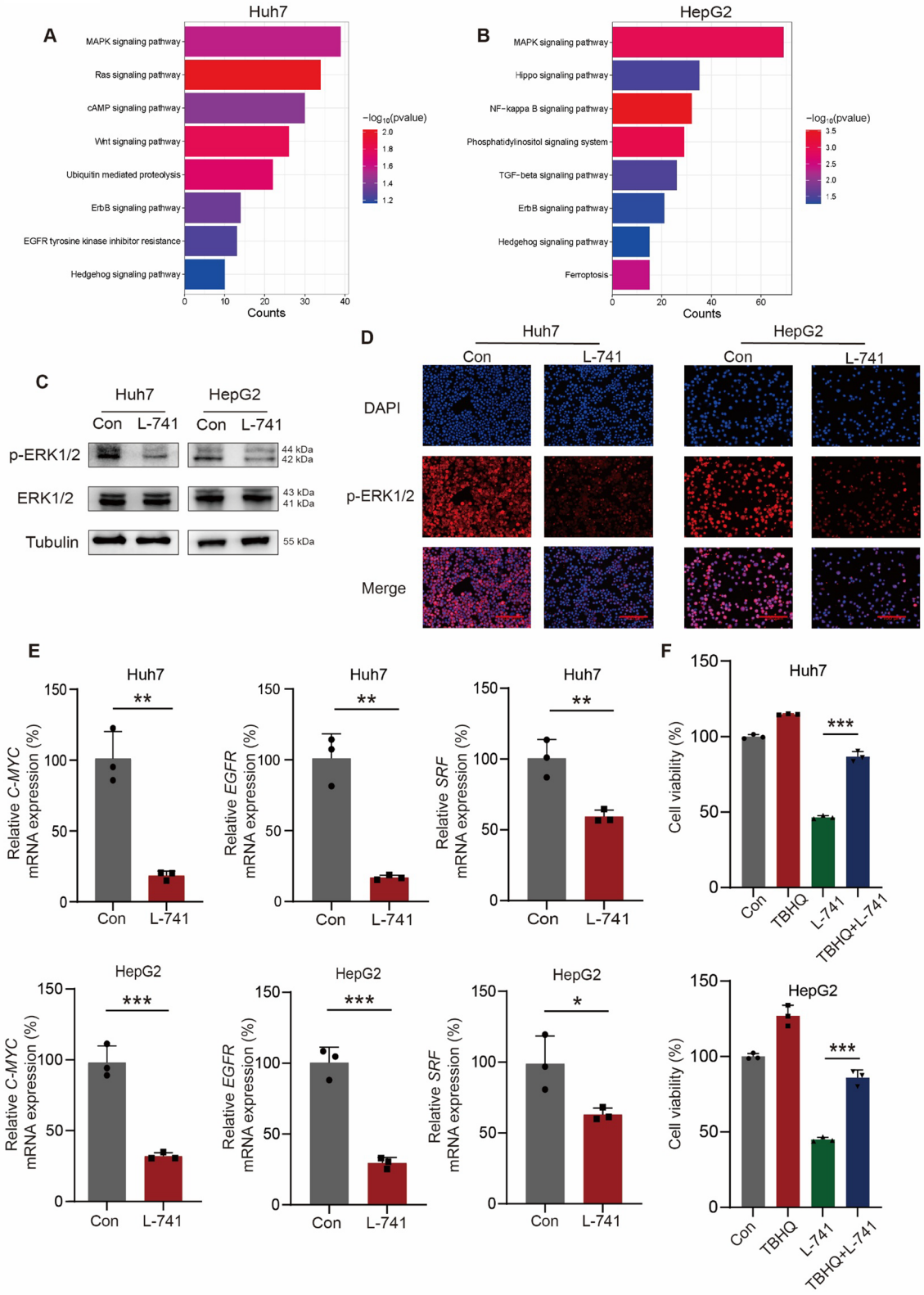
Fig. 2 **A**, **B** KEGG pathway enrichment analysis was performed on Huh7 and HepG2 cells treated with L-741626. **C** Western blot analysis of ERK1/2 and p-ERK1/2 protein levels in Huh7 and HepG2 cells treated with or without L-741626. **D** The expression level of p-ERK in Huh7 and HepG2 cells treated with L-741626 was detected with an immunofluorescence assay (scale bar: 100 μm). **E** RT–qPCR was used to measure the mRNA expression levels of C-MYC, EGFR, and SRF, key components of the MAPK/ERK signalling pathway, in Huh7 and HepG2 cells treated with L-741626. **F** Huh7 and HepG2 cells were treated with TBHQ, a MAPK/ERK signalling pathway activator, L-741626, or their combination for 48 h, and cell viability was assessed using a CCK8 assay. The data are presented as means ± SDs; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



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Molecular docking

Using AutoDock tools, we performed molecular docking studies. The crystal structure of Ref-1 (PDB: 5WN2) was obtained from the Protein Data Bank (PDB) (https://www.rcsb.org/), and the structure of L-741626 (CID: 133,633) was sourced from the PubChem Compound Database (https://pubchem.ncbi.nlm.nih.gov/).

DARTS assay

Huh7 cells were collected and digested with trypsin. After cell lysis, 2 μL of L-741626 (100 μM) and 2 μL of sterile water were added separately to two centrifuge tubes containing 60 μL of cell lysate (protein concentration: 100 μg/μL). The mixtures were incubated for 2 h to ensure adequate compound–protein interactions. The solution in each centrifuge tube was then equally divided into three separate tubes, forming three groups. Each group was treated with 0.05%, 0.025%, or 0.0125% proteinase K for 30 min. Subsequently, 4×loading buffer was added, and the samples were boiled for 5 min followed by western blot analysis for experimental validation. The specific principles and experimental procedures have been described previously [30].

Co-IP assay

One milligram of total protein was mixed with 1 μg of antibody and incubated overnight at 4 °C. Protein A/G PLUS-agarose beads were then added, and the mixture was incubated again overnight at 4 °C. After washing with RIPA buffer and PBS, the precipitate was suspended in SDS loading buffer and analyzed by Western blotting.

In Vivo animal experiments

A total of 2.0×10^6 Huh7 cells were injected subcutaneously into the dorsal flanks of male BALB/c nude mice. One week later, when the tumour volume had increased to a certain size, the mice were weighed and the tumour volume was measured every two days, followed by intraperitoneal injection of the drug. After two weeks, the mice were sacrificed and the tumours were isolated and weighed.

Results

L-741626 inhibits the growth of HCC cells

To verify the inhibitory effect of L-741626 on HCC, we conducted in vitro experiments on three HCC cell lines: Huh7, HepG2, and Hep3B. Through the CCK8 assay, we confirmed that L-741626 inhibits the proliferation of HCC cells, with its inhibitory effect intensifying as both the concentration and exposure time increase (Fig. 1A). Further verification through colony formation assays and Ki-67 immunofluorescence assays demonstrated that L-741626 inhibited the proliferation of the Huh7, HepG2, and Hep3B cell lines (Fig. 1B, C). Additionally, Transwell assays revealed that L-741626 inhibited the migratory ability of Huh7, HepG2, and Hep3B cells (Fig. 1D). Apoptosis levels were assessed using Annexin V/7AAD staining, which revealed that treatment with L-741626 significantly increased the apoptosis rates of Huh7, HepG2, and Hep3B cells (Fig. 1E).

L-741626 exerts antitumour effects by inhibiting the MAPK/ERK signalling pathway

To understand the mechanism by which L-741626 inhibits HCC cell proliferation, we performed RNA sequencing on untreated Huh7 and HepG2 cells, as well as L-741626-treated Huh7 and HepG2 cells (Fig. S1). KEGG pathway enrichment analysis revealed that the MAPK signalling pathway was suppressed in both L-741626-treated Huh7 and HepG2 cells (Fig. 2A, B). To confirm these findings, we used western blotting to examine the expression levels of key factors in these pathways, ERK1/2 and p-ERK1/2, in both Huh7 and HepG2 cells (Fig. 2C). Our results showed that L-741626 decreased the expression levels of p-ERK1/2, further confirmed through immunofluorescence experiments (Fig. 2D). Additionally, we used qPCR assays to determine the mRNA levels of the ERK pathway-related molecules C-MYC [31], EGFR [32], and SRF [33], before and after L-741626 treatment, and found that these key proteins had significantly reduced mRNA levels following treatment (Fig. 2E). Finally, we conducted rescue experiments using TBHQ, an ERK pathway activator [34], and found that the use of TBHQ was able to reduce the antitumour effect of L-741626 (Fig. 2F). In light of these findings, we conclude that L-741626 exerts

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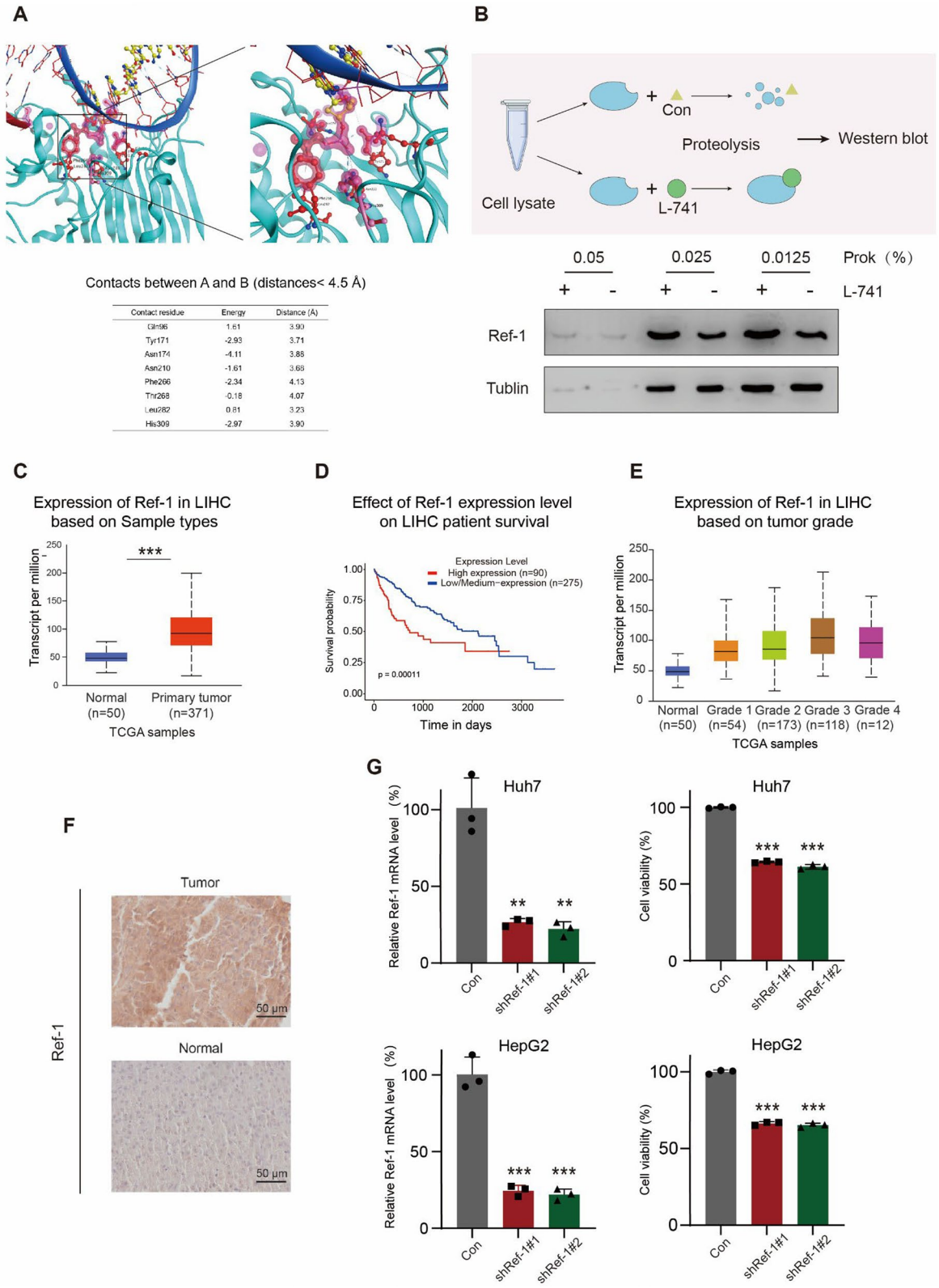
Fig. 3 **A** Molecular docking analyses revealed that L-741626 binds to Ref-1. **B** DARTS assay demonstrated the binding of L-741626 to the target protein Ref-1 in Huh7 cells. **C**–**E** Analysis of the TCGA database revealed that Ref-1 was highly expressed in hepatocellular carcinoma (HCC) and was associated with both patient prognosis and disease stage. **F** Immunohistochemistry of tumour and adjacent tissue sections from HCC patients confirmed that Ref-1 is highly expressed in the tumour tissues. **G** Ref-1 was knocked down in Huh7 and HepG2 cells and the knockdown efficiency was assessed using RT–qPCR. Cell viability was measured using a CCK-8 assay. The data are represented as means ± SDs; * $P<0.05$; ** $P<0.01$; *** $P<0.001$



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its antitumour effects through the MAPK/ERK signalling pathway.

L-741626 exerts antitumour effects by targeting Ref-1

L-741626 is a selective antagonist of D2-like dopamine receptors including DRD2, DRD3, and DRD4. However, analysis using the TCGA database revealed that these receptors are scarcely expressed in HCC tissues, suggesting that L-741626 does not exert its effects through D2-like dopamine receptors in this context (Fig. S2). To elucidate the mechanism by which L-741626 inhibits HCC cell viability, we used the SuperPred database for target protein prediction and found that L-741626 could be molecularly docked with multiple amino acid sites at the N-terminus of redox factor-1 (Ref-1) (Fig. 3A), which is associated with its redox function [35]. To confirm the direct interaction between L-741626 and Ref-1, we utilized a drug affinity responsive target stability (DARTS) assay, demonstrating that Ref-1 is indeed a direct target of L-741626. As anticipated, in the presence of L-741626, Ref-1 exhibited resistance to proteolytic degradation by proteinase K at various concentrations (Fig. 3B). Data from the TCGA database showed that Ref-1 is highly expressed in HCC and is significantly correlated with tumour stage and patient survival rates (Fig. 3C-E). Immunohistochemical analysis of clinical samples from HCC patients confirmed that Ref-1 expression is markedly higher in HCC tissues than in adjacent nontumorous tissues (Fig. 3F). Furthermore, we constructed Ref-1-knockdown Huh7 and HepG2 cell lines. Cell viability assays using CCK-8 revealed that Ref-1 knockdown inhibited the proliferation of Huh7 and HepG2 cells (Fig. 3G).

In summary, our results indicate that L-741626 exerts its antitumour activity by inhibiting Ref-1.

Ref-1 positively regulates the MAPK/ERK pathway by maintaining CRAF function

To further investigate the mechanism by which L-741626 inhibits HCC via the MAPK/ERK signalling pathway, we treated Ref-1 knockdown and non-knockdown Huh7 and HepG2 cell lines with L-741626. As anticipated, Ref-1 knockdown reduced the sensitivity of Huh7 and HepG2 cells to L-741626 (Fig. 4A, B). Furthermore, western blot analysis demonstrated that Ref-1 knockdown inhibited the activity of the MAPK/ERK signalling pathway in these cells (Fig. 4C). These findings further clarify that L-741626 exerts its antitumour effects by targeting Ref-1. Previous studies have shown that in papillary thyroid carcinoma Ref-1 can bind to BRAF, an upstream molecule of ERK, and maintain the function of BRAF through its redox activity [36]. CRAF and BRAF belong to the same family of proteins and CRAF is highly expressed in HCC and promotes its progression [37, 38]. We speculate that Ref-1 can also bind to CRAF and maintain its function. Analysis using the GEPIA 2 database revealed a significant positive correlation between the expression levels of Ref-1 and CRAF in HCC (Fig. 4D). We conducted Co-IP experiments, which demonstrated a direct interaction between Ref-1 and CRAF (Fig. 4E). These results led us to conclude that L-741626 inhibits N-terminal redox activity by targeting Ref-1, thereby disrupting the Ref-1 and CRAF interaction and subsequently inhibiting MAPK/ERK signalling pathway activity (Fig. 4F).

L-741626 synergistically enhances the antitumour effect of sorafenib

Sorafenib, a multitarget tyrosine kinase inhibitor, effectively inhibits multiple targets including the RAF family, C-Kit, FLT-3, VEGFR, and PDGFR. It is a classic drug used in the treatment of HCC [39]. However, the presence of tumor stem cells is a key factor in the development of resistance to sorafenib. We validated through sphere formation assays that L-741626 can suppress tumor stemness in Huh7 and HepG2 cells in vitro (Fig. 5A). Further analysis of the spheres collected and subjected to Western blotting revealed that L-741626 treatment resulted in a reduction in the expression of the tumor stemness marker CD133 (Fig. 5B). These results suggest that L-741626 may work synergistically with sorafenib to enhance antitumor effects. We conducted both in vitro and in vivo experiments to investigate the combined effects of sorafenib and L-741626. In vitro CCK8 assays revealed that compared with monotherapy the combination of sorafenib and L-741626 had a more significant antitumour effect on Huh7 and HepG2 cell lines (Fig. S3).

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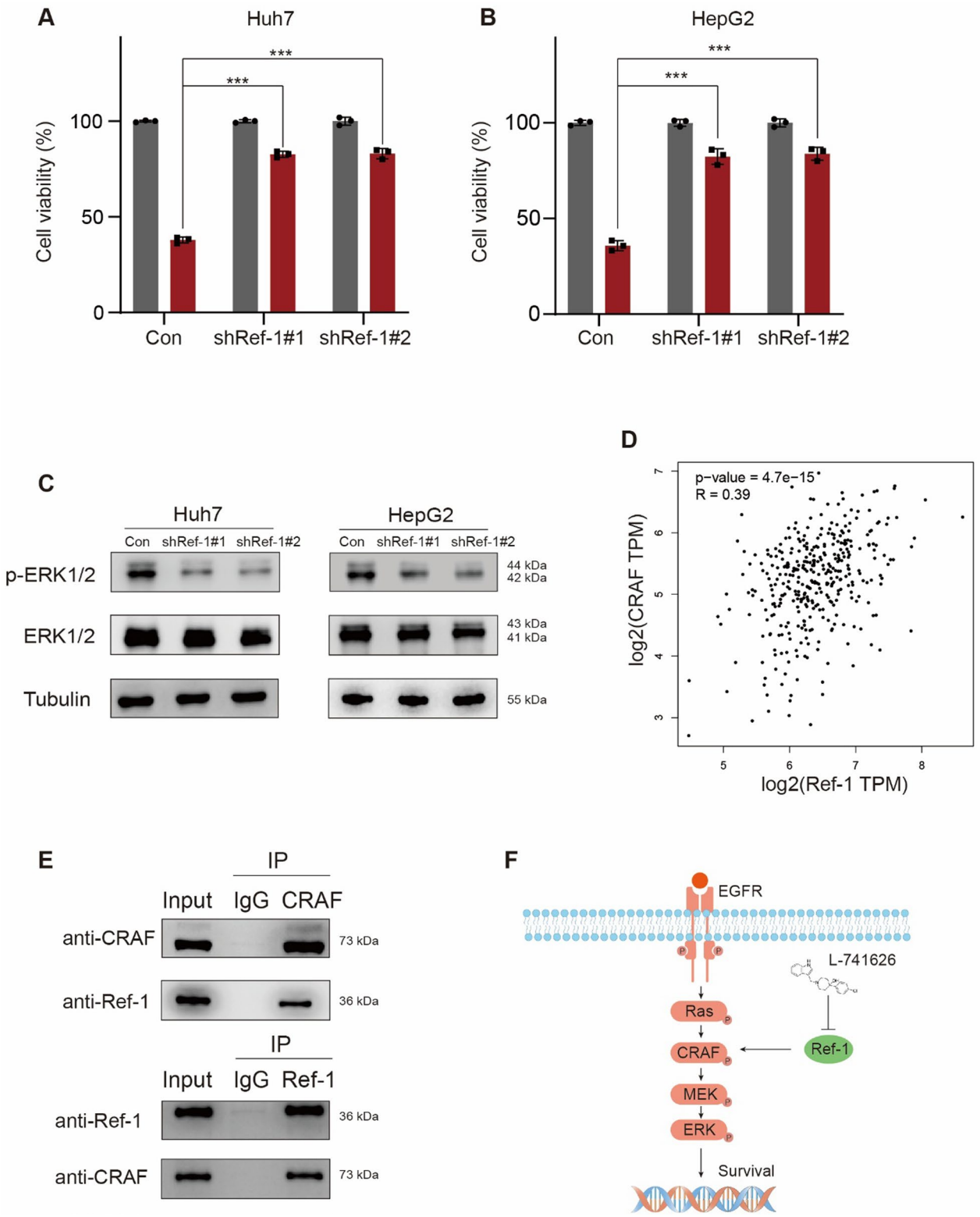
Fig. 4 **A**, **B** Huh7 and HepG2 cells with Ref-1 knockdown were treated with L-741626. Compared with the nonknockdown control group the sensitivity of Huh7 and HepG2 cells to L-741626 was reduced following Ref-1 knockdown. **C** western blot experiments were used to detect the expression levels of ERK1/2 and p-ERK1/2 in Huh7 and HepG2 cells with Ref-1 knockdown. **D** Analysis of the correlation between Ref-1 and CRAF protein expression in HCC was conducted using the GEPIA2 database. **E** Coimmunoprecipitation (co-IP) experiments demonstrated that Ref-1 and CRAF can directly bind to one another. **F** L-741626 targets Ref-1 and modulates the interaction between Ref-1 and CRAF, thereby inhibiting the MAPK/ERK signalling pathway. The data are presented as means ± SDs; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



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To further validate the synergistic effects of sorafenib and L-741626 in vivo we established a Huh7 xenograft mouse model. Nude mice bearing Huh7 tumours were intraperitoneally injected with sorafenib (20 mg/kg), L-741626 (20 mg/kg), a combination of both drugs, or an equivalent volume of PBS as a control every other day (Fig. 5C). After two weeks, although sorafenib or L-741626 monotherapy reduced tumour volume and weight to different extents, the combination therapy resulted in a significantly greater reduction in both tumour volume and weight, leading to the highest tumour inhibition rate (Fig. 5D-G). Moreover, neither monotherapy nor combination therapy caused changes in mouse body weight (Fig. S4A) and subsequent H&E staining of visceral organs did not reveal noticeable toxic side effects (Fig. S4B).

Discussion and conclusion

Hepatocellular carcinoma (HCC) remains a significant global health challenge [1, 40]. The MAPK/ERK signalling pathway is a critical target for intervention in HCC, with overactivation observed in more than 50% of patients [26, 41]. The level of activation of the MAPK/ERK signalling pathway is a prognostic indicator for HCC patients [37]. The advent of tyrosine kinase inhibitors (TKIs) such as sorafenib has marked a new era in HCC treatment [42]. However, TKIs face issues such as reduced drug sensitivity and limited long-term efficacy for patients [43, 44]. HCC patients who benefit from sorafenib treatment typically develop resistance after 6 months [12]. Therefore, novel treatment options for HCC are crucial.

Targeting tumours with small molecule inhibitors has emerged as a promising therapeutic strategy. A variety of small molecule inhibitors targeting cancer have either received clinical approval or are currently undergoing clinical trials [45]. In this study, we discovered that the small-molecule compound L-741626 inhibits the proliferation of hepatocellular carcinoma (HCC) cells by suppressing the activity of the MAPK/ERK signalling pathway. Through molecular docking and DARTS experiments L-741626 has been shown to bind to multiple amino acid sites on the N-terminus of the Ref-1 protein, which possesses redox activity. Previous studies have demonstrated that Ref-1 can regulate the activity of several signalling pathways including p53, NF-κB, AKT, and STAT3, through its redox function [46–49]. Ref-1 is highly activated in multiple cancer types including hepatocellular carcinoma, prostate cancer, pancreatic cancer, ovarian cancer, and lung cancer, and is closely associated with tumour aggressiveness. Targeting Ref-1 represents an effective strategy for combating tumours [19]. Recent research has shown that Ref-1 can bind to BRAF and sustain its function in papillary thyroid tumours [36]. In our study, Co-IP experiments revealed that Ref-1 can also bind to CRAF in HCC. Knockdown of Ref-1 in HCC cell lines significantly inhibited the MAPK/ERK signalling pathway. Therefore, we propose that L-741626 may exert its antitumour effects by blocking the interaction between Ref-1 and CRAF, thereby inhibiting the activation of downstream MAPK/ERK signalling. Although sorafenib also exerts its antitumor effects by inhibiting the MAPK/ERK signalling pathway, the presence of tumor stem cells often leads to resistance to sorafenib [50]. We found that L-741626 can inhibit tumor stemness in HCC cell lines, suggesting the potential for combining L-741626 with sorafenib. Our results indicate that this combination significantly enhances the sensitivity of HCC to sorafenib, resulting in a more pronounced antitumor effect. However, several limitations exist in our study. Although we have demonstrated that L-741626 effectively inhibits the progression of HCC, this evidence is solely based on in vitro and murine animal experiments, with no clinical data supporting the dosage, safety, and efficacy of L-741626 in humans. While we have shown that L-741626 targets Ref-1 in HCC and disrupts the interaction between Ref-1 and CRAF, the binding sites and underlying mechanisms of interaction between L-741626 and Ref-1, as well as between Ref-1 and CRAF, require further investigation. Additionally, nanoparticle-based delivery of small molecule compounds has been shown to enhance the antitumor activity of such compounds [51], and the nanoparticle delivery of L-741626 warrants further exploration.

In summary, we identified a novel pharmacological effect of the compound L-741626. L-741626 targets redox factor-1 (Ref-1), which is overexpressed in HCC and linked to poor patient prognosis. L-741626 disrupts the binding of Ref-1 to CRAF, thereby inhibiting the MAPK/

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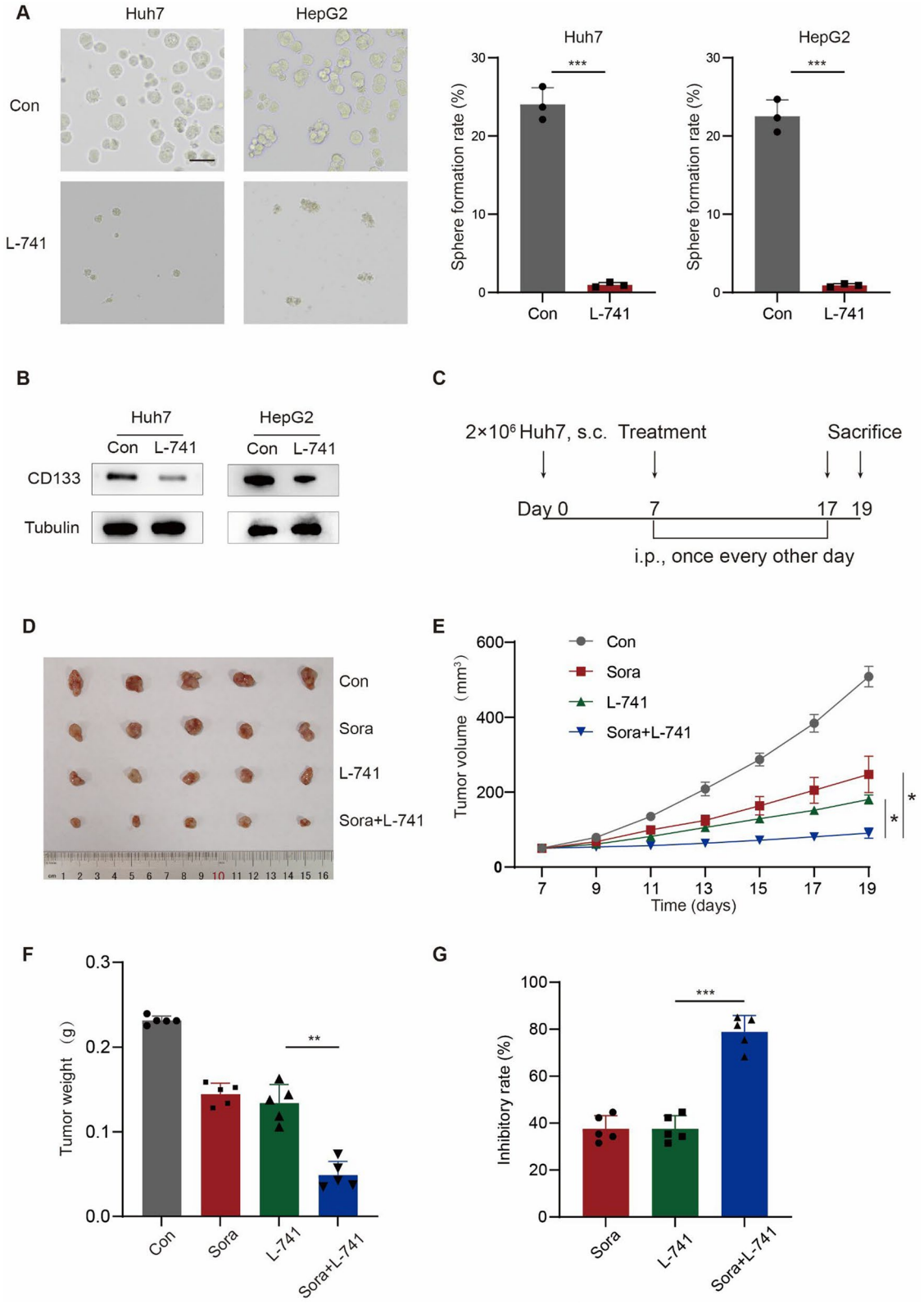
Fig. 5 **A** Sphere formation assays were performed on the control group and L-741626-treated Huh7 and HepG2 cell lines, and the sphere formation rate was calculated (scale bar: 50 μm). **B** Spheres from both the control and L-741626-treated groups were collected, and proteins were extracted for Western blot analysis to assess the expression levels of the tumor stemness marker CD133. **C** Schematic diagram of the construction and treatment of the Huh7 tumour-bearing mouse model. **D** Tumour tissue images from different treatment groups on Day 19 (n = 5). **E** Changes in tumour volume across each treatment group during the treatment period. **F** Tumour weights in different treatment groups. **G** Tumour inhibition rates of Huh7 tumour-bearing mice in each treatment group. The data are presented as means ± SDs; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



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ERK signalling pathway. These findings suggest a new potential therapeutic strategy for targeted intervention in HCC.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-025-00624-z.

Additional file1 (DOCX 632 KB)

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Author contributions
SJ, QZ, and XS conducted the experiments and drafted the manuscript. JS, PW, PL, and JG prepared the figures. YZ revised the manuscript. SJ, HZ, and XG initiated the study and provided guidance. All authors contributed to the manuscript and approved the final version. SJ, QZ and XS are co-first authors of this paper, contributing equally.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All methods were performed in accordance with relevant regulations and approved by the Ethics Review Committee of the School of Life Sciences of Zhengzhou University (approval number: ZZUIRB2020-54). Human hepatocellular carcinoma samples were obtained from the First Affiliated Hospital of Zhengzhou University with approval from the Institutional Review Board and informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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