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4-Hydroxyisoleucine inhibits tumor growth by triggering endoplasmic reticulum stress and autophagy

Peng Li [a,c,d,1], Yonghui Yang [a,c,d,1], Lu Qiu [a,c,d], Guangming Wan [e], Baomei Yuan [a], Yahong Wu [a,c,d], Yanfeng Gao [a,b], Guodong Li [a,c,d,*]

[a] School of Life Sciences, Zhengzhou University, Zhengzhou, 450001, China
[b] School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou, 510006, China
[c] Henan Key Laboratory of Bioactive Macromolecules, Zhengzhou University, Zhengzhou, 450001, China
[d] International Joint Laboratory for Protein and Peptide Drugs of Henan Province, Zhengzhou University, Zhengzhou, 450001, China
[e] The First Affiliated Hospital of Zhengzhou University, China

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ABSTRACT

4-Hydroxyisoleucine (4-HIL) is a non-protein amino acid that is able to reduce obesity and improve insulin sensitivity in mice, and recently emerged as a drug candidate against hypoglycemia. For the first time, we found that 4-HIL exhibits a potent anti-tumor activity in various cancer cell lines in *vitro* and in *vivo*. Most importantly, 4-HIL has no cytotoxic effect on normal or non-malignant cells. Proteomic data analysis revealed changes in endoplasmic reticulum stress (ERS) related protein and autophagy related protein. Western blot revealed that molecular components of the ERS pathway were activated, including phosphorylation of perk and EIF2a increased, while levels of GRP78 reduced, the cellular process of ERS potentially contributed to the activation of autophagy, Transmission electron microscopy revealed the formation of autophagic vesicles under 4-HIL treatment, and LC3B was increased. Meanwhile, activation of ERS inhibits intracellular protein synthesis rate, our results suggest that 4-HIL exhibits anti-tumor activity in various cancer cell lines by increasing ERS and triggering autophagy responses without causing damage to normal cells.

1Introduction

Breast cancer is one of the leading causes of cancer-related death in women worldwide. It is a highly invasive and malignant primary tumor with a high proliferation rate (Liang et al., 2020; Woolston, 2015). Despite advances in combined therapy, including radiotherapy and chemotherapy after surgical resection, the reported prognosis is poor, and the recurrence rate is high. Accordingly, more effective treatment approaches are needed.

An accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) leads to stress conditions. To mitigate such circumstances, stressed cells activate a homeostatic intracellular signaling network cumulatively called the Unfolded protein response (UPR), which orchestrates the recuperation of ER function (Rashid et al., 2015). Mild to moderate ERS-induced UPR signaling is seen as a compensatory mechanism, whereas severe and chronically prolonged ERS deteriorates cellular functions and switches from an adaptation program to apoptosis to remove irreversibly injured cells (Szegezdi et al., 2006; Rutkowski et al., 2006). In addition, under conditions of ERS, the pre-autophagosomal structure is assembled, and transport of autophagosomes to the vacuole is stimulated in an ATG protein-dependent manner.

Autophagy is a highly conserved cellular pathway critical for maintaining homeostasis and resistance to anticancer therapies (Klionsky et al., 2016). Tumor cells can enhance their ability to survive in the environment by moderate autophagy reaction. However, excessive autophagy can remove damaged organelles and excess protein in cells and degrade cell-specific proteins or cycle proteins, thereby preventing proliferation, invasion and metastasis of tumor cells, thus exerting a tumor-suppressive effect (Barnard et al., 2016; Guo et al., 2013; White, 2012). Activation of autophagy in tumors inhibits the proliferation of tumor cells and regulates immune responses in the tumor microenvironment (Rao et al., 2014; Ma et al., 2013). This is because dying tumor cells can induce effective immune recognition and anticancer immune

* Corresponding author. School of Life Sciences, Zhengzhou University, Zhengzhou, 450001, China.
E-mail address: ligd@zzu.edu.cn (G. Li).
[1] co-first authors.

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responses through autophagy responses (Ko et al., 2014; Michaud et al., 2011).

Major advances in monoclonal antibodies and controversial CAR-T cell therapies have made traditional small molecule drugs seem less competitive in recent years. However, traditional drug therapy is still necessary considering the economic benefit, malignant metastasis, and recurrence frequency (Torre et al., 2016). Due to their low toxicity and side effects, drugs derived from natural plants are often used as synergistic agents in cancer treatment (Ren et al., 2016). 4-HIL is a non-proteinogenic amino acid present in fenugreek seeds (Ogawa et al., 2011) that inhibits JNK, MAPK, and NF-KB activation by increasing AKT phosphorylation, thereby reducing blood glucose and cholesterol levels (Avalos-Soriano et al., 2016). However, Our study found that 4-HIL exerted excellent tumor suppressive ability in various tumor models. To the best of our knowledge, this is the first study to confirm the inhibitory effect of 4-HIL on 4T1 cell proliferation and anti-metastasis ability through in vitro and in vivo experiments. Here, we investigated the chemotherapeutic potential of 4-HIL in 4T1 cell populations in vitro and in vivo. It shows that 4-HIL may potentially be an effective therapeutic strategy for 4T1.

2Methods

2.1Cell lines and cultures

B16 and MC38 murine colorectal cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM). The 4T1 murine breast cancer cell line, the human umbilical vein endothelial cell line (HUVEC), and the MDA-MB-231 human breast cancer cell line were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. All media (GIBCO, Grand Island, USA) were supplemented with 10% fetal bovine serum (FBS) (BI, USA), 100 U/mL Penicillin and 100 U/mL Streptomycin (Solarbio, P1400 China). The cells were cultured under standard cell culture conditions (37 °C, 5%CO_2).

2.2Cell viability and proliferation assays

Possible cytotoxic effects of the indicated compounds on cancer cells were determined by an MTT assay. For this, MC38 cells were seeded into 96-well plates at a density of 3000 cells/well and allowed to adhere. Vehicle controls were treated with PBS as a negative control or with serial dilutions of the indicated compound at concentrations ranging from 0.5 to 30 mM. Following exposure for 24, 48 or 72 h, the cells were treated with 5 mg/mL MTT solution (SigmaM2003) dissolved in PBS and incubated at 37 °C for 4 h. Formazan crystals formed by the viable cells were dissolved in 150 μL DMSO(Solarbio, D8371 China) and MTT reduction was quantified by measuring the absorbance at 490 nm.

2.3Apoptosis assay

4T1 cells were seeded into 6-well plates at a density of 1×10^5 cells/mL (2 mL per well), followed by a 48 h incubation with 4-HIL dissolved in PBS. The cells were treated with RNase and Propidium iodide staining solution and incubated in the dark for at least 30 min at room temperature according to the manufacturer's instructions. Subsequently, the samples were analyzed by flow cytometry within 1 h. In order to quantify the apoptosis rate after treatment, the cells were stained with Annexin V-FITC and PI (MultiSciences #70-101-100) for 30 min at room temperature according to the manufacturer's instructions and the apoptosis rate was quantified by flow cytometry.

2.4Cell cycle

4T1 cells were seeded into 6-well plates at a density of 1×10^5 cells/mL (2 mL per well), followed by a 48h incubation with 4-HIL, The cells were treated with RNase and propidium iodide staining solution and incubated(MultiSciences #70-CCS012), in the dark, for at least 30 min at room temperature according to the manufacturer's instructions. Subsequently, the samples were analyzed by flow cytometry within 1 h.

2.5Colony forming assay

For the colony forming assay, the 4T1 cells were seeded into 6-well plates at a density of 5×10^4 cells per well. After cell attachment, culture media was added along with different concentrations of 4-HIL after which the cells were cultured for 1 week. The resultant colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Solarbio #G1061). All colonies were counted and each single colony was evaluated under the microscope.

2.6Western blot analysis

According to a protocol described previously, 20 μg protein of cell lysates were Western imprinted (Wang et al., 2016) and the membrane was co-incubated with the following antibodies: β-Actin (Abcam, #AC004, UK), eIF2α (Cell Signaling Technology, #ab242148, USA), P-EIF2α (Ser51, Cell Signaling Technology, #ab32157, USA) GRP78 (Cell Signaling Technology, #3177S, USA), PERK (Abcam,ab229912,UK), p-PERK(Affinity, DF-7576), LC3B (Abcam, ab192890, UK), Puromycin (Kerafast, EQ0001,USA). The band density was measured (Quantity One software) and normalized to β-Actin.

2.7Transmission electron microscopy

The cells were treated with 4-HIL for 48 h and then fixed with 4% glutaraldehyde for 2h and sent to a service provider for further analysis. The ultrastructure of 4T1 cells were photographed by transmission electron microscope (JEM-HT7700 model, Hitachi, Japan)

2.8Intracranial xenograft model and drug therapy

In vivo-experiments were performed with 6-week-old female C57BL/6J, BALB/c, and BALB/c nude mice (15–20g), respectively, and were purchased from Beijing Vitong Lihua Experimental Animal Technology Co., LTD. The animals were separately kept in ventilated cages in standard animal houses with a temperature of 26 °C and a humidity of 50% for 12 h without restriction of diet and drinking water. A total of 75 mice was included in the study. All animal experiments were conducted in strict accordance with the National Institute of Health guidelines for the care and use of laboratory animals (Kilkenny et al., 2010).

For tumor size measurements, six-week-old female BALB/c mice were subcutaneously (s.c.) injected in the right flank with 2×10^5 syngeneic B16 cells contained in 200 μL PBS. After one week, the tumor-bearing mice were randomized into control (normal saline) and a 4-HIL treatment group. The mice were treated with 200 μL PBS or 4-HIL dissolved in PBS through daily intraperitoneal (i.p.) injection for two weeks. C57BL/6 mice were subcutaneously injected in the right flank with 2×10^5 syngeneic B16 or MC38 cells. Eight days later, tumor-bearing mice were randomly grouped and treated with normal saline or 150 mg/kg 4-HIL by daily intraperitoneal injection for two weeks. For the 4T1 orthotopic breast tumor model, 1×10^5 4T1 cells contained in 100 μL PBS were injected into the fourth mammary fat pad of female BALB/c mice. Beginning at day 7, 4-HIL (50 or 150 mg/kg in 200 μL normal saline) was administered to the mice by daily i.p. injection for a period of 23 days. Metastatic nodules in each lung were counted on day 30 following tumor cell injection. Tumor sizes were measured using a digital caliper and tumor volumes were calculated with the formula:

$$V = 1 / 2 \times a\ (\text{length}) \times b\ (\text{width}) \times c\ (\text{height})$$

2.9Immunohistochemistry

Hematoxylin and eosin (H&E) stain was purchased from the Wuhan Servicebio Technology Co. Ltd. (Wuhan, China). At this point, we would like to strengthen that in all animal experiments normal saline was used as the carrier and the route and dosage of administration were performed according to Jiali Yang et al. (2020).

2.10Statistical analysis

Statistical analysis using was performed with paired or unpaired Student's t-test for analyzing differences between groups. Data were represented as means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

3Results

3.1Effect of 4-HIL on 4T1 cell proliferation in vitro

To verify the inhibitory effect of 4-HIL on the viability of cancer cells, 4T1 cells were treated with different concentrations of 4-HIL for different time. Decreases in cell viability (60%–80%) relative to untreated cells were statistically significant in 10 mM 4-HIL for different time (Fig. 1A). To further test the toxicity of 4-HIL on cell growth, we conducted cell colony formation assays to determine the colony formation ability of 4T1 cells. Crystal violet staining results showed that 4-HIL reduced the colony formation of 4T1 cells, and the effect of 4-HIL on colony formation was dose-dependent (30%–40%, 4 Mm–10mM) (Fig. 1B). Next, we verified the effect of 4-HIL on tumor metastasis and examined the effect of 4-HIL on cell migration by the trans-well assay. The migration ability of the 4T1 cell line was inhibited (30%–50%, 4 Mm–10mM) (Fig. 1C), and the same results were found during the scratch assays (25%–50%, 4 Mm–10mM) (Fig. 1D). These results indicate that 4-HIL can inhibit the proliferation and migration of 4T1 cells in vitro.

3.2Effect of 4-HIL on 4T1 cell protein synthesis

KEGG enrichment pathway analysis revealed enrichment of different pathways, including amino acid transport, metabolism and stress response pathways (Fig. 2A). Proteomics data analysis found that 4-HIL increased non-essential amino acids (NEAA) biosynthesis, amino acid transport-related proteins expression (Fig. 2B). Further studies showed that 4-HIL inhibited protein synthesis (Fig. 2C), while amino acid transport and stress reaction pathways were upregulated, most likely due to a compensatory elevation caused by intracellular deficiency of amino acids. The endoplasmic reticulum (ER) is an important site for protein synthesis, and proteomic data analysis revealed changes in multiple signal proteins during the ERS response (Fig. 2B). Western blot analysis was performed to identify the key participants of ERS. After 4-HIL treatment, we observed decrease in GRP78 expression and increased perk and eIF2α phosphorylation. To confirm that 4-HIL is synthesized by ERS suppressor protein, we co-incubated the drug with the ERS inhibitor 4PBA and then measured the protein synthesis rate. It is widely acknowledged that EIF2 α is a key factor affecting mRNA translation

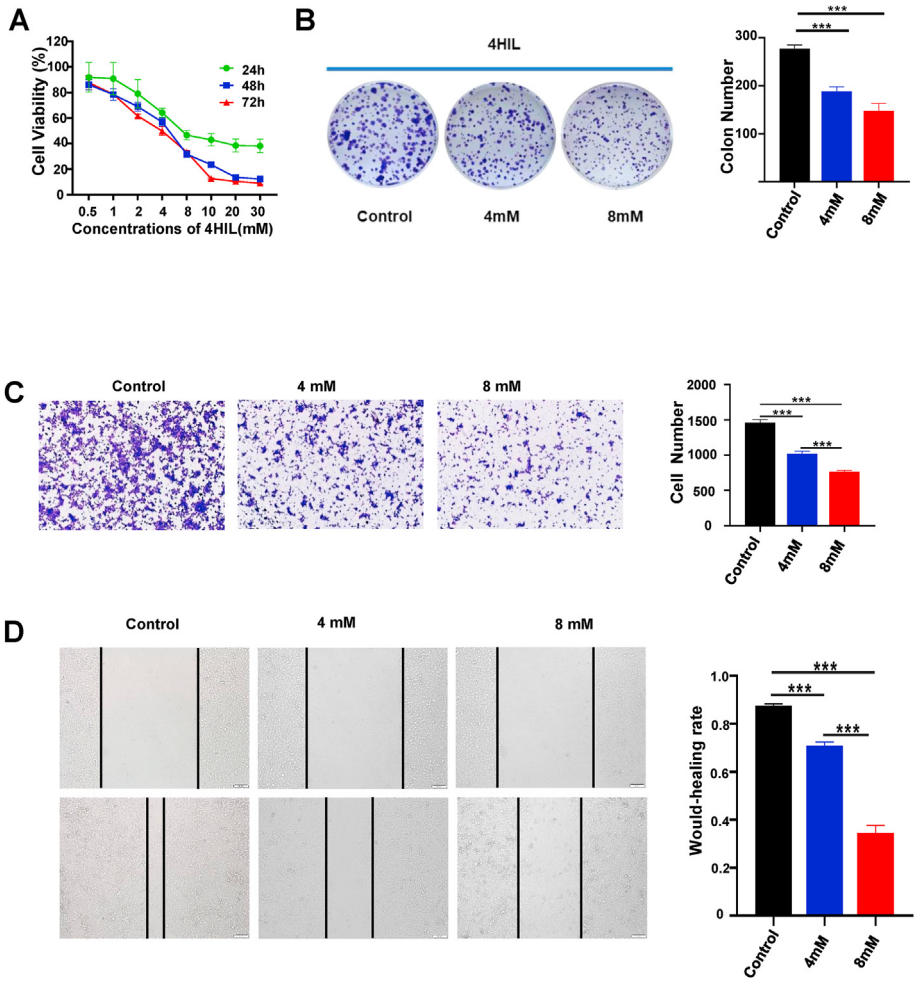

Fig. 1. Effects of different concentrations of 4-HIL on the proliferation of 4T1 cells after treatment with different time (A) Effects of different concentrations of 4-HIL on the Colony formation of 4T1 cells, (B) invasion of 4T1 cells and migration of 4T1 cells,(C) All values are mean ± SD; (n = 3) *P < 0.05, **P < 0.01, ***P < 0.001.

during ERS. We found that the phosphorylation level of eIF2α was recovered in the presence of 4PBA (Fig. 2D), and the protein synthesis rate was recovered (Fig. 2E). These results suggest that 4-HIL can induce ERS and inhibit protein synthesis.

3.3Impact of 4-HIL on autophagy and apoptosis

It is well-established that ERS is closely related to autophagy (Ylä-Anttila et al., 2009; Yorimitsu and Klionsky, 2007), the lack of nutrients can activate autophagy to synthesize biological macromolecules by degrading intracellular proteins and lipids. To determine whether 4-HIL induced autophagy in 4T1 cells, transmission electron microscopy (TEM) was used to visualize autophagy vesicles. TEM analysis showed that the number of autophagosomes increased after 4T1 cells were treated with 8 mM 4-HIL for 48h (Fig. 3A). LC3B is a well-known autophagy marker, and western blot analysis prepared from lysed cells showed a dose and time-dependent increase of LC3B in 4T1 cells (increases of 80% relative to untreated cells) (Fig. 3B). Autophagy flux was further used to evaluate autophagy induced by 4-HIL. We co-treated 4T1 cells with 4-HIL and autophagy inhibitors Lsy05 and Spautin-1, which blocked the downstream and upstream steps of the process, respectively. Western blot analysis showed that co-incubation of cells with 4-HIL and Lsy05 for 24h increased the ratio of LC3B-II/LC3B–I(30%, 4HIL + Lsy05 vs Lsy05) (Fig. 3C). Co-incubation of 4-HIL and Spautin-1(S1) also led to

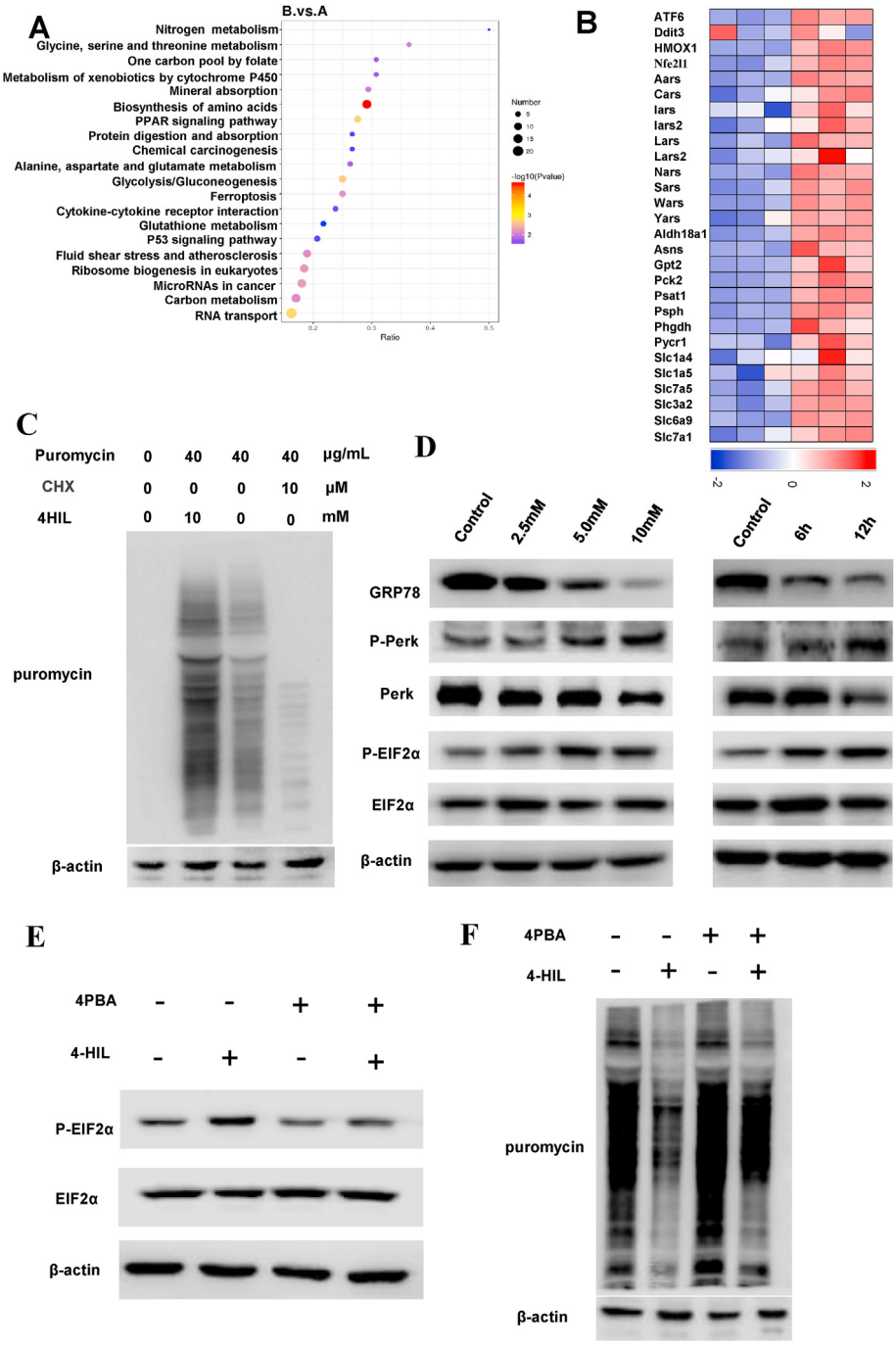

Fig. 2. Proteomics Enrichment Analysis of 4T1 cells treated with 4-HIL or PBS for the indicated time points (A) Heatmap of expression of key genes within protein synthesis signature.(B) Effect of 4HIL on intracellular puromycin levels (C) and the levels of GRP78, P-perk, perk, P-EIF2α, EIF2α and β-Actin,(D) Effect of 4-HIL or 4-PBA on levels of P-EIF2α, EIF2α , β-Actin (E) and puromycin (F). All values are mean ± SD; (n = 3) *P < 0.05, **P < 0.01, ***P < 0.001.

an increase in the ratio of LC3B-II/LC3B–I (40%, 4HIL + S1 vs S1) (Fig. 3D). These results showed that 4-HIL induced autophagy. In addition, electron microscopy showed typical signs of apoptosis, including nuclear shrinkage (increases of ~100% relative to untreated cells) (Fig. 3E). These results suggest that 4-HIL induces autophagy responses and apoptosis responses.

3.4Impact of 4-HIL on tumor in vivo

To verify whether 4-HIL exerts an antitumor effect in vivo, we conducted a series of experiments using a homogenic tumor model. The results showed that 4-HIL inhibited the growth of 4T1 tumors without reducing the body weight of the mice (Fig. 4A–E) and improved the survival rate of tumor-bearing mice (50 Days vs 39 Days, 150 mg/kg vs Control) (Fig. 4F). An important characteristic of 4T1 breast cancer cells is their ability to metastasize. Therefore, mice were sacrificed 30 days after inoculation to check for lung metastases. We found that a large number of tumor nodules were observed in the lungs of the control mice, while fewer nodules were observed in the lungs of 4-HIL treated mice (10 vs 25, 150 mg/kg vs Control) (Fig. 4G). Moreover, 4-HIL treatment enhanced the recruitment of $CD8^+$ in the 4T1 tumor microenvironment (increases of 1300% relative to untreated cells) (Fig. 4H). In addition, the marker for autophagy, LC3B, was increased in tumor cells, while GRP78, a marker for ERS response was decreased(Fig. 4I).The above findings demonstrate that 4-HIL exerts excellent antitumor effects in vivo.

3.5Effects of 4-HIL on a variety of tumors in vivo

Considering that endoplasmic reticulum stress and autophagy flux increase during the development of several tumor types, we sought to ascertain whether 4-HIL could induce the death of other types of tumor cells. Finally, we found that 4-HIL could inhibited cell proliferation in vitro in three non-breast cancer cell lines. Decreases in cell viability (60%–90%) relative to untreated cells were statistically significant in 10 mM 4-HIL for different time (Fig. 5A). Meanwhile, the results showed that 4-HIL inhibited the growth of 4T1 tumors(inhibition of 50%–60% relative to untreated cells)without reducing the body weight of the mice(Fig. 5B–C).

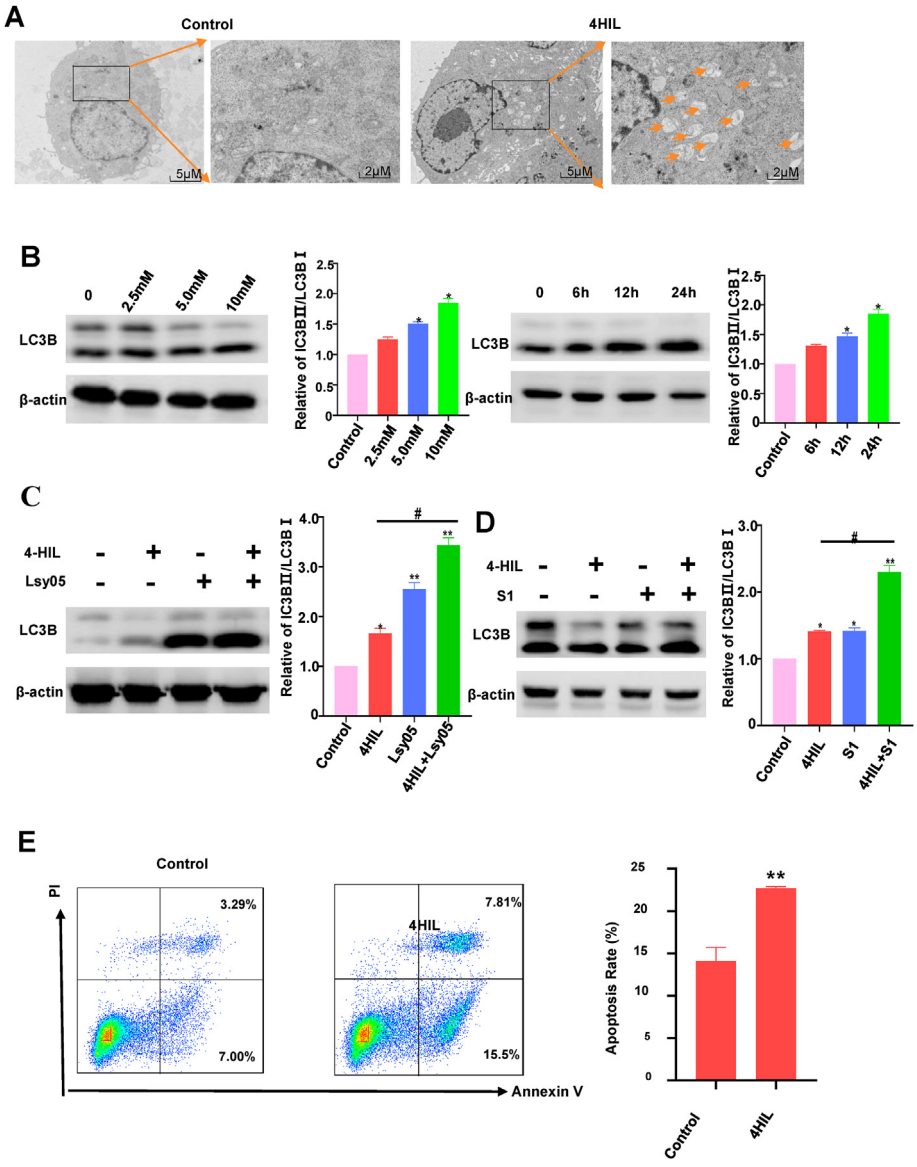

Fig. 3. Effect of 4HIL on the number of intracellular autophagosomes (A). Changes in LC3BII/LC3BI ratio in 4T1 cells after treatment with different concentrations of 4HIL or 10 mM 4HIL for different times (B). Effect of 4HIL(10 mM) or Lsy05 (10 μM) (C) and S1(10 μM) (D) on intracellular the levels of LC3BII/LC3BI. All values are mean ± SD; (n = 3) *P < 0.05, **P < 0.01, ***P < 0.001. #P < 0.05.

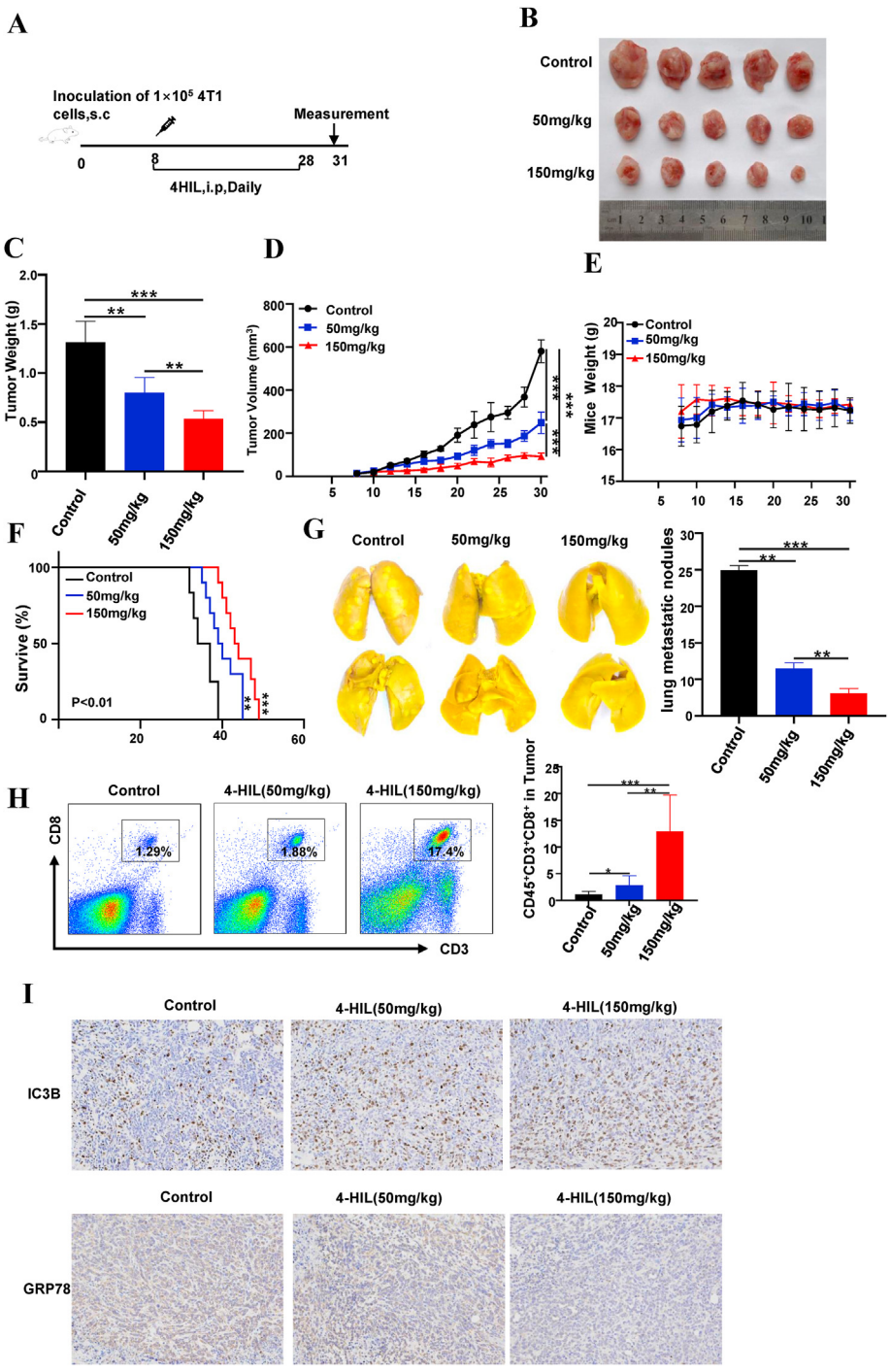

Fig. 4. Schematic diagram of animal model (A) and Tumor photogram (B). Effects of 4HIL on tumor weight (C), volume (D) and metastasis (G), body weight (E) and survival of mice. (F). Effect of 4HIL on $CD8^+$ level in tumor microenvironment (H) and the levels of GRP78, LC3B in tumor (I). All values are mean ± SD; (n = 5) *P < 0.05, **P < 0.01, ***P < 0.001.

3.64-HIL safety assessment

The toxic and side effects of antitumor drugs have attracted much attention during the drug screening process. To assess whether 4-HIL exerts toxic and side effects on normal cells, we first detected the effects of different concentrations of 4-HIL on the proliferation of normal HUVEC, 293T and 3T3L1 cells in vitro at different times by MTT assay. We found that 4 HIL did not affect the cell proliferation (Fig. 6A), and the immune cells taken from the spleen after 4 HIL incubation, although the drug does not promote cell proliferation, but the impact on immune cell proliferation is not (Fig. 6B), the body after the experiment, we for the apple of mice spleen kidney H&E staining. The results showed normal histological morphology (Fig. 6C). These results indicated that 4-HIL is not toxic to normal cells and does not cause organ damage while exerting an antitumor effect.

4Discussion

Drug therapy has become a mainstay of tumor treatment; however, it is associated with high toxicity rates, emphasizing the need for highly

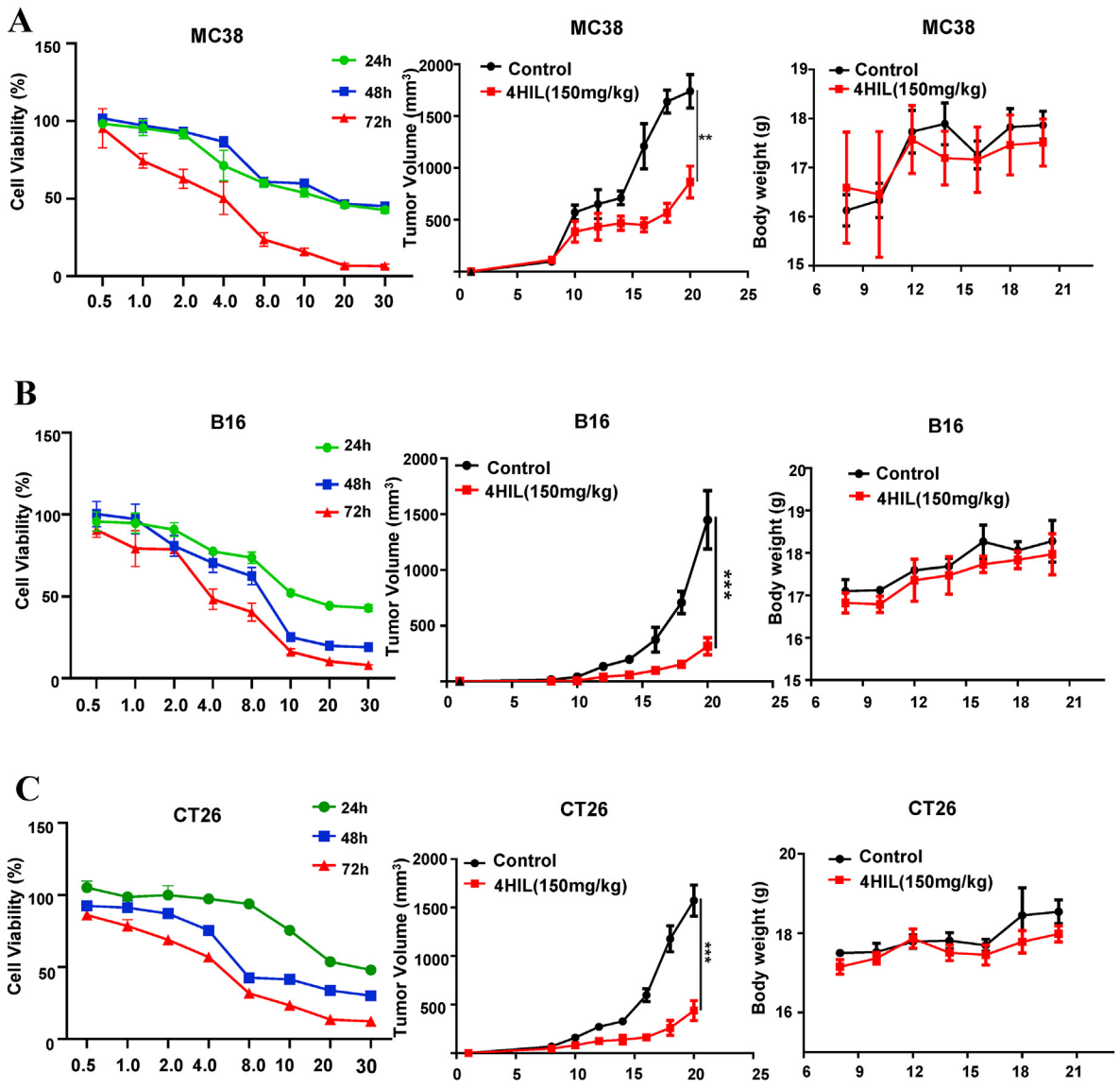

Fig. 5. Effect of 4HIL on proliferation of MC38 (A), B16 (B) and CT26 (C) in vitro and in vivo. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 50 μm.

effective and low toxicity anticancer drugs at the clinical level. This study corroborated that 4-HIL possesses strong antitumor and anti-metastasis ability both in vivo and in vitro and exerts no obvious toxic effect on normal cells and mice at an effective therapeutic dose, suggesting that 4-HIL has huge prospects as an antitumor drug with high efficiency and low toxicity. In this study, we found that 4-HIL could inhibit the proliferation of a variety of tumors in vitro and vivo. Meanwhile, our results suggest that 4-HIL exhibits anti-tumor activity in various cancer cell lines by increasing ERS and triggering autophagy responses without causing damage to normal cells. It is widely acknowledged that the endoplasmic reticulum is an important site for protein synthesis in cells. When amino acid deficiency occurs, ERS activates UPR signaling networks to reduce protein synthesis to counter the ERS and restore ER homeostasis, thereby promoting cell survival (Hwang and Qi, 2018; Sitia and Braakman, 2003). EIF2α is a key protein in regulating mRNA translation and the autophagy response by mediating the expression of downstream transcription factor C/-EBP homologous protein (CHOP), an important mediator between autophagy and ERS (Zecchini et al., 2019; Yang et al., 2021).

Autophagy is a process that degrades macromolecules and organelles and provides nutrients to cells (Meijer and Codogno, 2004). It is activated under stress conditions and is considered a self-protective mechanism derived from cells under adverse conditions. However, under sustained stress conditions, autophagy may lead to cell death (Scarlatti et al., 2009). In this study, nuclear shrinkage was observed, an important feature of cell apoptosis (Saraste and Pulkki, 2000; Jeong and Seol, 2008). Therefore, we speculated that the autophagy state could determine the degree of cell apoptosis.

Furthermore, autophagy represents an important cellular intrinsic mechanism and shapes cellular immunity (Levy et al., 2017). For example, autophagy during anthracycline treatment promotes DAMP release, and autophagy-deficient mouse colonic tumors exhibit reduced T cell recruitment and activation (Michaud et al., 2011, 2014; Wang et al., 2013), which can explain the increased CD8+T cell recruitment in the 4T1 tumor microenvironment during 4-HIL treatment.

Our results showed that 4-HIL exerts no significant toxic effects on normal cells and mice and has antitumor activity, suggesting that 4-HIL can selectively target tumor cells. This finding may be accounted for by the fact that, compared with normal cells, the basal level of autophagy is higher in tumor cells, and nutrient acquisition and metabolism pathways are reprogrammed in tumor cells (Dower et al., 2018), making them more sensitive to nutrient deprivation. This may be a major factor accounting for 4-HIL-specific antitumor activity.

However, there are some limitations to our study. First, although

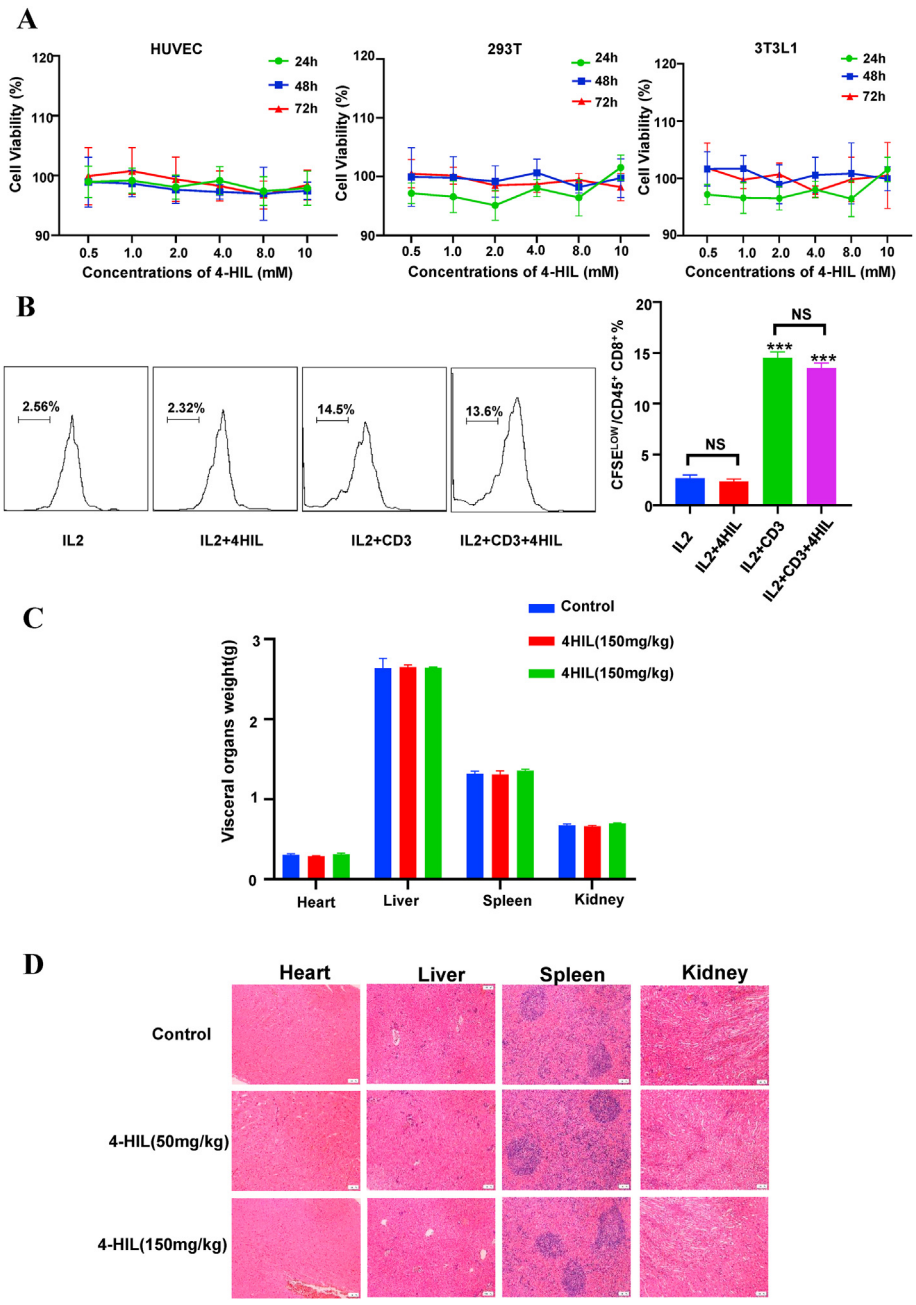

Fig. 6. Effect of 4HIL on the proliferation of HUVEC, 293T, 3T3L1 (A) and mouse spleen cells (B) in vitro. Histopathologic analysis of HE-stained tissue section from heart, liver, spleen, lung and kidney of tumor-bearing mice after the indicated treatment. (C) Scale bar: 50 μm.

autophagy and endoplasmic reticulum stress affect the proliferation and metastasis of tumor cells, it is unknown whether 4-HIL affects the proliferation and metastasis of tumor cells in other ways. Moreover, the effect of drugs on immune cell recruitment through autophagy has not been validated in mouse models.

In conclusion, we demonstrated that 4-HIL inhibits tumor growth by endoplasmic reticulum stress and autophagy response. Considering the inhibitory effect of 4-HIL on tumor growth and metastasis in mice and the lack of toxicity to normal cells, this study provides compelling evidence to support the future evaluation of 4-HIL in clinical trials and reinforces the idea that drugs can be highly selective for tumor cells.

Ethics approval and consent to participate

All mice experimental procedures were approved by the Ethics Committee of Zhengzhou University.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

GL, PL and YY conceived the research and designed the experiments; YY, PL conducted the experiments, acquired and analyzed the data with the critical assistance from YG, LQ, BY; all authors contribute to writing the manuscript; Prof.YG, GW revised the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Peng Li: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. **Yonghui Yang:** Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. **Lu Qiu:** Data curation. **Guangming Wan:** Visualization, Investigation. **Baomei Yuan:** Visualization, Investigation. **Yahong Wu:** Resources, Supervision. **Yanfeng Gao:** Funding acquisition, Resources. **Guodong Li:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relation ships that could have appeared to influence the work reported in this paper.

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Not applicable.

List of abbreviations

MTT	3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide;
4-HIL	4-Hydroxyisoleucine
AAR	amino acid Starvation response
BCAA	branched-chain amino acids
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ile	isoleucine; Leu: leucine; Val: valine
AA	amino acid

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