Original Research

Inhibitory effect of hydnocarpin D on T-cell acute lymphoblastic leukemia via induction of autophagy-dependent ferroptosis

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Impact statement

T-ALL is an aggressive clonal disease characterized by the abnormal proliferation of lymphocytes which requires novel therapy. Natural anticancer agents emerge as potential multi-target cancer therapy with low cytotoxicity in normal cells. Here, we investigated the efficacy of hydnocarpin D, a flavonolignan first isolated from *Hydnocarpus wightiana*, in T-ALL. Our study discovered that hydnocarpin D suppressed T-ALL proliferation *in vitro*, via induction of cell cycle arrest, subsequent apoptosis, and cytotoxic autophagy. Interestingly, the activation of autophagy triggered ferroptosis, a novel form of cell death characterized by the accumulation of iron and lipid peroxidation. Our results proposed hydnocarpin D as a multi-target agent which triggered diverse cell death processes and displayed potent activity against T-ALL cells *in vitro*.

Abstract

Hydnocarpin D (HD) is a bioactive flavonolignan compound that possesses promising antitumor activity, although the mechanism is not fully understood. Using T cell acute lymphoblastic leukemia (T-ALL) cell lines Jurkat and Molt-4 as model system, we found that HD suppressed T-ALL proliferation *in vitro*, via induction of cell cycle arrest and subsequent apoptosis. Furthermore, HD increased the LC3-II levels and the formation of autophagolysosome vacuoles, both of which are markers for autophagy. The inhibition of autophagy by either knockdown of ATG5/7 or pre-treatment of 3-MA partially rescued HD-induced apoptosis, thus suggesting that autophagy enhanced the efficacy of HD. Interestingly, this cytotoxic autophagy triggered ferroptosis, as evidenced by the accumulation of lipid ROS and decrease of GSH and GPX4, while inhibition of autophagy impeded ferroptotic cell death. Our study suggests that HD triggers multiple cell death processes and is an interesting compound that should be evaluated in future preclinical studies.

Keywords: Hydnocarpin D, T-ALL, apoptosis, ferroptosis, autophagy

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Introduction

T-ALL is an hematological malignancy caused by the uncontrolled transformation of T-cell lymphoblastoid cells, which accounts for about 15% of total ALL, with a total incidence rate of 25%.[1,2] In 2017, 5970 new cases were reported in the United States, with 1400 cases died of this cause.[3] T-ALL patients in clinical manifestations are usually immature T cells, diffuse infiltration of lymphoblastic cells, high leukocyte counts, mediastinal masses with pleural effusion. With novel treatment options including improved glucocorticoids, asparaginase, and central nervous system guided-treatments proposed in recent years, the outcome of T-ALL has been significantly improved, with a cure rate of 75% in children and 50% in adults.[1,4] However, due to the development of chemotherapy resistance and refractory relapse, adult T-ALL remains a challenge, with a five-year survival rate lower than 45%–55%.[5] Therefore, new drug therapies for T-ALL is still imminent.

Multi-target cancer therapy has recently been advocated due to its abolition of advanced cancer, thus prolonging disease-free survival. The use of some natural anticancer agents can somewhat resolve this problem because they can target diverse processes, including cell cycle, apoptosis, and autophagy, while presenting lower/no cytotoxicity for normal cells.[6] The genus *Hydnocarpus* (*Flacourtiaceae*) is mainly used as a treatment for leprosy and other skin diseases in traditional Chinese medicine. It was also used for helminth infection, blood diseases, constipation, and inflammation.[7] Hydnocarpin D (HD) is a flavonolignan first isolated from *Hydnocarpus wightiana* in India in 1973,[8] and it also exists in herbs such as *Malloy* and *Bruceajavanica*.[9] Studies have found that HD possesses good free-radical scavenging activity, bacteriostatic ability, and antineoplastic activity both *in vitro* and *in vivo*, without apparent toxicity *in vivo*.[9,10] Namely, HD can suppress the proliferation of human colon cancer cells by inhibiting Wnt/beta-catenin signal transduction,[11] and the combination of HD can significantly enhance the inhibitory effect of vincristine on B-ALL 697 cells.[12] Nevertheless, few studies in the literature explored the role and exact mechanism of HD in T-ALL.

Autophagy[13] is a conservative self-degradation process, which is responsible for the cellular maintenance and the recycling of the breakdown products.[14] Over the last few years, numerous papers have emphasized the dichotomous role played by autophagy in acute leukemia cells. It can either promote or inhibit cell growth and survival, depending on the context.[15] Silibinin, a well-studied flavonolignan isolated from milk thistle seeds with structural similarity as that of HD,[16] is reported to possess a significant anti-tumor efficacy in a variety of cancer models by induction of cytotoxic autophagy.[6] However, whether HD is equally capable of inducing autophagy in T-ALL and its underlying mechanisms are yet to be clarified.

Therefore, this study aims to evaluate biological activities of HD *in vitro*, and explore its molecular mechanisms, focusing on the autophagic pathway. Our results showed that HD inhibited T-ALL cell proliferation *in vitro* by promoting apoptosis, cell cycle arrest, and autophagy-dependent ferroptosis. Thus, our study implicate that HD is a pleiotropic compound with multitarget activity and provides a novel theoretical basis for further in-depth investigation of HD.

Materials and methods

Chemicals and reagents

HD is an isolated compound provided by Dr. Guozheng Huang (Anhui University of Technology, Anhui, China) and was dissolved by DMSO. The purity was further confirmed by HPLC to be over 98%. MTT, DAPI, and acridine orange (AO) were obtained from Sigma (MO, USA). C11-BODIPY, Chloroquine (CQ), Ferrostatin-1 (Fer-1), and 3-MA were acquired from Selleck (TX, USA). Antibodies against ATG5 (#2630), PARP (#9532), cleaved caspase-3 (Asp175, #9664), Bcl-2 (#2870), SQSTM1/p62 (#5114), c-Myc (#9402), LC3B (#3868 s), ATG7 (#8558), Bcl-XL (#2764), FTH1 (#4393), Beclin-1 (#3738), p-cdc2 (#4539), cdc2 (#9116), GAPDH (#5174), β-tubulin (#2128) were purchased from Cell Signalling Technologies (MA, USA). And antibodies against p27 (sc-1641) and ACSL4 (sc-365230) were obtained from Santa Cruz (CA, USA), while antibodies against CDO1 (ab232699) and GPX4 (ab125066) were purchased from Abcam (Cambridge, UK).

Cell culture

T-ALL cell lines Jurkat, Molt-4, and human lymphocytic cell line CAM-191 were acquired from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured with RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA). Penicillin (100 U/mL) and streptomycin (100 ug/mL) were also added. Cells were split before reaching the density of 3×10^6 cells/mL and maintained at a concentration between 1×10^5 and 1×10^6 viable cells/mL. Cells were used before 10 passages.

Cell viability assay

HD cytotoxicity was determined by MTT analysis. The cells were plated in 96-well at a density of 1.0×10^4 cells per well and exposed to various concentrations of HD for 48 h. Equal volume of MTT solution (0.5%) was added to each well at the end of the treatment. After incubating for another 4 h, DMSO was used to dissolve formazan crystals, and then the plates were detected using a microplate reader (Bio-Tek, CA, USA) at 570 nm.

Detection of cell apoptosis

Apoptosis analysis was performed by double staining with Annexin V-FITC/7-AAD (BD Biosciences). Briefly, T-ALL cells were seeded in six-well plates (1×10^6 cells per well) and incubated for 48 h. The collected cells were resuspended and stained with Annexin V-FITC (5 μL) and 7-AAD (5 μL) in dark for 10 min and detected by flow cytometry using Guava Easy Cytometer (Merck, Germany).

Cell cycle analysis

Jurkat and Molt-4 cells were plated into six-well plates at 1×10^6 cells per well. After treated with 7.5 or 15 μM HD for 48 h, cells were added to 70% ethanol for fixation and stored at −20°C for 12 hours. After staining with 5 μg/mL PI, cells were then analyzed by Guava Easy Cytometer, and Modfit software was used for DNA quantification.

Acridine orange (AO) staining

Briefly, 5×10^5 cells/well were seeded in six-well plates. After incubation with 7.5, 15 or 30 μM HD for 48 h, cells were stained with AO (5 μg/mL) for 15 min. Images were visualized using a Nikon fluorescence microscope.

Glutathione synthesis and lipid peroxidation analysis

For GSH levels detection, cells were seeded in six-well plates at 1×10^6 cells/well and incubated with various concentrations of HD for 24 h. The GSH content of T-ALL cells

was analyzed by GSH detection kit (No. S0053, Beyotime, China).

After treated with HD for 24 h, cells were dyed with C11-BODIPY (5 μM) and DAPI (5 μM) for another 30 min and imaged at ×40 magnification using a Nikon fluorescence microscope.

For flow cytometry analysis, T-ALL cells were incubated with different concentrations of HD or DMSO as blank control for 30 min. After further incubation with 5 μM C11-BODIPY in dark for 30 min, cells were filtered into single-cell suspensions and analyzed on a Guava Easy Cytometer equipped with a 488 nm laser for excitation.

RT-PCR

Total RNA was extracted from T-ALL cells using Trizol agent (Invitrogen, Carlsbad, CA). Reverse transcription was carried out according to PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) instructions. After cDNA was mixed with SYBR Green (Bio-Rad, Berkeley, CA), quantitative PCR reactions were performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Triplicate samples per condition were analyzed. Then data were analyzed by $2-\Delta\Delta Ct$ method and compared with GAPDH for normalization of the samples. The primers used were as follows

GPX4-F: GAGGCAAGACCGAAGTAAACTAC
GPX4-R: CCGAACTGGTTACACGGGAA
GAPDH-F: GGAGCGAGATCCCTCCAAAAT
GAPDH-R: GGCTGTTGTCATACTTCTCATGG

Western blotting analysis

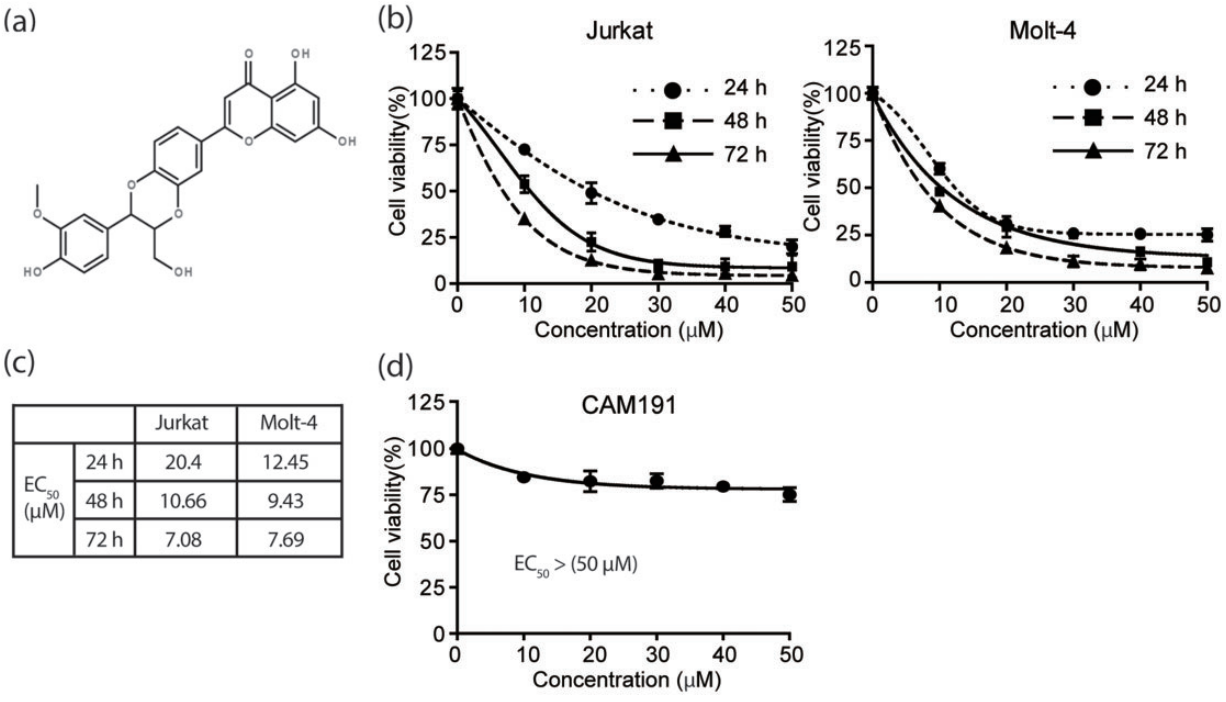
Jurkat and Molt-4 (2×10^6) cells were seeded in 6 cm^2 dishes. Cells were collected after indicated treatment following lysing with RIPA buffer. BCA protein detection kit (Beyotime, Jiangsu, China) was used to measure total protein concentration. Protein sample (40 μg) were separated by 10% or 12% SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked and then incubated with the corresponding primary antibody overnight at 4°C. The next day, after three washes with TBST and incubated with corresponding second antibodies for 2 h at 20°C, the signals on the membrane were visualized by ECL (BIO-RAD, USA).

DAPI staining assay

Cells were plated in six-well plates at a density of 5.0×10^5 cells per well. After indicated treatments, T-ALL cells were washed with PBS following staining with DAPI (5 μM) for 15 min. The stained cells were photographed and counted under a fluorescent microscope (Nikon, Tokyo, Japan).

Knockdown of ATG5 and ATG7

For the downregulation of ATG5 and ATG7 expression, shRNAs targeting either ATG5 or ATG7 (purchased from GeneCopoeia, Cat. No. HSH065365 and HSH061981) with the oligomer-Lipofectamine™ 2000 complex was added to the medium. The cells were harvested after another 48 h. Cells added with scramble shRNA were used as controls.



		Jurkat	Molt-4
EC_{50} (μM)	24 h	20.4	12.45
	48 h	10.66	9.43
	72 h	7.08	7.69

Figure 1. HD suppresses the proliferation of T-ALL cells. (a) Chemical structure of HD. (b) T-ALL cells were exposed to various concentrations of HD (0, 10, 20, 30, 40, 50 μM) for 24, 48 and 72 h. MTT analysis was used to determine the cell viability. (c) EC_{50} values of HD in T-ALL cell lines were calculated by GraphPad Prism. The data represent three independent experiments. (d) Normal human lymphocyte cell CAM-191were treated with different concentrations of HD (0, 10, 20, 30, 40, 50 μM) for 48 h. The cell viability was examined by MTT assay.

Statistical analysis

EC_{50} values were calculated by GraphPad Prism Software version 5.01 (CA, USA). Independent experiments were conducted three times. Data were presented as mean ± SD. Statistical significance was analyzed using Student's *t*-test. The statistical significance was defined as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

HD suppresses the proliferation of T-ALL cells in vitro

To ascertain whether HD can inhibit proliferation of T-ALL cells *in vitro*, we first treated Jurkat and Molt-4 with various concentrations of HD (Figure 1(a)), ranging from 10 to 50 μM for up to 72 h. MTT analysis was performed to measure the cell viability. We found that HD can suppress the proliferation of both cell lines in a concentration-dependent manner (Figure 1(b)). The IC_{50} values ranged from 7 to 20 μM (Figure 1(c)). To determine whether HD specifically inhibits T-ALL cells, we investigated the effect of HD on CAM-191, a normal human lymphocyte cell line. MTT assay confirms that HD shows no significant toxicity to CAM-191 (Figure 1(d)). These results suggested that HD can specifically inhibit T-ALL cell proliferation *in vitro.*

HD induces G2/M phase arrest in T-ALL cells

In order to determine whether the decrease of cell viability of HD-treated T-ALL cells is attributed to the induction of cell cycle arrest, flow cytometry was applied to analyze the impact of HD on cell cycle distribution. The results showed that the treatment of either 7.5 or 15 μM HD significantly increased the portion of cells arrested at the G2/M phase, as compared with the control group (Figure 2(a) and (b)). Concurrently, HD treatment elevated the expression of p-cdc2 and p27, while decreased oncogenic c-Myc expression (Figure 2(c)). Together, these results showed that HD

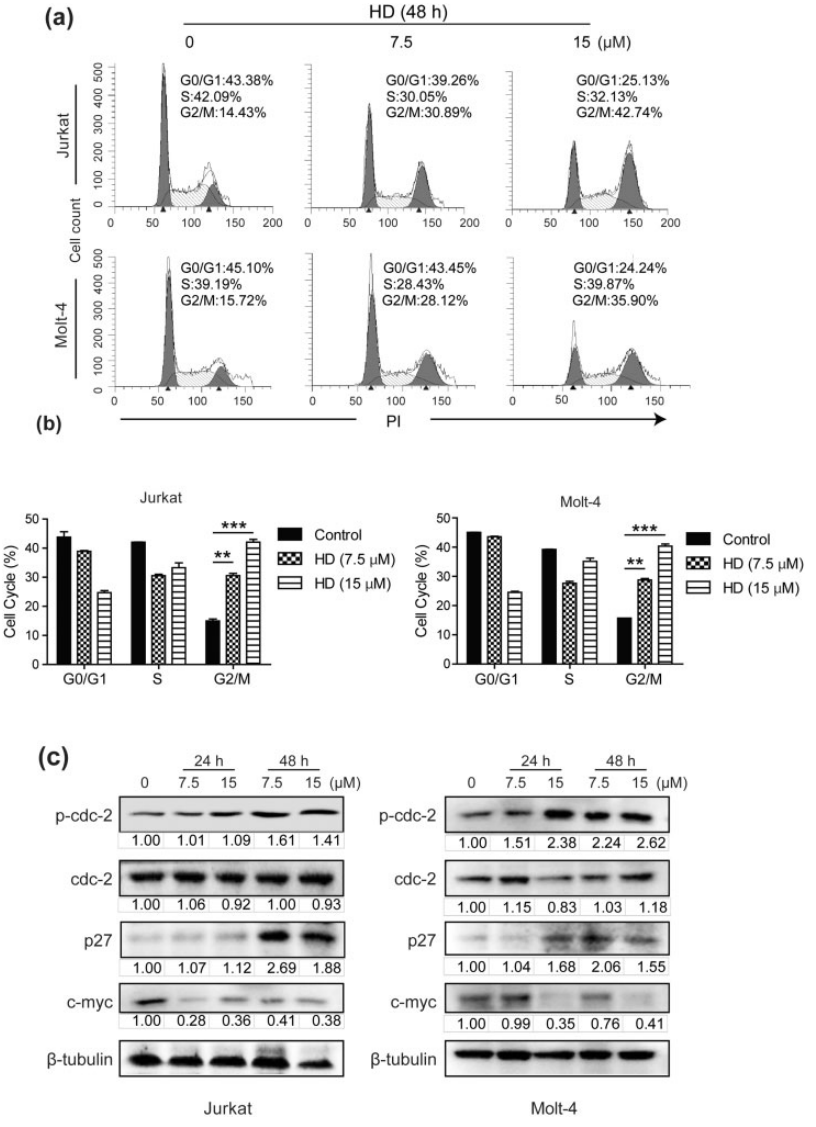


Figure 2. HD induces T-ALL cell cycle arrest at the G2/M phase. (a) Both cells were treated with HD (0, 7.5, and 15 μM) for 48 h, followed by PI staining and flow cytometry analysis of cell cycle distribution. (b) Cell cycle distribution analysis of (a). HD vs. control, $^{**}p < 0.01$, $^{***}p < 0.001$. (c) The expression levels of cell cycle-related proteins after treatment with HD (0, 7.5, and 15 μM) for 24 and 48 h, with β-tubulin as loading control.

could arrest T-ALL cells at the G2/M phase, thereby reducing T-ALL cell viability.

HD induces apoptosis in T-ALL cells

To investigate whether apoptosis is involved in HD's anti-leukemic activity, we treated both cell lines with 7.5, 15, or 30 μM HD for 48 h. Flow cytometry were carried out to detect the percentage of apoptotic cells. As shown in Figure 3(a) and (b), HD induced concentration-dependent apoptosis in both T-ALL cell lines, with apoptosis rate reaching 66.25% and 52.14% in Jurkat and Molt-4 cells, respectively. For further confirmation, we monitored the change of nuclear morphology. As expected, after treatment with HD for 48 h, cell apoptosis-related phenotypes including chromatin condensation was significantly increased (Figure 3(c)). In addition, HD increased the cleavage and activation of PARP and caspase-3, while Bcl-2 and Bcl-xL were considerably downregulated (Figure 3(d)). Altogether, the results clearly implied that HD induced caspase-dependent apoptosis in T-ALL cells.

HD induces cytotoxic autophagy in T-ALL cells

Autophagy and apoptosis are two vital forms of programmed cell death, both of which participate in the regulation of cell death through complex protein intersecting networks.[17] We next studied whether autophagy is related to HD-induced proliferation inhibition. AO staining of HD-treated leukemic cells displayed yellow-orange fluorescence, a specific marker for the occurrence of autophagosomes formation (Figure 4(a)). The occurrence of autophagy was further confirmed by Western blotting analysis, as the expression of LC3-II was clearly elevated after the treatment of HD. Furthermore, HD upregulated Beclin-1 while downregulated p62 (Figure 4(b)). To further

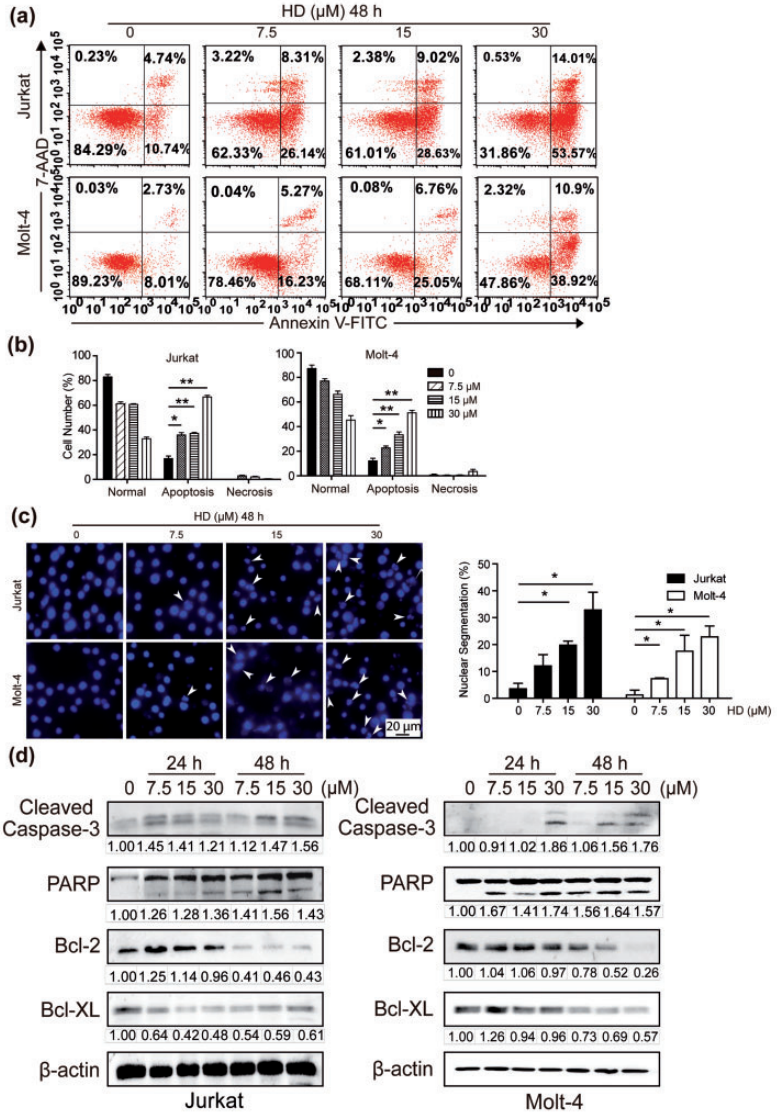


Figure 3. HD induces apoptosis in T-ALL cells. (a) Annexin V/7-AAD double staining and flow cytometry analysis were used to detect the apoptosis ratio of cells treated with HD (0, 7.5, 15, 30 μM) for 48 h. (b) Quantification of the number of apoptotic cells in panel A. HD vs. Control. *$p < 0.05$, **$p < 0.01$. (c) After DAPI staining, the nucleus morphology of T-ALL cells treated with HD was imaged and the number of apoptotic cells was quantified. Scale bar, 20 μm. (d) After HD treatment for 24 or 48 h, Western blotting analysis of apoptosis-related proteins was performed. (A color version of this figure is available in the online journal.)

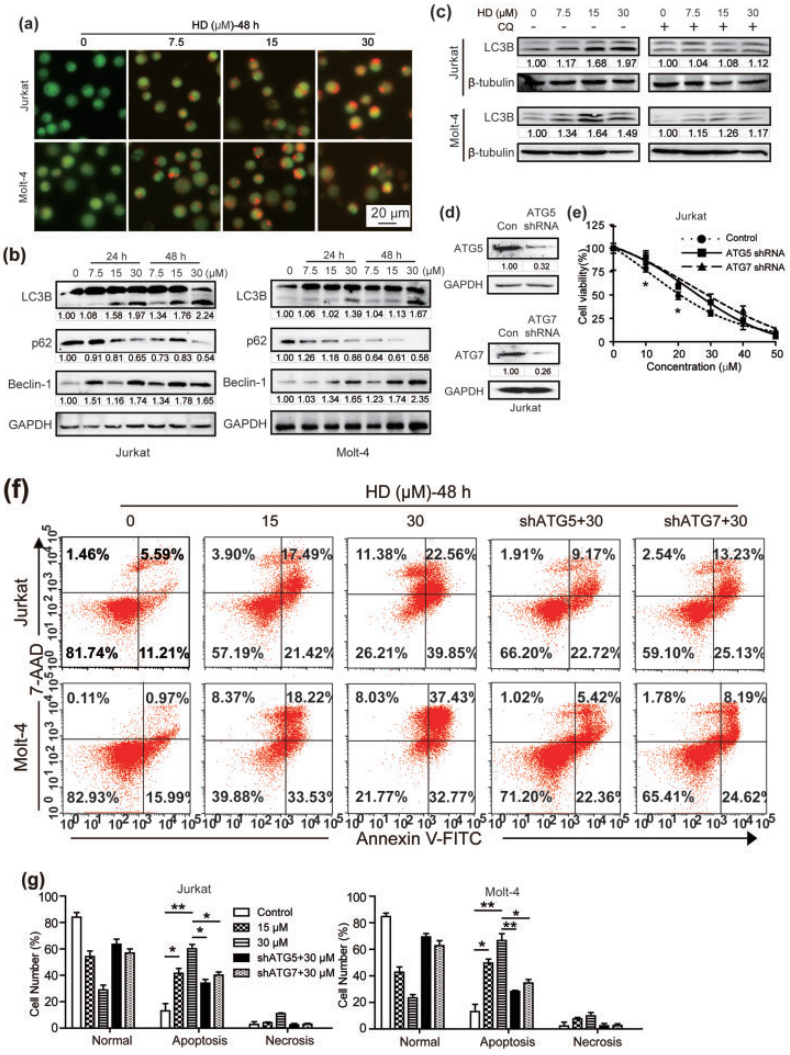


Figure 4. HD induces cytotoxic autophagy in T-ALL cells. (a) Representative images of AO staining in Jurkat and Molt-4 cells treated with various concentration of HD for 48 h were shown. Scale bar, 20 μm. (b) The expression levels of autophagy-specific proteins were detected using Western blotting analysis in cells treated with HD for 24 or 48 h. (c) T-ALL cells were incubated with various concentration of HD with or without CQ (10 μM). The expression of LC3-II was detected by western blotting. (d) Jurkat cells were transiently transfected with ATG5 or ATG7 specific short hairpin RNA (shRNA), and the expression levels of ATG5 and ATG7 were measured using western blotting analysis. (e) After the knockdown of ATG5 or ATG7, Jurkat cells were treated with HD and subjected to MTT analysis. ATG5 shRNA or ATG7 shRNA vs. scramble shRNA as control. (f) ATG5 or ATG7 knockdown cells was subjected to flow cytometry analysis of apoptosis. (g) Quantification of the number of apoptotic cells in panel f. $^{*}p < 0.05$, $^{**}p < 0.01$. (A color version of this figure is available in the online journal.)

confirm autophagy activation by HD, autophagic flux in T-ALL cells was evaluated by detection of LC3-II with or without the addition of chloroquine (CQ 10 μM). The data suggested that HD could enhance autophagic flux in T-ALL cells (Figure 4(c)). As autophagy is crucial in the survival of leukemic cells,[17] in order to clarify the exact function of HD-induced autophagy, we inhibited autophagy in Jurkat cells by knocking down ATG5 and ATG7, both of which are essential molecules for the induction of autophagy (Figure 4(d)). Results showed that inhibition of HD-induced autophagy partially rescued cell viability (Figure 4(e)). To test whether knockdown of ATG5 or ATG7 rescued cell viability via the apoptotic pathways, we assessed the percentage of apoptotic cells in ATG5/7 silenced cells treated with HD. The results showed that knockdown of ATG5 or ATG7 decreased HD-induced cell apoptosis (Figure 4(f) and (g)). These results suggest that HD induced a cytotoxic autophagy, which enhanced the anti-T-ALL efficacy of HD.

HD induces ferroptosis in T-ALL cells

Ferroptosis is recognized to be a programmed oxidative cell death accompanied by lipid peroxidation and iron accumulation, producing reactive oxygen species.[18] Recent studies have shown that autophagy can lead to ferroptosis through ferritin phagocytosis.[19] Thus, whether HD can trigger ferroptosis by autophagy is worth investigating. Upregulation of lipid ROS is a well-characterized ferroptosis marker[20]; therefore, we examined the effect of HD on lipid ROS levels in Jurkat and Molt-4 cells. The results showed that HD treatment induced rapid accumulation of lipid radicals even at a low concentration, as determined by both flow cytometry and fluorescent imaging using the C11-BODIPY probe (Figure 5(a) and (b)). GPX4 has been proven to

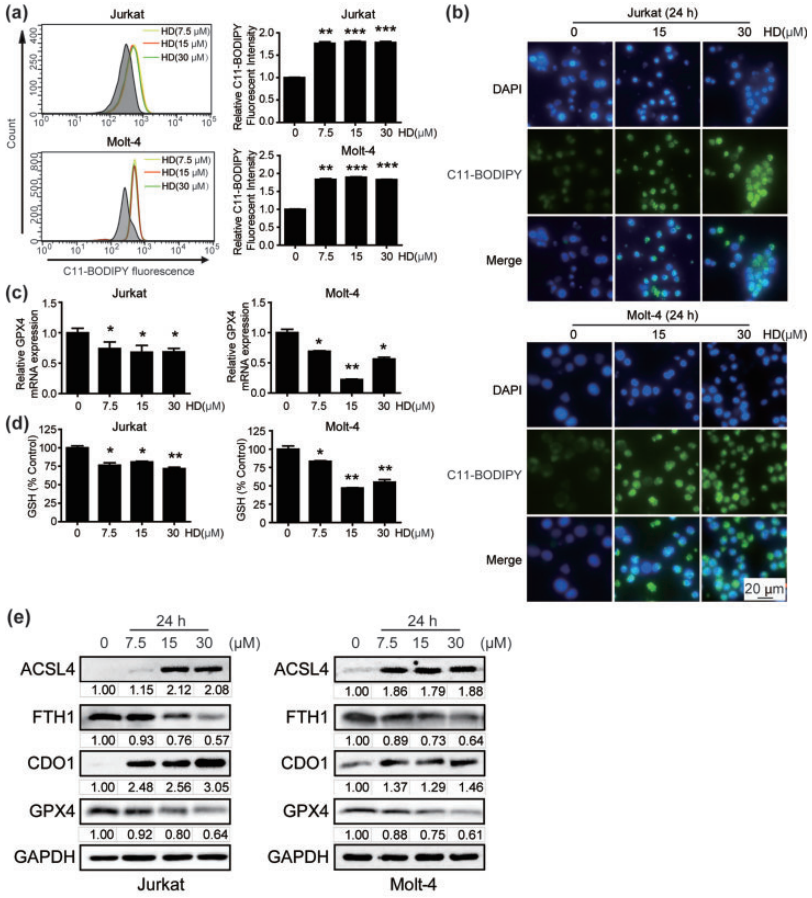


Figure 5. HD induces ferroptosis in T-ALL cells. Lipid ROS production after treatment with HD for 24 h was assessed by both flow cytometry (a) and fluorescent imaging (b) using the C11-BODIPY probe. Scale bar, 20 μm. (c) qRT-PCR was performed to measure the mRNA level of GPX4 after the treatment of HD. (d) The level of GSH was determined in T-ALL cells exposed to 7.5, 15 or 30 μM HD using a GSH assay kit. (e) The expression levels of ferroptosis-related proteins were evaluated by Western blotting. Representative data of three independent experiments is shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (A color version of this figure is available in the online journal.)

participate in the ferroptosis process by scavenging hydroperoxides.[21] Thus, we postulate that HD may induce ferroptosis by inhibiting GPX4. As expected, transcription of GPX4 was decreased by the treatment of HD (Figure 5(c)). Under condition of ferroptosis, GSH depletion plays a crucial role by modulating lipid peroxide level that is normally triggered by decrease of GPX4.[22] Therefore, we investigated GSH level in HD-induced ferroptosis. Unsurprisingly, the GSH level in all HD-treated groups was significantly reduced compared to control (Figure 5(d)). Western blotting analysis further confirmed a concentration-dependent decrease of GPX4 and FTH1, and a concomitant increase of CDO1 and ACSL4, all of which are specific markers of ferroptosis (Figure 5(e)).[23] Collectively, these data confirmed the involvement of ferroptotic cell death in HD-induced proliferation inhibition of T-ALL cells.

Inhibition of autophagy reduces HD-induced ferroptosis in T-ALL cells

Studies have provided evidence that autophagy contributes to ferroptosis through NCOA4-mediated ferritinophagy, and that autophagy is closely linked to the induction of ferroptosis by regulation of cellular ROS generation.[19,24] Thus, to further clarify the crosstalk between autophagy and ferroptosis in HD-treated T-ALL, we first analyzed cell viability after the treatment of HD, in the presence or absence of autophagy inhibitor 3-MA or ferroptosis inhibitor Fer-1. In accordance with the data of Figures 4 and 5, suppression of either autophagy or ferroptosis dramatically abolished HD-induced proliferation inhibition (Figure 6(a)), and suppressed HD-induced T-ALL cells apoptosis (Figure 6(b) and (c)). Collectively, these results further supported the notion that both autophagy and ferroptosis contributed to the suppression of T-ALL *in vitro*. Next, we investigated whether HD-induced autophagy promotes ferroptosis in T-ALL cells. Data illustrated that the induction of lipid ROS by HD treatment is abolished by the inhibition of autophagy (Figure 6(d) and (e)). Consistently, HD-induced expression of ACSL4 and CDO1 were downregulated in the presence of 3-MA, whereas autophagy inhibition up-regulated GPX4 expression (Figure 6(f)). Altogether, our results suggest that HD-induced ferroptosis is autophagy dependent.

Discussion

HD has been proven to possess an anti-cancer capacity in human colon cancer cells and ALL cells by suppression of Wnt/beta-catenin pathway and P-gp, respectively.[11,12]

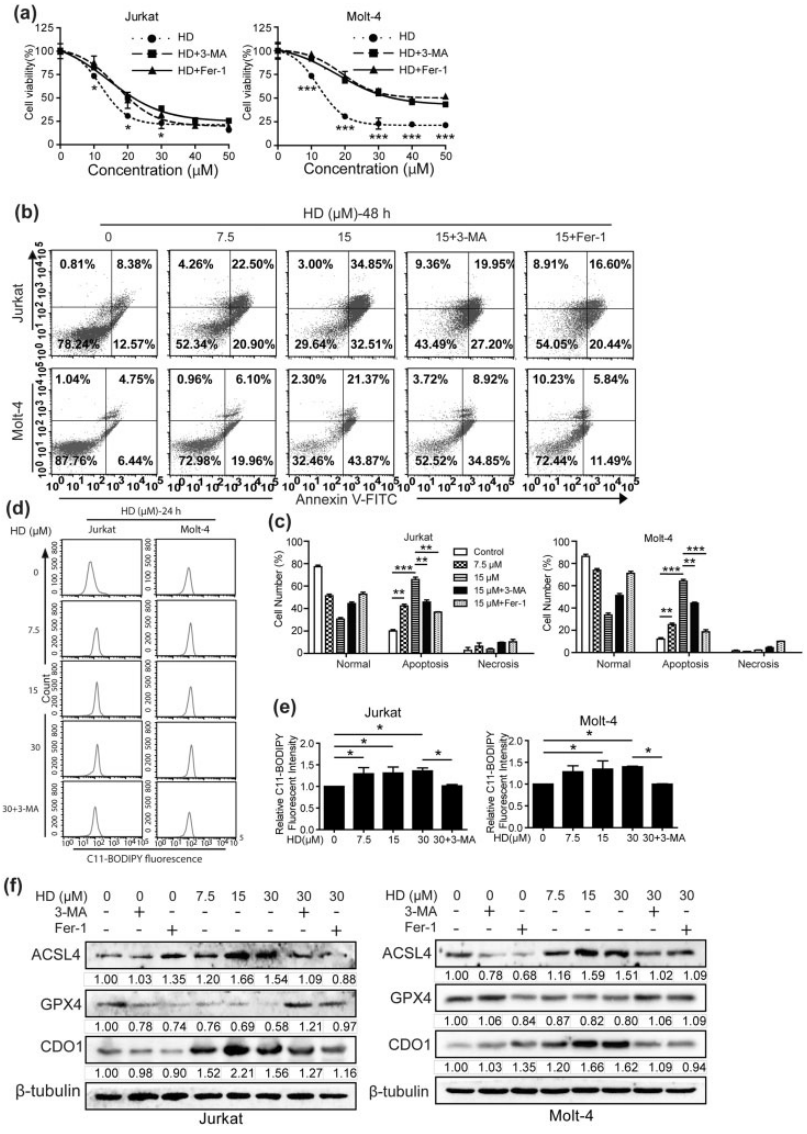


Figure 6. Inhibition of autophagy reduces HD-induced ferroptosis in T-ALL cells. (a) After treated with HD in the present or absent of autophagy inhibitor 3-MA (2 mM) or ferroptosis inhibitor Fer-1 (10 μM), cell viability was determined by MTT assay. (b) After pre-treatment with either 3-MA (2 mM) or Fer-1 (10 μM) in combination with HD, cell apoptosis ratio was detected by Annexin V/7-AAD double staining and subsequent flow cytometry analysis. (c) Quantification of the number of apoptotic cells in panel b. $^{**}p < 0.01$, $^{***}p < 0.001$. Lipid ROS production after treatment with HD with or without 3-MA (2 mM) for 24 hours was assessed by flow cytometry (d) and quantified in panel e using the C11-BODIPY probe. $^{*}p < 0.05$. (f) Western blotting analysis of ferroptosis-related proteins in T-ALL cells after pre-treatment with 3-MA (2 mM) or Fer-1 (10 μM) for 2 hours, and then incubated with HD for another 24 h.

In the present study, we first documented that HD inhibited T-ALL cell proliferation *in vitro*, partially via arresting cells at the G2/M phase and inducing apoptosis. By upregulating the expression of p-cdc2, HD caused delayed progression of T-ALL cells from G2 into mitosis. The down-regulation of c-Myc, an oncogene responsible for promoting cell cycle by activating cyclin and CDK while suppressing p21/p27,[25,26] also contributed to the cell cycle arrest and the increase of CDK inhibitor p27. Furthermore, our results showed that HD induced cleavage of PARP and caspase 3, both of which are key executioners of apoptosis specifically cleaved during apoptosis.[27,28] At the same time, anti-apoptotic proteins including Bcl-2 and Bcl-xL were significantly downregulated in HD-induced apoptosis. Thus, HD activated the apoptosis pathway to eradicate T-ALL cells that failed to complete the G2/M transition.

Apart from activation of cell cycle arrest and apoptosis, HD also induced cytotoxic autophagy, as evidenced by the up-regulation of autophagy marker Beclin-1 and lipidated LC3-II, as well as the degradation of autophagy substrate p62/SQSTM1. The occurrence of autophagy subsequently contributed to the accumulation of lipid peroxidation and induction of ferroptosis. The term ferroptosis was described for the first time in 2012 to depict a form of cell death activated by iron oxidation.[29] As a caspase-independent form of cell death, ferroptosis is not commonly associated with apoptosis. However, emerging evidence suggests that ferroptosis often shares common pathways with apoptosis. Few studies have reported an interrelationship between ferroptosis and apoptosis: switching apoptosis to ferroptosis[30] and ferroptotic agent-mediated sensitization of apoptosis.[31] Moreover, reports have

shown that the product of lipid peroxidation could activate MAPKs pathway through association with ERK, JNK and eventually induce apoptosis.[32] Thus, as a lipid peroxidation inhibitor,[33] Fer-1 may inhibit apoptosis by decreasing lipid ROS in T-ALL cells, as shown in Figure 6(b) and (c). Recent evidence suggests that autophagy is closely related to ferroptosis. By degradation of ferritin in fibroblasts and cancer cells, autophagy increases cellular unstable iron levels through the NCOA4-mediated pathway, thus disrupting cellular iron homeostasis and contributes to ferroptosis.[24] Inhibition of autophagy by knockdown of either ATG5, ATG7, BECN, or LC3B limited erastin-induced lipid peroxidation and ferroptotic cell death.[19,24,34] Our study also confirmed that inhibition of autophagy abrogated HD-induced lipid peroxidation and reduced the expression of ferroptosis-related proteins. However, more in-depth investigations are required to elucidate the molecular mechanisms governing the interplay between HD-induced autophagy and ferroptosis.

Flavonolignans are the structural combination of flavonoids and phenylpropanoids (lignans), and have attracted much attention as potent anticancer agents due to their multi-target capacity and minimal adverse effects. The first group of flavonolignans is silymarin complex extracted from milk thistle (*Silybum marianum*), which has been intensively studied for its hepatoprotection, antioxidant, and anti-cancer properties.[35] Hydnocarpin-type compounds are formally dehydrated analogs of silymarin flavonolignans with flavanone-3-ol (3-hydroxyflavanone) structure. But unlike silybins that can be completely synthesized,[36] hydnocarpin-type flavonolignans are not synthetically available,[8] which largely hindered their scientific study. Our previous finding of converting silibinin to HD under Mitsunobu reaction condition lay the foundation for highly effective and convenient preparation of HD,[16] which shall greatly facilitate the scientific research and clinical application of HD.

In summary, we provide evidence for the first time that, by concurrently targeting multiple pathways mediating cell cycle, apoptosis, and autophagy-dependent ferroptosis, the natural flavonolignan HD possesses anti-leukemic activities in T-ALL cells. Our findings significantly contribute to the understanding of the various biological functions of flavonolignan in T-ALL cells and lay the foundation for future experiments.

AUTHORS' CONTRIBUTIONS

HJZ and SYL designed the experiments; HHW, GH, LLX, SST and GYK performed the experiments; GZH and LM supplied Hydnocarpin D. HHW and SYL did the statistical analysis; HHW, ZZH and SYL wrote the paper. The final version of the manuscript has the approval of all authors.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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