7-Deoxynarciclasine shows promising antitumor efficacy by targeting Akt against hepatocellular carcinoma

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Akt is a promising therapeutic target for cancer treatment. In our study, we have identified that 7-deoxynarciclasine (7-DONCS) is a potential inhibitor of Akt, which results in the repression of multiple oncogenic processes in hepatocellular carcinoma (HCC). We have found that 7-DONCS suppresses the growth of HCC by inducing the apoptotic and autophagic capacities, as well as by inhibiting epithelial–mesenchymal transition (EMT) in vitro and in vivo. Pretreatment of cells with specific autophagy inhibitor (Bafilomycin A1) or knockdown of endogenous LC3B by siRNA strongly abrogates 7-DONCS-regulated apoptosis and EMT. Consequently, we have found that 7-DONCS selectively inhibits phospho-Akt (Ser473), and subsequent molecular docking reveals that 7-DONCS directly binds to the C-terminal domain of Akt. Overexpressing Akt significantly blocks these effects via 7-DONCS in HCC cells. Furthermore, 7-DONCS, by targeting Akt, exhibits a promising therapeutic effect in orthotopic hepatocellular tumors. Finally, higher p-Akt expression is associated with poor prognosis, and higher level of Akt was positively correlated with the enrichment of both apoptosis and autophagy downregulation, and EMT upregulation in HCC patients. These studies suggest that 7-DONCS serves as an attractive drug candidate by targeting Akt for future HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed and leading causes of cancer-related death globally.1,2 It is estimated that there are 782,500 new liver cancer cases and 788,000 deaths each year in the world.3 China alone accounts for about half of the new cases and deaths. Although there are novel therapeutic strategies against HCC, such as resection, transplantation, local ablation and chemotherapy, the 5-year recurrence rate exceeds 70% and often leads to death.4,5 Among the chemotherapeutic agents, sorafenib, a small molecule multikinase inhibitor, is considered as a standard treatment for patients with advanced HCC. However, the survival time of patients with advanced HCC exceeds 70% and often leads to death.4,5

Key words: 7-deoxynarciclasine, Akt, hepatocellular carcinoma

Abbreviations: 7-DONCS: 7-deoxynarciclasine; BA1: Bafilomycin A1; CETSAs: cellular thermal shift assays; CQ: chloroquine; DARTS: drug affinity responsive targets stability; DMEM: Dulbecco’s Modified Eagle Medium; E-cad: E-cadherin; EMT: epithelial–mesenchymal transition; FBS: fetal bovine serum; GEO: gene expression omnibus; GSEA: gene set enrichment analysis; H&E: hematoxylin and eosin; HCC: hepatocellular carcinoma; IHC: immunohistochemistry; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N-cad: N-cadherin; PBS: phosphate buffered saline; PCD: programmed cell death; TEM: transmission electron microscopy; VIM: Vimentin

Additional Supporting Information may be found in the online version of this article.

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What’s new?

7-deoxynarciclasine (7-DONCS), an active compound isolated from the bulbs of *Lycoris radiata* (Amaryllidaceae), exhibits various biological effects including anti-cancer activities. The defined targets or molecular mechanisms underlying 7-DONCS-regulated tumorigenesis remain to be unveiled, however. This study elucidates that 7-DONCS induces apoptosis and autophagy in hepatocellular carcinoma (HCC) and inhibits epithelial-mesenchymal transition (EMT) by targeting Akt, which in turn prevents tumor growth. The findings indicate that 7-DONCS may serve as a potential inhibitor of Akt and a promising candidate for blocking tumorigenesis in HCC.

is only extended by approximately 2–3 months.6 Therefore, there is an urgent need to develop novel and promising strategies against advanced HCC.

Natural products possessing diverse chemical structures and biological functions usually serve as major new leads source of pharmaceutical development, and approximately half of the pharmaceuticals in drug discovery today are derived from natural products and their derivatives.7-9 7-deoxynarciclasine (7-DONCS), an active narciclasine alkaloid from *Lycoris radiata* (Amaryllidaceae), exhibits various biological effects, including anticancer, plant growth-inhibitory, anti-Alzheimer’s disease and antiparasitic activities. The various biological activities and low toxicity of 7-DONCS make it a promising candidate for clinical development.10-14 Previous studies have reported that 7-DONCS potentially suppresses the proliferation of various cancer cells, such as ascites cancer, breast cancer, colon cancer, leukemia, lung cancer, pancreas cancer and prostate cancer.11,14-16 However, the defined targets or molecular mechanisms underlying 7-DONCS-regulated tumorigenesis inhibition still remain unveiled.

In our study, we have demonstrated that 7-DONCS suppresses the growth of HCC by inducing the apoptotic and autophagic capacities, as well as by inhibiting epithelial–mesenchymal transition (EMT) *in vitro* and *in vivo*. We have found that 7-DONCS is an effective inhibitor of Akt by directly interacting with the C-terminal domain of Akt, leading to repression of multiple oncogenic processes in HCC. Overexpression of Akt significantly blocks these effects by 7-DONCS in HCC cells. Furthermore, by targeting Akt, 7-DONCS exhibits a promising therapeutic effect in orthotopic hepatocellular cancers. Finally, clinical studies have shown that higher phospho-Akt (Ser473) expression is associated with poor prognosis.

Taken all together, these results suggest that 7-DONCS serves as an attractive drug candidate by targeting Akt for future HCC therapy.

Materials and Methods

**Cell culture and cell treatments**

Human HCC cell lines HepG2 and HuH-7 were obtained from ATCC (Manassas, VA). The hepatic stellate cell line LX-2 was obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). Reinstated cells were used within 1 month. The cell lines were determined by a short tandem repeat analysis. Mycoplasma contamination was excluded in these cell lines. Cells were cultured at 37°C in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator under 5% CO₂. Expression vectors of human Akt were designed and purchased from Servicebio Technologies (Wuhan, China). For siRNA knockdown, siRNA oligos against LC-3B were obtained from Hanbio Biotechnology (Shanghai, China). Bafilomycin A1 (BA1) and chloroquine (CQ) were obtained from Selleck (London, ON, Canada). 7-DONCS was isolated and purified (>95%) by our laboratory. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell viability assay**

Cell viability was detected by MTT assay. In brief, human HCC cells (5 × 10⁴ cells/well) were treated with 7-DONCS at different time for 15 μM, and further maintained with MTT solution at 37°C for 4 hr. Then, medium was removed. About 100 μl DMSO was added and determined by a microplate reader at 490 nm. For blocking study, cells were precultured with 10 nM BA1 for 2 hr and then treated with 15 μM 7-DONCS for 48 hr.

**Annexin V/PI staining**

After treatment, cells were harvested and washed twice with cold PBS, then resuspended in 100 μl of binding buffer. Each sample was added with Annexin V-FITC (5 μl) and PI staining reagents (5 μl). The samples were incubated for 10 min at room temperature away from light. About 400 μl of binding buffer was added to each sample, and the samples were detected by flow cytometry.

**Colonies formation assay**

Colonies formation assay was performed as described above.17 Human HCC cells were seeded into six-well plates and cultured overnight. Cells were then treated with different time of 7-DONCS. On day 14, colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of colonies was counted in indicated time periods.

**Orthotopic mouse model**

H22 cells (1 × 10⁶ in 0.2 ml PBS) were inoculated subcutaneously (*vía* s.c. injection) into Kunming mice. Then, subcutaneous tumors were peeled from subcutaneous mouse models after Schedule 1 killing with a longitudinal diameter of 1 cm. Tumor tissues were washed in 0.9% NaCl and were cut into about 1 mm³ pieces. Two to three pieces were implanted in the left lobe of the liver in recipient mice under anesthesia. The orthotopically implanted tumors were allowed to grow for 2 weeks. Then, the mice were randomly divided into four groups (n = 9, per group) and received vehicle or...
7-DONCS (1 mg/kg twice a week) and followed by CQ (20 mg/kg) via i.v. injection for 33 days. The mice were sacrificed to collect major target organs (liver and lung). The numbers of lung tumors were counted under a microscope and determined by routine histopathological analysis. All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of Tianjin University of Traditional Chinese Medicine. No specific exclusion or inclusion used for animal experiments.

**Immunofluorescence assay**
Immunofluorescence (IF) staining was performed as described above.18 In brief, human HCC cells were fixed with 4% paraformaldehyde for 30 min, conducted by incubation with 0.5% Triton X-100, and blocked with 5% BSA for 30 min. The slides were incubated with anti-LC-3B, anti-E-cadherin, anti-N-cadherin and anti-Vimentin (VIM) antibodies overnight at 4°C, followed by incubation with Alexa-Fluor 488-conjugated goat antiserum IgG or Alexa-Fluor 555-conjugated goat antirabbit IgG antibody for 1 hr at room temperature. Nuclei were stained with DAPI and observed with an inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

**Database of HCC cancer patients**
Clinical data can be obtained via the publically available TCGA datasets. The phosphorylation level of Akt (Ser473) in HCC patients was analyzed by Kaplan–Meier estimate.

**Immunohistochemistry assay**
Immunohistochemistry (IHC) analysis was performed as reported above.19 The prepared sections were incubated with anti-PARP, anti-p-Akt and anti-VIM antibodies overnight at 4°C, followed by biotin-conjugated secondary antibody. Images were obtained on a Leica DM4000B microscope (Leica, Wetzlar, Germany).

**Analysis of autophagic flux**
To determine autophagic flux, cells were transfected with a tandem mRFP-GFP-tagged LC-3 according to the instruction of the manufacturer (GeneChem, Shanghai, China). The transfected cells were treated with 15 μM of 7-DONCS for 48 hr. Cells then were fixed in 4% paraformaldehyde and washed in PBS. Finally, the GFP/mRFP images were acquired with a confocal scanning microscope (Olympus FV1000, Tokyo, Japan).

**Transmission electron microscopy**
Transmission electron microscopy (TEM) was determined as described above.20 In brief, human HCC cells were treated with 15 μM of 7-DONCS for 48 hr and fixed with 2% glutaraldehyde. The ultrathin (50 nm) sections were cut with an ultramicrotome, contrasted with uranyl acetate/lead citrate and obtained with electron microscope Hitachi H-7650 (Hitachi, Tokyo, Japan).

**Western blot assays**
Standard western blot assays were conducted as described previously.18,19 Antibodies against SQSTM1/p62 (D5E2), LC-3B (D11), phospho-Akt (Ser473), phospho-Akt (Thr308), total Akt, E-cadherin, N-cadherin and VIM were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin (A5441) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Full scans of western blot assays are shown in Supporting Information Figures S6–S10.

**Cellular thermal shift assays**
CETsAs were conducted to detect the direct binding between 7-DONCS and Akt in cellular. Briefly, cells were pretreated with DMSO or 7-DONCS for 48 hr, chilled on ice, washed with PBS plus protease inhibitor cocktail and then transferred into 1.5 ml PCR tubes and heated for 3 min at appropriate temperature. Subsequently, cells were lysed using liquid nitrogen and two repeated cycles of freeze–thaw. Precipitated proteins were separated, and the corresponding index was detected by western blot assays.

**Drug affinity responsive targets stability assay**
The DARTS assay was conducted as described above.21 To prepare DARTS samples for mass spectrometry analysis, 1 × 10⁷ HCC cells (HepG2 and HuH-7) were lysed in 2.4 ml M-PER buffer with protease inhibitors, centrifuged and then added 10x TNC buffer. Lyases were equally divided into two parts for 1 hr at room temperature with DMSO or 7-DONCS and incubated with 1 mg/ml pronase at room temperature for 30 min. The reaction was stopped by adding protease inhibitors, and samples were stored at −80°C standby.

**Molecular docking**
Indrsw software was used to draw the 2D structure of the 7-DONCS. The structure of p-Akt (PDB code: 3MV5, the 3D crystal structure of p-Akt simulated protein phosphorylation by replacing amino acids in position 473) was obtained from the protein data bank (http://www.rcsb.org/pdb). Docking simulations of 7-DONCS and p-Akt to CDOCKER Experiment were carried out with Discovery Studio 2017 R2. Interaction energies were calculated for predicting docking positions and selecting the binding pose that had the lowest binding energy (kcal mol⁻¹).

**Gene set enrichment analysis**
To identify Akt correlated biological process of Hepatocarcinoma, we used the GSEA 3.0 software (http://www.broadinstitute.org/gsea/) to subject to Gene Set Enrichment Analysis (GSEA). Global mRNA expression profiles were obtained from the Gene Expression Omnibus (GSE116174) and the matrix was ranked according to expression level of Akt. Association of Akt and apoptotic, autophagy, EMT gene sets was considered significant, based on the resulting parameters of the enrichment analysis: |NES| >1, NOM <0.05 and FDR <0.25.

**Statistical analysis**
The data were presented as mean ± SD. The statistically significant differences were conducted to analyze the results of animal
experiments by the one- or two-way ANOVA and the unpaired Student’s t-test. All other p values were performed using Student’s t-test (unpaired, two-tailed). Survival analysis was determined using the Kaplan–Meier estimates and the log-rank test. Experiments were performed in at least three independent experiments and the variation (p < 0.05) was considered statistically significant.

**Results**

**7-DONCS inhibits growth and promotes apoptosis in HCC cells**

The chemical structure of 7-DONCS is shown in Figure 1a. The MTT assay was used to assess the growth inhibitory effects of 7-DONCS against two typical HCC cell lines (HepG2 and HuH-7), as well as a nontumorigenic human liver cell line (LX-2). As shown in Figure 1b, cell viability of HCC cell lines was markedly reduced time-dependently by 7-DONCS (0–72 hr) for 15 μM. While, 7-DONCS did not affect the cell viability of LX-2 cells (Supporting Information Fig. S1A). Consistently, as evidenced by decreased clonogenicity (Figs. 1c and 1d), 7-DONCS strongly inhibited cell proliferation in HCC cells. Furthermore, 7-DONCS treatment significantly promoted cell death (Fig. 1e). To explore cell apoptosis regulation effect of 7-DONCS on HCC cells, we performed western blot assays, along with activation of Cleaved Caspase 3 and PARP by 7-DONCS time-dependently. Moreover, 7-DONCS treatment showed upregulation of Bax and a down-regulation of Bcl-2, which led to a dose-dependent increase in the

![Figure 1](https://example.com/figure1.png)

**Figure 1.** 7-DONCS inhibits growth and promotes apoptosis in HCC. (a) Chemical structure of 7-DONCS. (b) Cell viability was detected using MTT assay treated with 7-DONCS at indicated time. (c, d) Colony formation analysis of HCC cells treated with or without 7-DONCS. (e–g) HCC cells were treated with indicated time of 7-DONCS. (e) The phase-contrast photomicrographs show the morphology. (f) The protein levels of Cleaved Caspase 3, PARP, Bcl-2 and Bax were determined by western blot assays. (g) The percentage of apoptotic cells was detected by flow cytometer. For b–d, data are shown as mean ± SD (n = 3); *p < 0.05; **p < 0.01; ***p < 0.001 compared to control (Student’s t-test). [Color figure can be viewed at wileyonlinelibrary.com]
The apoptotic effects were further confirmed by employing Annexin V/PI staining by treating HCC cells with 7-DONCS (Fig. 1g). Collectively, these results suggest that 7-DONCS inhibits growth and promotes apoptosis of HCC cells.

7-DONCS induces autophagy in HCC cells

Autophagy is an alternate mode of programmed cell death and plays an essential role in cancer progression. In this present study, we first investigated the protein levels of LC-3B and p62, two classical autophagy markers. As shown in Figure 2a, the result demonstrated that 7-DONCS elevated the expression of LC-3B-II, while significantly reduced the expression of p62 in a time-dependent manner. To further verify whether 7-DONCS promotes autophagy, we investigated the cellular ultrastructure of HCC cells by transmission electron microscopy. As shown in Figure 2b, there was a marked accumulation of double-membrane vesicles containing subcellular materials in 7-DONCS-treated cells. Using a tandem mRFP-GFP-tagged LC-3, we found strong green fluorescent (GFP-tagged LC-3), with red dots (indicating autolysosomes) and yellow dots (indicating autophagosomes) generated. Treatment with 7-DONCS resulted in an increased LC-3-II/LC3-I, promotion of LC-3 lipidation and puncta and congestion of autophagosomes (Figs. 2c and 2d). Taken together, these results suggest that 7-DONCS induces autophagy in HCC cells.

7-DONCS inhibits EMT in HCC cells

EMT is a highly conserved biological process that has attracted more and more attention in enhancing the invasion and migration of tumor cells. In the present study, by western blot assays, we first detected the protein levels of two specific EMT markers, E-cadherin and N-cadherin. As shown in Figure 3a, there result demonstrated that 7-DONCS significantly increased the expression of E-cadherin, while decreased the expression of N-cadherin; the similar trends were further confirmed by IF staining (Figs. 3b, 3c, 3d, and 3e).
To further verify 7-DONCS-regulated EMT, we investigated the expression of VIM, the EMT prototypical epithelial cell marker. As shown in Figures 3a and 3b, the result demonstrated that 7-DONCS inhibited the protein expression level of VIM by western blot and IF staining. Collectively, these results suggest that 7-DONCS inhibits EMT in HCC cells.

**Blocking autophagy enhances promotion of apoptosis and inhibition of EMT by 7-DONCS**

Previous studies have reported that manipulation of autophagy may play a key role in regulating cancer cell survival via maintaining apoptosis, metastasis and EMT. To investigate the relationship between autophagy and cancer cell survival regulated by 7-DONCS on HCC cells, Bafilomycin A1 (BA1), an autophagy–lysosomal inhibitor, was used to block autophagy. And then the effect of 7-DONCS on apoptosis and EMT was detected. HCC cells were treated with 7-DONCS with and without BA1 treatment respectively. As shown in Figures 4a–4d and Supporting Information Figure S2A, the apoptotic and growth inhibitory effects of 7-DONCS were enhanced when 7-DONCS was combined with BA1 in comparison to the treatment with 7-DONCS alone. Moreover, we have found that BA1 further enhanced the inhibition of EMT by 7-DONCS in HCC cells by IF staining (Figs. 4e and 4f and Supporting Information Figs. S2B and S2C). Consistent results were obtained when the autophagy function was blocked by using siRNA targeting LC-3B.

Figure 3. 7-DONCS inhibits EMT in HCC cells. (a) The protein expression of E-cadherin, N-cadherin and VIM was determined treated with indicated time of 7-DONCS by western blot assays. (b–d) The expression of E-cadherin, N-cadherin and VIM was measured by IF staining. Right, relative level of E-cadherin, N-cadherin and VIM. For b–d, data are shown as mean ± SD (n = 3); ***p < 0.001 compared to control (Student’s t-test). Scale bar: 50 μm. [Color figure can be viewed at wileyonlinelibrary.com]
Knockdown of endogenous LC-3B by siRNA further enhanced 7-DONCS-induced apoptosis and growth inhibitory effects in HCC cells (Fig. 4g and 4h and Supporting Information Figs. S3A–S3C). In addition, knockdown of endogenous LC-3B also markedly enhanced inhibition of EMT by 7-DONCS (Supporting Information Figs. S4A and S4B). Taken together, these results strongly indicate that inhibition of autophagy could enhance promotion of apoptosis and inhibition of EMT by 7-DONCS.
7-DONCS promotes the apoptotic and autophagic capacities and suppresses EMT via Akt inactivation

To determine whether Akt is a direct target of 7-DONCS, we performed two types of biochemical experiments. Cellular thermal shift assays (CETsAs) and drug affinity responsive targets stability (DARTs) assays were performed to determine the direct binding between Akt protein and 7-DONCS in cellulo. As shown in Figures 5a and 5b, 7-DONCS treatment obviously shifted the Akt melting curve compared to control. Furthermore, our DARTS data indicated that 7-DONCS binds to Akt, protecting it from degradation by proteinase (Fig. 5c). To search the potential target for 7-DONCS, the interaction between 7-DONCS and Akt was analyzed by molecular docking simulation with Discovery Studio 2017 R2. The CDOCKER docking result showed that 7-DONCS can be docked with Akt Ser473 (PDB code: 3MV5), and be extended into the accessible pocket in Akt, surrounded by key residues (LEU239, ARG243, GLU341, GLY345, ARG346 and LEU347). The compounds occupied the Akt kinase domain so as to inhibit the activity function of Akt Ser473 (Figs. 5d and 5f).

To further determine the Akt inhibitory effect, we determined the constitutive activation of Akt in HCC cells using the specific antibodies against phospho-Akt Ser473. As shown in Figure 5g, 7-DONCS markedly downregulated the phosphorylation level of Akt (Ser473), but there was no noticeable difference in phospho-Akt (Thr308) and Akt total expression in HCC cells. Next, we found that ectopic Akt expression in HCC cells blocked 7-DONCS-induced apoptosis and autophagy and regulated EMT related protein expressions (Figs. 5h and 5i). And Akt overexpression strongly attenuated the inhibitory effect of 7-DONCS on cell viability (Fig. 5j). In addition, ectopic Akt expression in HCC cells clearly recovered 7-DONCS-suppressed EMT and 7-DONCS-induced autophagy (Figs. 5k and 5l and Supporting Information Figs. 5a and 5b). Collectively, these results suggest that 7-DONCS promotes apoptosis and autophagy and suppresses EMT through targeting Akt.

7-DONCS inhibits growth and development in an orthotopic mouse model of HCC

To investigate whether 7-DONCS inhibits HCC growth and development in vivo, we established an orthotopic mouse model by injecting H22 cells subcutaneously into Kunming mice. As shown in Figure 6a, macroscopically, the size of 7-DONCS-treated tumors was markedly reduced compared to that of the control group. Combination of 7-DONCS with CQ, an autophagy inhibitor, further decreased the size of tumors compared to 7-DONCS treatment alone. Furthermore, pretreatment of CQ further inhibited lung metastasis of HCC compared to 7-DONCS treatment alone (Fig. 6b). In addition, combination of 7-DONCS with CQ further decreased VIM and enhanced cleavage of PARP by IHC staining and western blot (Figs. 6c–6f). Moreover, IHC staining demonstrated that positive staining of phosphorylated Akt was much stronger in control orthotopic tumors compared to that in 7-DONCS-treated orthotopic tumors (Fig. 6g). Accordingly, 7-DONCS treatment attenuated the phosphorylation level of Akt in H22 orthotopic tumors (Fig. 6h).

Clinical significance of the p-Akt in HCC

To further evaluate the clinical correlation of p-Akt in cancer patient tissues, we subjected them to Kaplan–Meier survival analysis in the publically available dataset of TCGA. Data showed that cancer patients with higher phosphorylation level of Akt displayed poorer percent progression-free survival (PFS) rate (Fig. 6i). Furthermore, we examined whether Akt was linked to apoptosis, autophagy and EMT in HCC. According to the level of Akt from GSE116174, we estimated that higher level of Akt was positively correlated with enrichment of downregulation of apoptosis and autophagy, as well as upregulation of EMT (Fig. 6j). Collectively, these results suggest that Akt could be a prognosis marker for poor survival in HCC patients.

Taken together, our results suggest that the Akt plays a key role in 7-DONCS-regulated apoptosis, autophagy and EMT in HCC.

Discussion

Natural products are the mainstay of cancer chemotherapy in the past decade.28 7-DONCS, an active compound isolated from the bulbs of Lycoris radiata (Amaryllidaceae) in our laboratory, has been reported to possess potential anticancer activities in several cancer cell lines.11,14–16 However, the defined molecular mechanism of 7-DONCS on HCC remains unclear. In our study, we have for the first time clarified that 7-DONCS represses the growth of HCC by inducing the apoptotic and autophagic capacities as well as by inhibiting EMT via targeting Akt in vitro and in vivo.

Apoptosis is a genetically coded programmed cell death (PCD) that represents an efficient and evolutionarily conserved cellular suicide pathway.29 Apoptosis plays an integral part in tumor prevention, growth and progression, and its disorder is thought to be closely related to many diseases including cancer.30,31 Caspases-family cysteine proteases are the central engines of the apoptotic pathway which trigger apoptosis by cleaving numerous substrates that implement cell dissolution.30 Previous studies have demonstrated that many chemotherapeutic agents trigger apoptotic machinery through the activation of caspases.31,32 In our study, we have found that 7-DONCS treatment markedly promotes...
Figure 5. 7-DONCS promotes the apoptotic and autophagic capacities and suppresses EMT via Akt inactivation. (a, b) Cells were conducted by CETSAs, and the protein expression of Akt was detected by western blot assays. Right, relative band intensity of Akt. (c) Cells were conducted by DARTS assay, and the Akt protein levels were tested by western blot assays. (d-f) Docking model of 7-DONCS with Akt. (d) The interaction pattern of 7-DONCS with the residues. (e) 7-DONCS binding with the pocket is composed of hydrogen bonds. (f) 2D diagram between the receptor and ligand. (g) HepG2 and HuH-7 cells were treated with indicated time of 7-DONCS for 48 hr. The protein levels of p-Akt were detected by western blot assays. Total Akt expressions were determined as the internal control. (h-l) Cells transfected with Akt (Akt Vec) or empty vector (Control Vec) followed by 7-DONCS treatment. (h, i) The protein levels of Akt, VIM, PARP and p62 were detected by western blot assays. (j) The cell viability was measured by MTT assay. (k) The VIM was detected by IF analysis. Right, relative level of VIM. (l) The LC-3B was detected by IF analysis. Right, total number of endogenous LC-3B puncta per cell. For j-l, data are shown as mean ± SD (n = 3); **p < 0.001 compared to Vector control; ###p < 0.001 compared to Vector control transfected cells treated with 7-DONCS (Student’s t-test). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 6. Legend on next page.
HCC apoptosis by employing Annexin V/PI staining. Furthermore, 7-DONCS treatment markedly augments Cleaved Caspase 3 and PARP. The mitochondrial pathway, one of the major caspase-dependent apoptotic pathways, is strictly controlled by Bcl-2 family members. Numerous anticancer drugs show proapoptotic activity by modulating the expression of Bcl-2 family proteins. We also have found that 7-DONCS treatment shows upregulation of Bax and a downregulation of Bcl-2, which leads to a dose-dependent increase in the ratio of Bax/Bcl-2. Collectively, these results suggest that 7-DONCS induces apoptosis via the mitochondrial pathway in HCC cells.

Autophagy, or type II PCD, is a conserved catabolic process which regulates the degradation and recycling of cellular proteins as well as cytoplasmic organelles under unfavorable conditions. In the 1950s, scientists first discovered autophagy by transmission electron microscopy (TEM). Even in recent years, TEM remains the golden standard to examine the formation of autophagic compartments. In the present study, we have found that 7-DONCS accumulates the formation of autophagic vacuoles (AVs) by TEM. In response to starvation or stress, LC3-I is conjugated with PE to form LC3-II (LC3-PE), which is involved in the formation of autophagosomes; as a specific marker. In our study, we have clarified that 7-DONCS markedly increases LC-3B-II/LC-3B-I ratio and promotes LC3-I lipidation and the autophagosomes formation. P62, a key autophagy adaptor, directly binds specific ubiquitinated substrates and delivers them to autophagic organelles. We have found that 7-DONCS markedly inhibits the protein expression level of p62. Altogether, these results significantly suggest that 7-DONCS promotes autophagy in HCC cells.

EMT is a highly conserved biological process that undergoes multiple biochemical steps, allowing polarized epithelial cells from the surrounding tissue to exhibit an active mesenchymal phenotype. In recent years, this important process has attracted more and more attention since it enhances the invasion and migration of tumor cells and increases the chemotherapeutic resistance to apoptosis. The changes in the expression of different cadherins have been used as a standard for monitoring the progression of EMT during cancer. When the epithelial cells become cancerous, cadherins switch from E-cadherin to N-cadherin and vice versa. In the present study, we have analyzed the changes in the expression of both E-cadherin and N-cadherin and have found that 7-DONCS treatment leads to an increased expression of E-cadherin and a decreased expression of N-cadherin by IF staining and western blot assays. Furthermore, a change in the expression of VIM is the EMT prototypical epithelial cell marker. VIM overexpression is accompanied by the promotion of EMT. Interestingly, in our study, we have found that 7-DONCS markedly inhibits the protein expression level of VIM by IF staining and western blot assays. Taken together, these results strongly suggest that 7-DONCS inhibits EMT in HCC cells.

Autophagy, as a cellular process, is very important in cancer research. Recent studies have reported that many anticancer agents kill tumors by inducing autophagic cell death, such as tamoxifen, temozolomide and etoposide. In our previous study, we have found that magnolin suppresses human colorectal cancer cell growth via activating autophagy and cell cycle arrest. Inhibition of autophagy can markedly block magnolin-regulated cell cycle arrest and significantly abrogate magnolin-inhibited proliferation. It seems that autophagy may serve as an essential anticancer role. Our previous studies have demonstrated that lycorine-promoted autophagy and apoptosis inhibit the growth of HCC cells. Disruption of autophagy markedly enhances the apoptotic effect by facilitating the switch from autophagy to apoptosis, thus further augmenting antitumor effects. Therefore, the role of autophagy in cell survival and death is complex and may have diametrically opposed effects depending on the situation.

Previous numerous lines of evidence have reported that manipulation of autophagy may play essential roles in regulating cancer cell survival via maintaining apoptosis, metastasis and EMT. In our study, we have demonstrated that 7-DONCS suppresses the growth of HCC by inducing the apoptotic and autophagic capacities and by inhibiting EMT. Our current studies have also suggested that autophagy undergoes a temporary survival mechanism after chemotherapies, and inhibition of autophagy further enhances 7-DONCS-induced apoptosis and ulcerator blocks 7-DONCS-regulated EMT. Collectively, 7-DONCS may regulate apoptosis and EMT via triggering autophagy, which results in inhibiting the growth of HCC in vitro and in vivo.

Akt is a serine/threonine kinase that serves as an essential node in the PI3K-Akt signaling pathway. This signaling axis often dysregulated and plays a key role in cell growth, proliferation and survival in cancer, leading to a significant increase in the clinical development of Akt inhibitors. Many of these targeted therapies have shown great promise for treating Akt-addicted tumors. Akt activity is typically dependent on two phosphorylated residues: threonine 308 (Thr308) and serine 473 (Ser473). Thr308 phosphorylation is located at the catalytic site and is mediated by the dependent phosphoinositide-dependent kinase-1 (PDK1) which
located on the plasma membrane. Phosphorylation of Ser473 at the C-terminal hydrophobic motif, which is regulated by mTOR, activates the Akt enzyme. In the present study, we have found direct binding between Akt and 7-DONCS by CETSA and DARTS assay. Moreover, we have found that 7-DONCS selectively inactivates phospho-Akt (Ser473) and directly binds to the C-terminal domain of Akt by molecular docking. The inhibition effects are further confirmed by western blot assays: 7-DONCS significantly decreases phospho-Akt (Ser473) in a time-dependent manner, but there is no obvious change in phospho-Akt (Ser308) and Akt total expression in HCC cells. Furthermore, we find that ectopic Akt expression markedly blocks 7-DONCS-regulated growth of HCC. Importantly, higher p-Akt expression is associated with poor prognosis, and higher level of Akt was positively correlated with the enrichment of apoptosis and autophagy downregulation, as well as EMT upregulation in HCC patients. Collectively, our results suggest that the Akt plays a key role in 7-DONCS-regulated apoptosis, autophagy and EMT in HCC.

In summary, our study elucidates that 7-DONCS induces apoptosis and autophagy and inhibits EMT through targeting Akt, which in turn prevents the tumor growth of HCC. These studies suggest that 7-DONCS serves as a potential candidate that blocks tumorigenesis in HCC.

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