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Hydroxypyridinone-Coumarin Inhibits the Proliferation of MHCC97 and HepG2 Human Hepatocellular Carcinoma Cells and Down-Regulates the Phosphoinositide-3 Kinase Pathway

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

Literature Search F

Funds Collection G

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Background: Worldwide, hepatocellular carcinoma (HCC) is one of the most commonly diagnosed malignant diseases and is the third leading cause of cancer-related death. This study aimed to investigate the effect of hydroxypyridinone-coumarin (HPC) on MHCC97 and HepG2 human HCC cells and the mechanisms involved.

Material/Methods: MHCC97 and HepG2 human HCC cells were cultured *in vitro*. An MTT cytotoxicity assay was used to assess cell viability and proliferation, with and without treatment with HPC. Cell autophagosomes were labeled with GFP-LC3 using confocal fluorescence microscopy. Western blot was used to measure protein expression.

Results: HPC significantly reduced the cell proliferation rate in a concentration-dependent manner, with 2 μ M of HPC resulting in a reduced proliferation rate of MHCC97 cells (by 36%) and HepG2 cells (by 29%) ($P < 0.02$). HPC significantly reduced autophagy in MHCC97 and HepG2 cells. Western blot showed that treatment with HPC significantly upregulated Atg5, beclin-1, LC3-phosphatidylethanolamine conjugate (LC3-II), and Atg-3, reduced p62 and Akt protein expression, and induced phosphorylation of ERK1/2. GFP-LC3B labeling in MHCC97 and HepG2 cells was increased following HPC treatment.

Conclusions: HPC induced autophagy and inhibited the proliferation of MHCC97 and HepG2 HCC cells *in vitro* and involved activation of ERK1/2 and down-regulation of the Akt pathway.

MeSH Keywords: **Apoptosis • Autophagy • Proteostasis Deficiencies**

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Background

Worldwide, hepatocellular carcinoma (HCC) is one of the most commonly diagnosed malignant diseases and is the third leading cause of cancer-related death [1,2]. Approximately 600,000 new cases of primary HCC are diagnosed worldwide [1,2]. The stage at diagnosis of HCC usually determines the prognosis [2].

Traditional chemotherapy agents for advanced-stage HCC may be ineffective. However, recent developments in surgical treatment for HCC have resulted in improvement in the five-year survival rate for patients [3]. When HCC is resistant to radiotherapy and conventional chemotherapy, metastasis to lymph nodes, adrenal glands, bone, and the lungs can occur [4]. Studies continue to understand the mechanisms associated with the genesis and progression of HCC [5–7]. However, effective novel compounds are still needed for the treatment of HCC.

Autophagy involves the intracellular degradation of misfolded proteins and cleavage of damaged organelles by lysosomes [8]. Autophagy plays an important role in cell survival, as well as the death of cells [9,10]. Several natural products exhibit anti-cancer effects by induction of cell autophagy [11,12].

Resveratrol is a natural product derived from plants, which has previously been shown to reduce the viability of U373 glioma cells and ovarian carcinoma cells by induction of autophagy [13]. Curcumin is another plant-based compound that induces autophagy by activation of the Akt/mammalian target of rapamycin (mTOR) signaling pathway [14]. In HCC cells, autophagy and apoptosis are activated by arenobufagin, a natural bufadienolide derived from toad venom, by targeting the phosphoinositide 3 kinase pathway [15].

Heterocyclic compounds have been widely used in drug discovery [16]. For example, imidazole is a structural constituent of many complex bioactive natural compounds and therapeutic molecules [17]. These heterocyclic compounds have therapeutic activity against microbes [18], tumor cells [19], and Alzheimer's disease [20]. Coumarin derivatives of hydroxypyridinone, including hydroxypyridinone-coumarin (HPC), have antioxidant effects and anti-tumor effects [19,20].

Therefore, this study aimed to investigate the effect of HPC on MHCC97 and HepG2 human HCC cells and the mechanisms involved.

Material and Methods

Cell culture

MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cell lines were provided by Biotechnology Co., Ltd., Shanghai, China. The two cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) with penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were incubated with 5% CO₂ at 37°C with and without increasing concentrations of hydroxypyridinone-coumarin (HPC).

MHCC97 and HepG2 cell proliferation

The MTT assay measured cell proliferation in MHCC97 and HepG2 cells cultured with and without HPC. MHCC97 and HepG2 cells placed in 96-well plates at 2×10⁶ cells per well and cultured at 37°C overnight. Fresh medium was mixed with 0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM, 1.25 µM, 1.5 µM, 1.75 µM, or 2 µM of HPC, and the plates were incubated for a further 72 h. Then, a 1 mg/ml solution of MTT was added to the wells and incubation was performed for a further 2 h at 37°C. Following removal of the culture medium, 100 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan precipitate. Measurement of absorbance for each plate was made in triplicate at a wavelength of 590 nm using an ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT, USA).

Analysis of cell autophagy

The induction of autophagy by HPC was assessed using acridine orange staining of the acidic vesicular organelles [21]. Briefly, MHCC97 and HepG2 cells were plated in six-well plates at 2×10⁵ cells per well to achieve confluence during 12 h. The cells were treated for 72 h with 1.0 µM and 2 µM of HPC at 37°C. The cells were stained with acridine orange at 37°C for 15 min and then trypsinized, followed by resuspending in phosphate-buffered saline (PBS). The BD Accuri™ C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for the measurement of green and red fluorescence of the cells at an excitation wavelength of 488 nm.

Scanning electron microscopy (SEM)

The MHCC97 and HepG2 cells were placed onto microscope slides at 2×10⁶ cells/well in the 24-well plates. The culture medium contained RPMI-1640 medium mixed with FBS (10%). The incubation of the cells was performed with 1.0 µM and 2 µM of HPC for 72 h at 37°C or with DMSO as the control. Following incubation, the cells were fixed initially for 1.5 h and then for 72 h at 4°C in a 2.5% solution of glutaraldehyde in sodium cacodylate buffer. The cells were incubated for 2 h in osmium tetroxide at 4°C followed by dehydration in graded acetone.

After dehydration, the cells were coated with gold and palladium alloy before examination by scanning electron microscopy (SEM) at a magnification of $\times 750$ using a Jeol JSPM-5200 electron microscope (JEOL, Tokyo, Japan).

Western blot

The MHCC97 and HepG2 cells at a concentration of 2×10^6 cells/well were cultured at 37°C with $1.0 \mu\text{M}$ and $2 \mu\text{M}$ of HPC for 72 h. After incubation, cell lysis was performed using HEPES buffer (50 mM), at pH 7.5, sodium chloride (150 mM), EDTA (1 mM), vanadate (1 mM), Na_2PO_4 (10 mM), sodium fluoride (10 mM), NP-40 (1%) and a protease inhibitor cocktail. The lysate was collected in Eppendorf tubes and centrifuged at 4°C for 25 min at $10,000 \times g$. The protein level in the supernatants was analyzed using a bicinchoninic acid (BCA) assay kit, according to the manufacturer's instructions.

Protein separation was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by loading $10 \mu\text{g}$ of protein sample per lane on an 8–10% SDS polyacrylamide gel. After separation, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 400 mA (Merck Millipore, Burlington, MA, USA). The membranes were incubated with blocking buffer for 2 h, followed by incubation at 4°C overnight with the primary antibodies at a dilution of 1: 1,000. The primary antibodies were to phosphoinositide-3 kinase (PI3K), Akt, p-PI3K, extracellular signal-regulated kinase (ERK)1/2, p-Akt, mammalian target of rapamycin (mTOR), p-mTOR, p-ERK1/2, c-Jun N-terminal kinase (JNK), p-JNK, Atg-5, Atg-3 and β -actin (Cell Signaling Technology, Inc., Danvers, MA, USA). After washing in PBS, the membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit secondary antibodies. Incubation of the immunoblots was performed for 5 min with Western-Bright enhanced chemiluminescence (ECL) reagent (Advansta, San Jose, CA, USA). The blots were visualized using Super RX-N film (Fujifilm, Minato, Tokyo, Japan) and quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Transfection with the GFP-LC3 expression vector and fluorescence microscopy

The MHCC97 and HepG2 cells were sampled at 1×10^5 cells per well in six-well plates and transfected with GFP-LC3 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) [10]. After 24 h, the cells were treated with $1.0 \mu\text{M}$ and $2 \mu\text{M}$ of HPC for 72 h at 37°C . The cells were fixed for 40 min with 4% paraformaldehyde at 37°C , followed by washing with PBS. The cells were stained with 1 mg/ml of 4',6-diamidino-2-phenylindole (DAPI) and were observed using a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany).

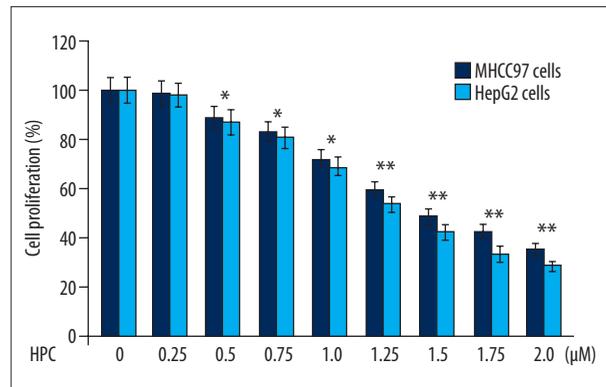


Figure 1. The effect of hydroxyppyridinone-coumarin (HPC) on the proliferation of in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells. The MHCC97 and HepG2 human HCC cells after treatment with $1.0 \mu\text{M}$ and $2 \mu\text{M}$ of HPC for 72 h underwent an MTT assay for the measurement of cell proliferation. * $P < 0.02$ and ** $P < 0.01$ vs. the untreated control cells.

Statistical analysis

Data were presented as the mean \pm standard deviation (SD). Analysis of variance (ANOVA) was followed by Tukey's post hoc test. GraphPad Prism software was used to analyze the data (GraphPad Software, La Jolla, CA, USA). A P -value < 0.05 was considered to be statistically significant.

Results

Hydroxyppyridinone-coumarin (HPC) inhibited the proliferation of MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells

The MTT assay assessed the MHCC97 and HepG2 cell proliferation after 72 h of exposure to $0.25 \mu\text{M}$, $0.5 \mu\text{M}$, $0.75 \mu\text{M}$, $1.0 \mu\text{M}$, $1.25 \mu\text{M}$, $1.5 \mu\text{M}$, $1.75 \mu\text{M}$, or $2 \mu\text{M}$ of HPC (Figure 1). The dose-dependent decrease in proliferation of both the tested cell lines was observed on HPC treatment compared with the control. The proliferation of MHCC97 and HepG2 cells was reduced to 36% and 29%, respectively, following treatment with $2 \mu\text{M}$ of HPC compared with 100% in the control group. There was no significant decrease in MHCC97 and HepG2 cell proliferation with treatment using HPC at $0.25 \mu\text{M}$.

HPC resulted in autophagy in MHCC97 and HepG2 cells

Treatment of MHCC97 and HepG2 cells with HPC at $2 \mu\text{M}$ significantly increased autophagy in comparison with the control cells ($P < 0.02$) (Figure 2). The autophagy induction was not observed in MHCC97 and HepG2 cell cultures on exposure to HPC at $0.25 \mu\text{M}$ for 72 h.

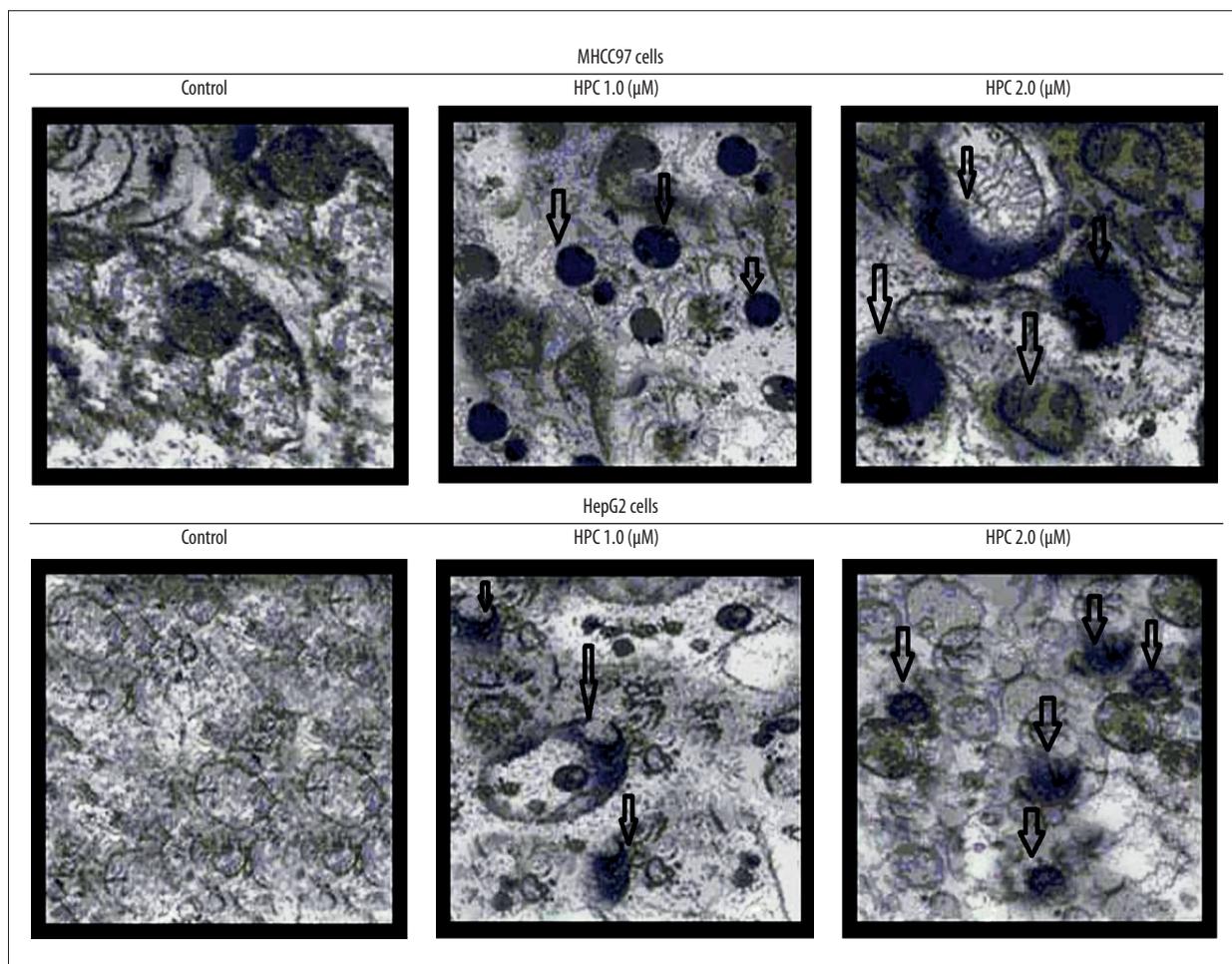


Figure 2. The effect of hydroxypyridinone-coumarin (HPC) on the induction of autophagy in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells. The MHCC97 and HepG2 human HCC cells after treatment with 1.0 μM and 2 μM of HPC for 72 h were examined for autophagy by flow cytometry. Magnification $\times 750$.

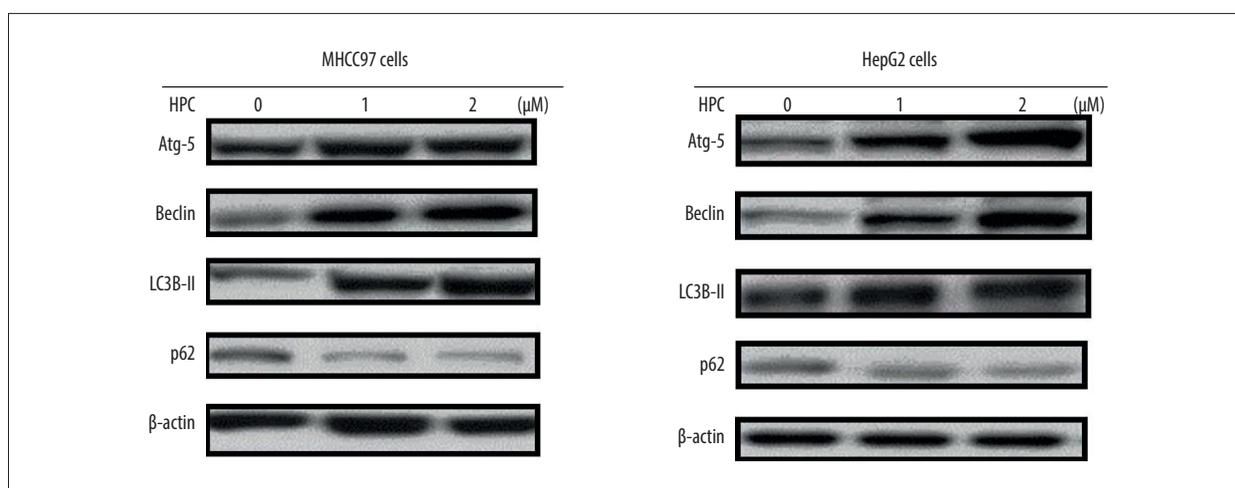


Figure 3. The effect of hydroxypyridinone-coumarin (HPC) on the expression of proteins associated with autophagy in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells. The MHCC97 and HepG2 cells after 72 h of treatment with 1.0 μM and 2 μM HPC underwent Western blot for the detection of autophagy-associated proteins, Atg 5, beclin-1, LC3-phosphatidylethanolamine conjugate (LC3-II), and Atg 3. β -actin expression was used as the internal loading control.

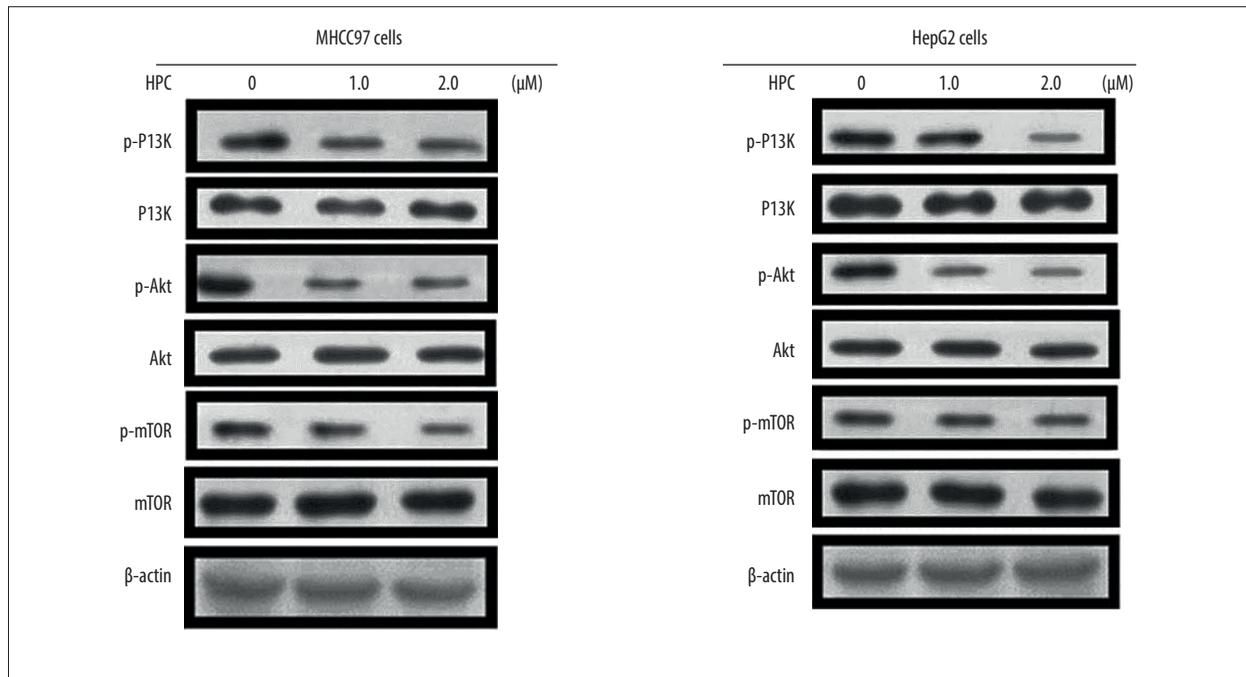


Figure 4. The effect of hydroxypyridinone-coumarin (HPC) on activation of the Akt pathway in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells. The MHCC97 and HepG2 human HCC cells after treatment with 1.0 μM and 2 μM of HPC for 72 h underwent Western blot for the detection of PI3k, Akt, and mTOR. β-actin expression was used as the internal loading control.

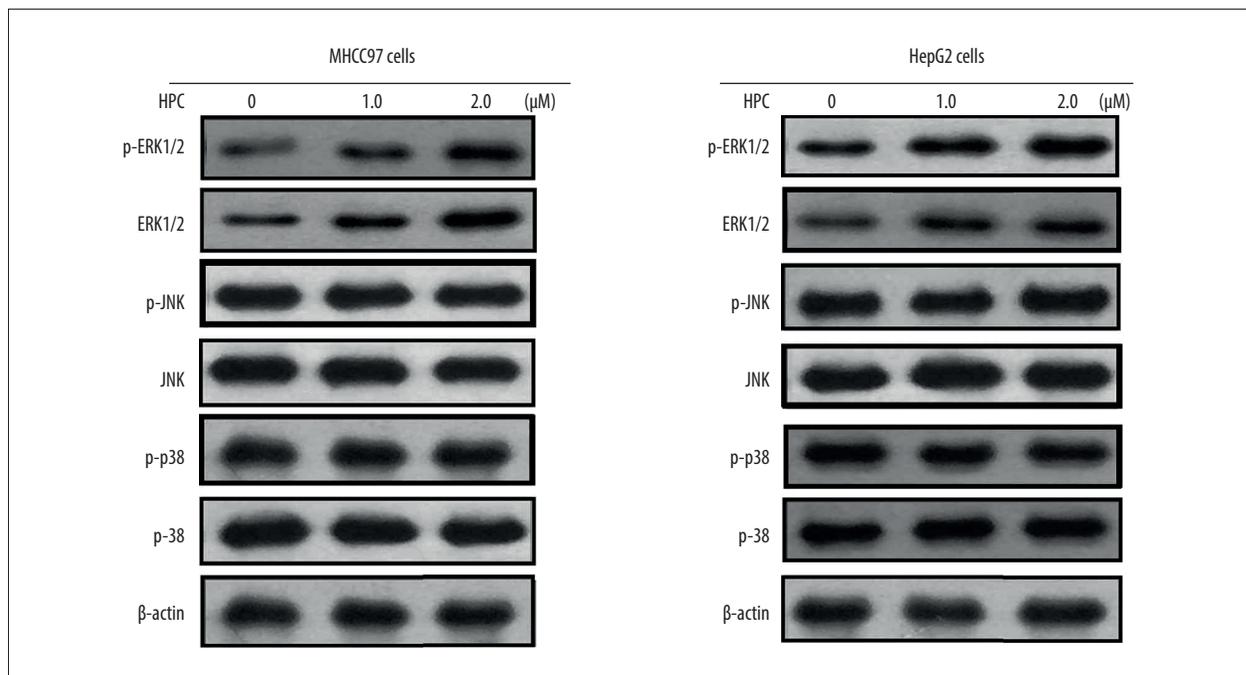


Figure 5. The effect of hydroxypyridinone-coumarin (HPC) on the expression of the mitogen-activated protein kinase (MAPK) pathway in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells. The MHCC97 and HepG2 human HCC cells after treatment with 1.0 μM and 2 μM of HPC for 72 h underwent Western blot to measure the expression levels of phosphorylated ERK1/2, JNK, and p38. β-actin expression was used as the internal loading control.

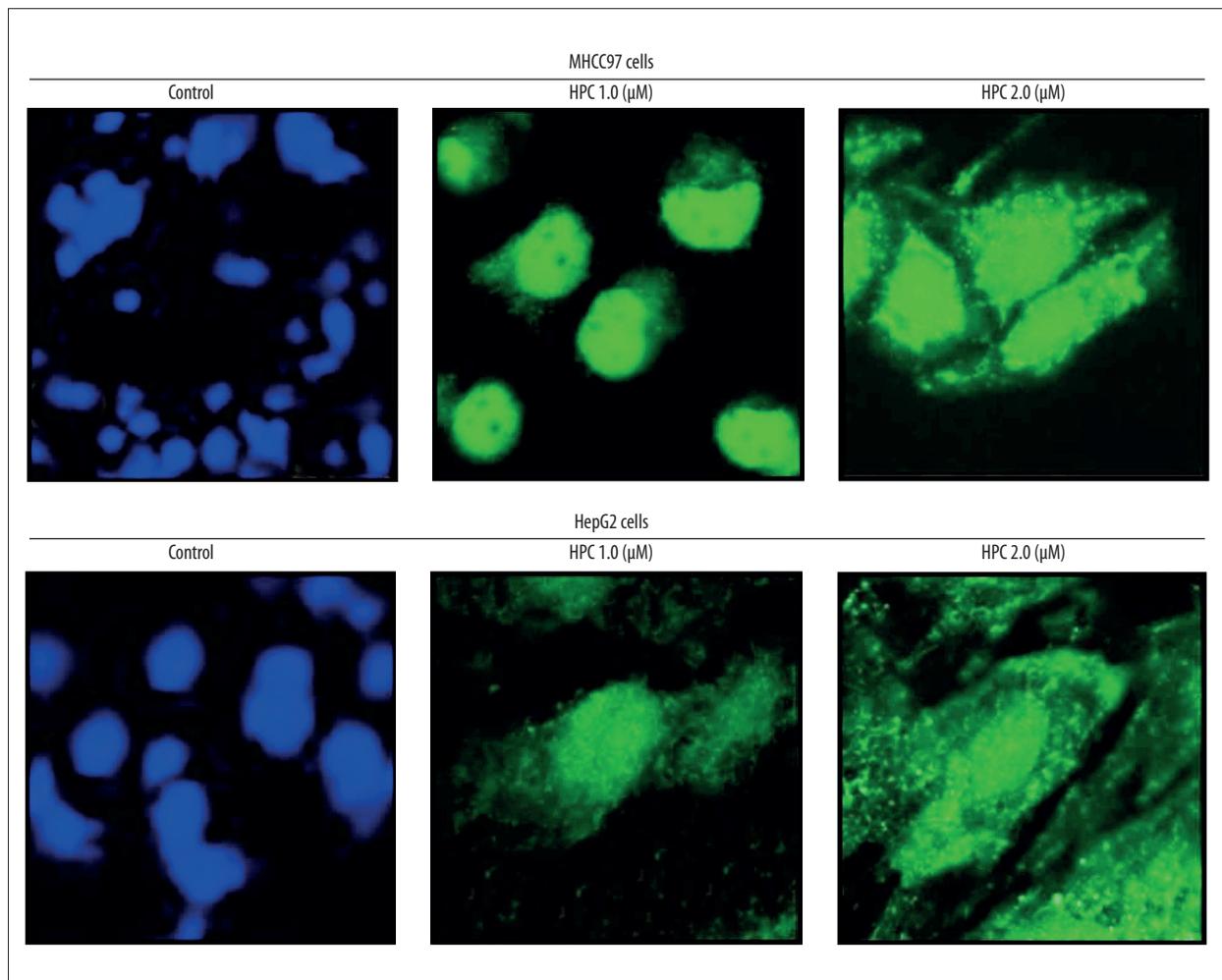


Figure 6. The effect of hydroxyppyridinone-coumarin (HPC) on GFP-LC3B labeling of autophagosomes in MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells using confocal fluorescence microscopy. The MHCC97 and HepG2 human HCC cells after treatment with 1.0 μM and 2 μM of HPC for 72 h underwent GFP-LC3B labeling. Autophagosomes were detected using confocal fluorescence microscopy with 4',6-diamidino-2-phenylindole (DAPI) staining. Magnification $\times 200$.

HPC altered the expression of autophagy-related proteins in MHCC97 and HepG2 cells

The expression of Atg-5, beclin-1, p62, and LC3-phosphatidylethanolamine conjugate (LC3-II) in MHCC97 and HepG2 cells was assessed by western blot assay at 72 h of exposure to 1.0 μM and 2 μM of HPC (Figure 3). The data showed a significant upregulation of Atg-5, beclin-1, and LC3B-II proteins in MHCC97 and HepG2 cells on exposure to HPC at 2 μM relative to control. The expression of p62 in both MHCC97 and HepG2 cells was suppressed by 2 μM of HPC.

HPC down-regulated the Akt pathway in MHCC97 cells

The Akt pathway proteins and their corresponding phosphorylated forms were determined in MHCC97 and HepG2 cells at 72 h of HPC (Figure 4). The data showed that HPC treatment

significantly reduced the expression of p-PI3K, p-Akt, and p-mTOR in MHCC97 and HepG2 cells. The PI3K, Akt, and mTOR proteins showed no significant change following treatment with HPC for 72 h.

HPC upregulated ERK1/2 activation in MHCC97 and HepG2 cells

The HPC treatment of MHCC97 and HepG2 cells at 2 μM significantly induced the phosphorylation of ERK1/2 when compared with the untreated cells (Figure 5). The expression of p-ERK1/2 was significantly increased in HPC (2 μM) treated MHCC97 and HepG2 cells at 72 h. However, the expression of p-JNK and p-p38 proteins was not changed in MHCC97 and HepG2 cells by treatment with 2 μM of HPC.

HPC promoted GFP-LC3B labeling in MHCC97 and HepG2 cells

The GFP-LC3B labeling was analyzed in MHCC97 and HepG2 cells at 72 h of exposure to 1.0 μM and 2 μM of HPC (Figure 6). The confocal fluorescent microscopy showed a significant increase in the population of GFP-LC3B-labeled MHCC97 and HepG2 cells following treatment with 2 μM of HPC. Treatment with 0.25 μM of HPC did not significantly increase the percentage of GFP-LC3B-labeled MHCC97 and HepG2 cells.

Discussion

The findings from the present study showed that hydroxypyridinone-coumarin (HPC) inhibited MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cell proliferation by inducing autophagy and that the PI3K/Akt/mTOR signaling pathway was down-regulated and ERK1/2 was upregulated. Previous studies have shown that chemotherapeutic agents, such as matrine and bufalin, which are steroid-like agents isolated from Chinese toad venom, inhibit the viability of HCC cells through the induction of autophagy [22,23]. The expression of genes that include LC3-II, which encodes LC3-phosphatidylethanolamine conjugate (LC3-II), have effects on the induction of autophagy in cells [24,25]. In the present study, HPC significantly inhibited cell proliferation of MHCC97 and HepG2 cells. This study also investigated the role of HPC on autophagy in MHCC97 and HepG2 cells, and the mechanism of the effects included inhibited inhibition of cell proliferation associated with autophagy when the dose of HPC was 2 μM . HPC significantly upregulated the expression of proteins associated with the regulation of autophagy, including Atg 5, beclin-1, Atg 3, and LC3B-II in MHCC97 and HepG2 cells. However, p62 expression was significantly reduced in both cell lines following treatment with HPC. These findings showed that HPC suppressed MHCC97 and HepG2 cell proliferation by activating autophagy in MHCC97 and HepG2 human HCC cells *in vitro*.

Cell autophagy may be induced in different types of cancers through various pathways following treatment with therapeutic agents [26–29]. Autophagy has a role in the suppression of tumor growth, and previous studies have shown that autophagy might be a potential target for the treatment of cancer [30–32]. In breast cancer cells, autophagy induced

by dihydroartemisinin involves the inhibition of nuclear factor- κB (NF- κB) nuclear translocation, while thiazolidinedione causes peroxisome proliferator-activated receptor- γ activation [27]. Curcumin treatment induces autophagy and inhibits the growth of malignant gliomas through the regulation of Akt and ERK signaling pathways [28]. In gastric cancer cells, E platinum treatment leads to the induction of autophagy by inhibiting mTOR phosphorylation [29]. The induction of autophagy also involves the regulation of the class I Akt pathway [33].

The PI3K pathway has a role in the control of several cellular physiological functions, including cell proliferation, cell viability, and the induction of autophagy and apoptosis [34]. Carcinogenesis and tumor progression are regulated by the mitogen-activated protein kinase (MAPK) pathway [35], which includes p38, MAPK, ERK1/2, and c-Jun N-terminal kinase (JNK) [36]. Previous studies have shown that chemotherapeutic agents, inflammatory factors, and reactive oxygen species (ROS) activate JNK and p38 pathways [37,38]. In the present study, the phosphorylation of Akt pathway proteins was inhibited in MHCC97 and HepG2 cells following treatment with HPC. However, no significant change was observed in the expression of PI3k, Akt, and mTOR proteins by HPC. Cell autophagy and apoptosis are also regulated by the activation of the JNK pathway [39]. In this study, HPC treatment resulted in phosphorylation of ERK1/2 in MHCC97 and HepG2 human HCC cells. The findings also showed increased expression of p-ERK1/2 following treatment of MHCC97 and HepG2 cells with HPC. However, HPC treatment did not significantly change the expression of p-JNK and p-p38 in MHCC97 and HepG2 human HCC cells studied *in vitro*.

Conclusions

This study aimed to investigate the effect of hydroxypyridinone-coumarin (HPC) on MHCC97 and HepG2 human hepatocellular carcinoma (HCC) cells and the mechanisms involved. HPC induced autophagy and inhibited the proliferation of MHCC97 and HepG2 HCC cells *in vitro* and involved activation of ERK1/2 and down-regulation of the Akt pathway.

Conflict of interest

None.

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