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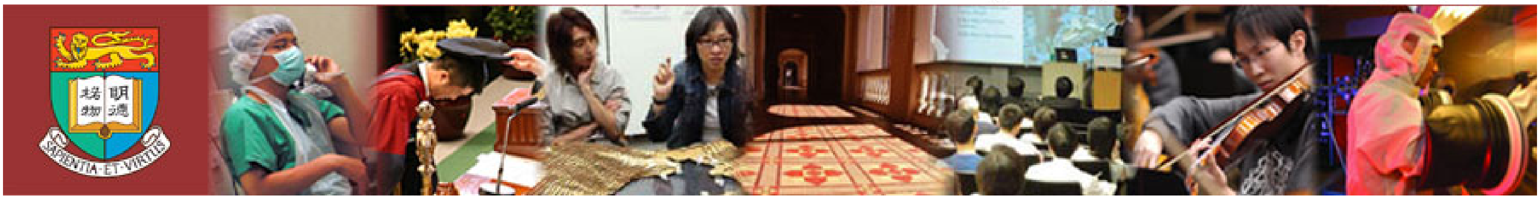

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Suppression of hypoxia inducible factor-1α (HIF-1α) by YC-1 is dependent on murine double minute 2 (Mdm2) ☆

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Abstract

Inhibition of HIF-1α activity provides an important strategy for the treatment of cancer. Recently, 3-(5′-hydroxymethyl-2′–furyl)-1-benzyl indazole (YC-1) has been identified as an anti-HIF-1α drug in cancer therapy with unclear molecular mechanism. In the present study, we aimed to investigate the effect and mechanism of YC-1 on HIF-1α in a hepatocellular carcinoma cell line under hypoxic condition, which was generated by incubating cells with 0.1% O_2. The phenotypic and molecular changes of cells were determined by cell proliferation assay, apoptosis assay, luciferase promoter assay, and Western blot analysis. YC-1 arrested tumor cell growth in a dose-dependent manner, whereas it did not induce cell apoptosis. Hypoxia-induced upregulation of HIF-1α was suppressed by YC-1 administration. YC-1 inhibited HIF-1α protein synthesis under normoxia and affected protein stability under hypoxia. YC-1 suppressed the expression of total and phosphorylated forms of murine double minute 2 (Mdm2), whereas this inhibitory effect was blocked by overexpression of Mdm2. In conclusion, YC-1 suppressed both protein synthesis and stability of HIF-1α in HCC cells, and its inhibitory effects on HIF-1α were dependent on Mdm2.

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Keywords: Hypoxia inducible factor-1α; YC-1; Murine double minute 2; Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the five most common malignancies in the world, with an increasing incidence in both Asian and Western countries [1]. Only a small proportion of patients are suitable candidates for liver transplantation, surgical resection or other surgical treatments due to the advanced stage of tumor or poor hepatic functional reserve. Transarterial chemoembolization is one of the major alternatives for the treatment of HCC patients with an advanced stage [2,3]. However, the long-term survival is unsatisfactory and the role of hypoxia in stimulating cancer growth is thought to be one of the reasons that lead to treatment failure [4].

Hypoxia is a common phenomenon in solid tumors, as oxygen supply usually does not meet the demand of tumor cells during progression [5]. The reduced oxygen levels in tumor tissues induce serial changes of hypoxia-related molecules that promote angiogenesis, among which hypoxia inducible factor-1α (HIF-1α) is the most predominant one [6,7]. Overexpression of HIF-1α was associated with angiogenesis, tumor invasion, and poor prognosis of various types of cancers [8–12]. In HCC, it was reported that activation of HIF-1α promoted upregulation of VEGF, a key player during angiogenesis [13,14]. In addition to hypoxic condition, HIF-1α could be upregulated by some therapeutic approaches, such as transarterial chemoembolization, resulting in treatment failure and poor outcomes [15]. Due to the importance of HIF-1α in tumor progression and angiogenesis,

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targeting HIF-1α becomes a potential approach of cancer therapy that has attracted great interest [12,16–18].

A number of chemicals and drugs have been discovered in recent years for targeting HIF-1α, one of which is 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1). YC-1 was first identified as an activator of platelet guanylate cyclase in 1994 and was used as a vessel dilator in circulation disorders [19]. Under hypoxic condition, YC-1 exhibited anticancer effects through inhibition of HIF-1α activity [20]. However, little is known about the possible mechanism of YC-1-mediated HIF-1α suppression. As the relationship between murine double minute 2 (Mdm2) and HIF-1α has been demonstrated by some studies, we designed the present study to investigate the potential role of Mdm2 in YC-1-mediated HIF-1α suppression.

Materials and methods

Cell lines. HepG2 human HCC cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin (Life Technologies, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell proliferation assay. Cell proliferation was determined by 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. The HepG2 cells (1×10^4) were inoculated into 96-well plates, and treated with 1% dimethylsulfoxide (DMSO) in 10% FBS-DMEM or different doses (1, 5, and 10 μM) of YC-1 (dissolved in 1% DMSO-10% FBS-DMEM), respectively, for 12 h before incubating in a humidified atmosphere of 95% N_2/5% CO_2 (the final oxygen content estimated to be 0.1%) for 24 h. MTT was then added into each well and the cells were incubated for another 4 h. The reaction was stopped with 0.04 M hydrochloride (in isopropanol) and measured at $_A$570–630 nm in a V_{max} kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The cell proliferation index was expressed as means ± SD.

Cytofluorometric apoptosis analysis. The HepG2 cells (5×10^5) were inoculated into each well of a six-well plate, and treated with 1% DMSO in 10% FBS-DMEM and different doses (1, 5, and 10 μM) of YC-1, respectively, in a hypoxic condition for 24 h. The cells were then labeled with Annexin V-FITC (BD Biosciences Pharmingen, San Diego, CA), and detected in a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Unstained cells were used as a negative control.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The TUNEL technique was performed to detect apoptotic cells using the in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN). Briefly, the HepG2 cells were cultured on cover slides with different treatments. After 24-h incubation, cover slides were fixed with 4% paraformaldehyde for 1 h and permeabilized by 0.1% Triton X-100 at 4 °C for 2 min. The slides were then incubated with TUNEL reaction mixture for 1 h at 37 °C. After washing, the slides were incubated with horse-radish peroxidase-conjugated anti-fluorescein antibody for 30 min at 37 °C. After substrate reaction, slides were counterstained with hematoxylin, and the number of apoptotic nuclei was examined under a light microscope with the magnification of 400.

Western blot. The HepG2 cells (5×10^5) were inoculated into each well of a 6-well plate, and treated with 1% DMSO in 10% FBS-DMEM and 10 μM of YC-1, respectively, for different time intervals under hypoxic condition according to the experimental design. After exposure of cells to the indicated agents and time courses, reactions were terminated by addition of lysis buffer (Cell Signaling Technology, Beverly, MA). The cell lysates were electrophoresized on 8–12% SDS–PAGE. The primary antibodies were anti-HIF-1α (Calbiochem, San Diego, CA), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mdm2 and anti-phosphorylated Mdm2 (P-Mdm2) (Cell Signaling Technology). The relative protein level was expressed by a ratio to β-actin.

HIF-1α protein synthesis and protein stability. In the protein synthesis experiment, to determine the optimal doses and time intervals of proteasome inhibitor, MG132 (Sigma–Aldrich, St. Louis, MO), at different doses, was added into the cell line, and incubated for different time periods, respectively. The expression of HIF-1α was examined by Western blot. Based on the findings of the above protocols, the dose of 40 μM MG132 and incubation time of 4 and 6 h was chosen for the following experiments. The HepG2 cells were pre-treated with 10 μM YC-1 for 12 h before adding 40 μM MG132 and incubated for 4 and 6 h, respectively, and the expression of HIF-1α was determined by Western blot. In the protein stability experiment, the HepG2 cells were incubated under hypoxic condition (0.1% O_2) for 4 h before administration of 100 μM protein synthesis inhibitor, cycloheximide (Sigma–Aldrich) with or without 10 μM YC-1, and incubated for another 30 and 60 min, respectively. Cells were lysed and protein was extracted for Western blot analysis of HIF-1α expression.

Cell transfection. Cytomegalovirus (CMV)-Mdm2 plasmid (a gift from Dr. Bert Vogelstein) [21] and empty vector were transfected for 24 h before being treated with 5 μM YC-1 under hypoxic condition. The levels of HIF-1α, Mdm2 and P-Mdm2 were also detected by the standard Western blot protocol.

Transfections and luciferase reporter assay. The HepG2 cells (1×10^5) were transfected with 1 μg of pGL3-Mdm2 reporter plasmid (a gift from Dr. Jason M. Shohet) [22] and 1 μg of pRL-TK (*Renilla* luciferase, Promega, Madison, WI) as a normalization control. Cell transfection was achieved by using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). The luciferase activities were measured by luminometer using the Dual-Luciferase Reporter Assay System according to the manufacturer's instruction (Promega).

Results

Under hypoxic condition, YC-1 exerted a dose-dependent inhibition of cell growth in the HepG2 cells with IC_{50} of 5 μM (Fig. 1A). To further examine whether the effect of YC-1 on tumor cells was cytostatic or cytotoxic, cytofluorometric apoptosis assay was performed. Under the same experimental conditions, YC-1 exhibited no significant effect on tumor cell death even with a concentration of 10 μM in a 24-h treatment (Fig. 1B). Similar to the results of Annexin-V staining, TUNEL assay did not identify any difference in the number of apoptotic cells between the groups with and without YC-1 treatment in the HepG2 cells, even with the highest dose tested (10 μM) (Fig. 1C).

When the tumor cells were pre-treated with 10 μM YC-1 for 12 h before incubating in 0.1% O_2 for another 4 h, the protein expression of HIF-1α was significantly decreased in the HepG2 cells, compared with that without YC-1 treatment (data not shown).

As HIF-1α protein is subjected to rapid degradation under normoxia by the process of pVHL-mediated ubiquitin-proteasome pathway, whereas the hypoxic condition blocks the effect of degradation and leads to accumulation of HIF-1α protein. A proteasome inhibitor, MG132, was used to prevent proteasome-mediated HIF-1α protein degradation under normoxia and the effect of YC-1 on HIF-1α protein synthesis was determined by measuring the accumulation of protein at certain time points using Western

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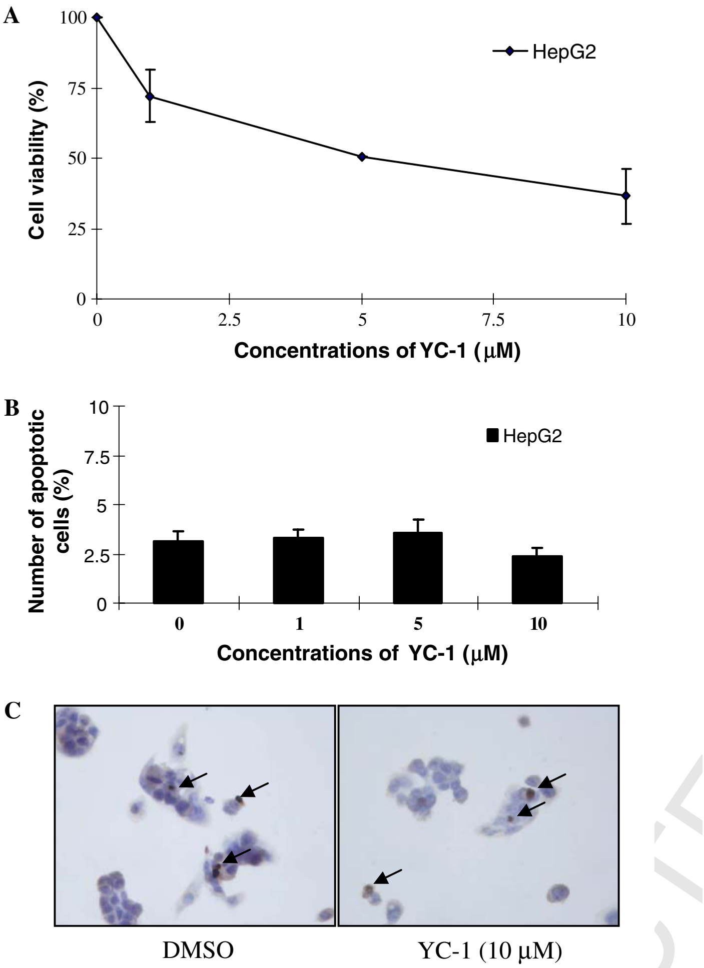

Fig. 1. YC-1 inhibited tumor cell growth under hypoxic condition. (A) The HepG2 cells were treated with different doses (1, 5, and 10 μM) of YC-1 for 12 h before incubating under 0.1% O_2 for another 24 h. The cell viabilities were assayed using MTT as described in the Materials and methods. The number of apoptotic cells was determined by (B) cytofluorometric apoptosis assay (Annexin V–FITC labeling) and (C) TUNEL assay. Under the conditions with or without YC-1 treatment, no significant difference in the number of apoptotic cells was detected by both assays. The percentage of Annexin V-FITC positive cells was expressed as means ± SD. Arrows pointed to the apoptotic nuclei. DMSO, dimethyl sulfoxide.

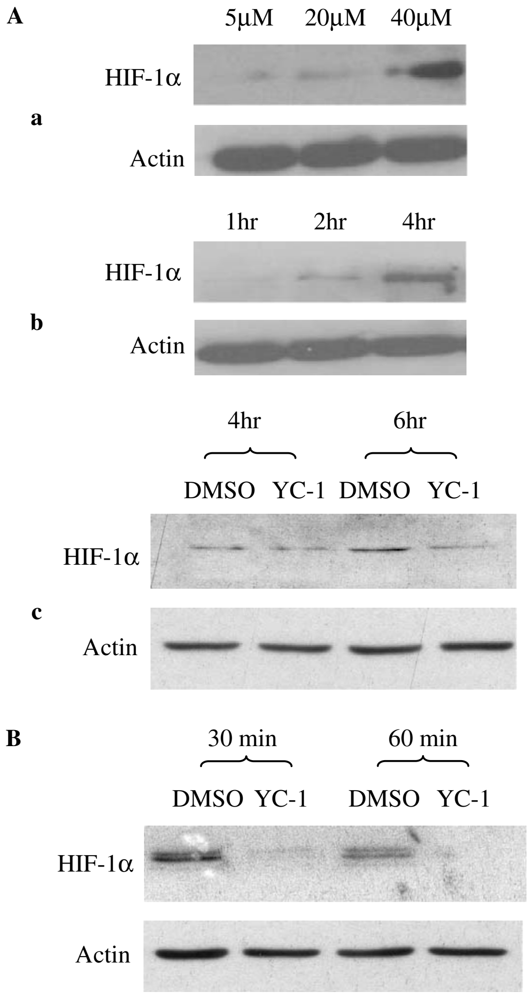

Fig. 2. (A) YC-1 inhibited HIF-1α protein synthesis under normoxic condition. To inhibit the HIF-1α protein degradation, a proteasome inhibitor, MG132, was used. (a) and (b) The HepG2 cells were treated with different doses (10, 20 or 40 μM) of MG132 for 4 h, or incubated for different time periods (1, 2 or 4 h) before determination of HIF-1α protein levels using Western blot. MG132 exhibited a dose and time dependent suppression of HIF-1α protein degradation. (c) After treated with 10 μM YC-1 and MG132 (40 μM) for 4- or 6-h, a downregulation of HIF-1α was detected. (B) YC-1 inhibited HIF-1α protein stability under hypoxic condition. The HepG2 cells were pre-treated with 0.1% O_2 for 4 h before cyclohexmide (100 μM) was added with or without YC-1 (10 μM), and incubated for 30 or 60 min. Cells were harvested and the HIF-1α protein levels were detected using Western blot. DMSO, dimethylsulfoxide. Representative of three independent experiments.

blot. The effect of MG132 on proteasome inhibition was in a dose and time dependent manner (Fig. 2A-a). As MG132 at the dose of 40 μM (Fig. 2A-a) and with the incubation time of 4 h (Fig. 2A-b) had the most significant inhibitory effect (with no obvious morphological changes of the cells), these dose and time point were chosen for the YC-1 experiment. Compared to the control groups, the protein synthesis of HIF-1α in the HepG2 cells was affected by YC-1 and a significant inhibitory effect was observed at the 6-h time point (Fig. 2A-c).

In addition to the effect of YC-1 on HIF-1α protein synthesis, its effect on protein stability was also tested. After incubating the cells under hypoxic condition for 4 h, a protein synthesis inhibitor, cycloheximide, was added into the culture medium with or without YC-1 treatment. It was found that the expression of HIF-1α protein in the DMSO control group was much higher than that in the YC-1 treated HepG2 cells (Fig. 2B).

As both the HIF-1α protein synthesis and stability could be affected by YC-1 in the HepG2 cells and Mdm2 was a potential upstream molecule that regulated HIF-1α expression, the possible link between Mdm2 and YC-1-mediated HIF-1α suppression was investigated. The HepG2 cells were treated with 10 μM YC-1 under hypoxia for 1, 2, and 4 h, respectively, and the expression of HIF-1α, total Mdm2, and P-Mdm2 was detected by Western blot. A concurrent downregulation of HIF-1α, total Mdm2, and

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P-Mdm2 was detected with YC-1 treatment for 2 and 4 h under hypoxic condition (Fig. 3A).

In order to further examine whether YC-1 mediated its effect on HIF-1α expression through suppression of Mdm2, under hypoxia, cells were transfected with CMV-Mdm2 plasmid for 24 h before DMSO or YC-1 was added. The transfection of Mdm2 induced a significant increase in the expression of total Mdm2 and P-Mdm2. In addition, the enhanced expression of Mdm2 by transfection could increase HIF-1α level despite the presence or absence of YC-1 treatment in the HepG2 cells (Fig. 3B).

The previous experiments revealed that YC-1 might mediate its inhibitory effect on HIF-1α expression by downregulation of Mdm2 protein. It was of interest to know whether YC-1 affected Mdm2 expression at the transcriptional level or protein level. Therefore, wild type Mdm2 promoter constructed in luciferase reporter plasmid was transfected before YC-1 administration. It was found that 10 μM YC-1 significantly suppressed Mdm2 transcription in hypoxic HepG2 cells by an average of 2-fold compared with DMSO control (Fig. 3C).

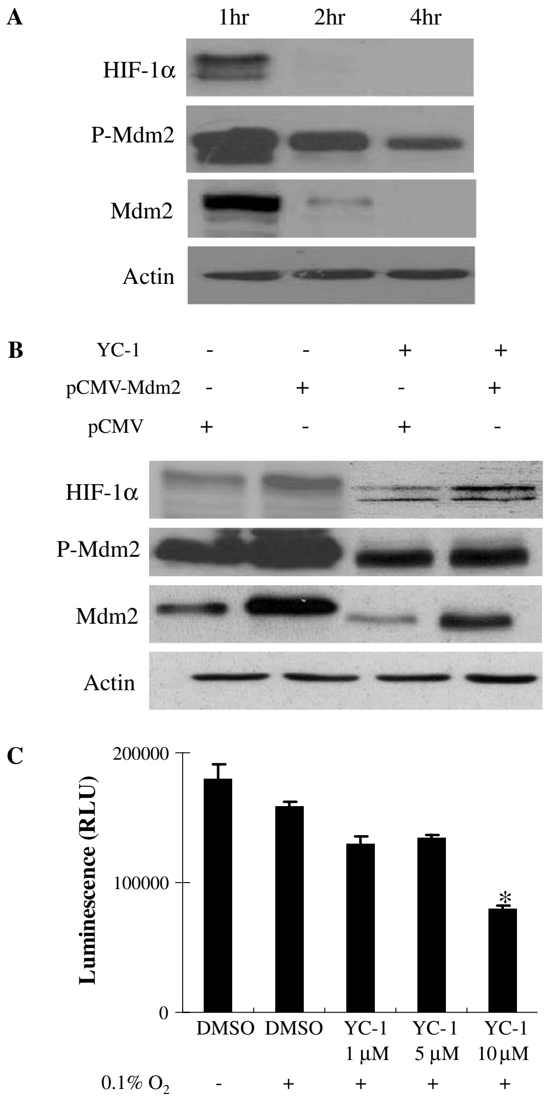

Fig. 3. (A) YC-1 suppressed the expression of HIF-1α, total and phosphorylated forms of Mdm2 under hypoxic condition in a time dependent manner. The HepG2 cells were treated with 10 μM YC-1 under hypoxia for different time intervals (1, 2 or 4 h). Cells were then harvested for the detection of HIF-1α, Mdm2, and P-Mdm2 expression using Western blot. (B) Upregulation of Mdm2 by transfection reversed YC-1-mediated HIF-1α suppression. The HepG2 cells were transfected with either empty vector or CMV-Mdm2 for 24 h. After transfection, the cells were treated with DMSO or 10 μM YC-1 and incubated under 0.1% O_2 for 4 h before determination of HIF-1α, Mdm2, and P-Mdm2 expression using Western blot. (C) YC-1 suppressed the promoter activity of Mdm2 in the HepG2 cells. Cells were co-transfected with 1 μg of pGL3-Mdm2 reporter plasmid and 1 μg of pRL-TK as a normalization control. The luciferase activity or *Renilla* luciferase activity was measured by luminometer using Dual-Luciferase Reporter Assay System according to the manufacturer's instruction. DMSO: dimethyl sulfoxide. The Firefly luciferase activity was normalized with *Renilla* luciferase activity. $^{*}P < 0.05$, compared with DMSO control under hypoxia (Student's *t* test). Representative of three independent experiments.

Discussion

In the present study, we demonstrated that YC-1 inhibited the growth of HCC cells. This was consistent with the study of Wang et al., [23], which suggested that YC-1 exhibited an anti-proliferative effect by arresting the cell cycle in the G0–G1 phase in HCC cells. Similar effect was also found in endothelial cells and mesangial cells [24,25]. However, our data did not support a previous finding in prostate cancer that YC-1 could induce apoptosis of tumor cells [26]. Even with the dose of 10 μM, YC-1 exhibited no effect on induction of cell apoptosis examined by both TUNEL assay and cytofluorometric apoptosis assay, suggesting that YC-1 inhibited the activity of HCC cells through a cytostatic pathway rather than a cytotoxic one.

Although the anti-HIF-1α effect of YC-1 has been well demonstrated in several studies, the molecular basis of YC-1-mediated HIF-1α suppression remains largely unclear. The present study revealed that YC-1 could affect both protein synthesis and protein stability of HIF-1α, suggesting dual effects of YC-1 on suppressing HIF-1α expression. To further explore the suppressive effect of YC-1 on protein synthesis, we performed another set of experiments to investigate whether this inhibitory effect was related to the mammalian target of rapamycin (mTOR) signaling pathway, as several downstream molecules of mTOR, such as ribosomal S6 kinase and eukaryote initiation factor 4E binding protein 1, were key regulators in protein translation and synthesis [27,28]. However, we did not detect any changes of these molecules after YC-1 treatment (data not shown), implying that YC-1-mediated inhibition of protein synthesis was independent of mTOR signaling pathway. Therefore, further studies are needed to explore other pathways that are related to protein synthesis.

Based on some studies demonstrating that Mdm2 might play a potential role in HIF-1α protein stability [29,30], we investigated the relationship among YC-1, HIF-1α, and

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Mdm2 in the present study. With the downregulation of HIF-1α, the protein level of Mdm2 was significantly decreased with YC-1 administration in a time dependent manner, indicating that Mdm2 might be involved in YC-1-mediated HIF-1α suppression. To further prove this hypothesis, we induced upregulation of Mdm2 in the HepG2 cells by transfection before DMSO or YC-1 administration, and found that the increased expression of Mdm2 could reverse the inhibitory effect of YC-1 on HIF-1α expression, suggesting that YC-1 regulated HIF-1α expression was Mdm2 dependent. To further explore whether YC-1 functioned on Mdm2 at a transcriptional level, we measured the promoter activity of Mdm2 under the conditions with or without YC-1 treatment, and found that YC-1 could decrease the promoter activity of Mdm2, suggesting that YC-1 might act on the transcriptional level of Mdm2. In addition, by detecting a downregulation of Fli-1, an upstream transcriptional regulator of Mdm2 [31], this study suggested that YC-1 functioned on the transcriptional level of Mdm2 in the cells with endogenous Mdm2.

In conclusion, YC-1 retarded cell growth and exhibited a cytostatic effect in the HCC cells under hypoxic condition. YC-1 downregulated HIF-1α expression by affecting both protein synthesis and stability, and the inhibitory effects of YC-1 on HIF-1α were dependent on Mdm2.

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