

Effects of YC-1 targeting hypoxia-inducible factor 1 alpha in oesophageal squamous carcinoma cell line Eca109 cells

Yadong Feng, Hong Zhu, Tingsheng Ling, Bo Hao, Guoxin Zhang and Ruihua Shi¹

Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, People's Republic of China

Abstract

HIF-1 α (hypoxia-inducible factor 1 alpha) is believed to promote oesophageal squamous tumour growth. Thus, an HIF-1 α inhibitor is viewed as a therapeutic target in treating oesophageal cancer. Recently, YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] has been widely used as a potential HIF-1 α inhibitor and is being developed as a novel anticancer drug. However, little is known about the effects of YC-1 in human oesophageal cancer. In the present study, we aimed to investigate these effects in an esophageal squamous cancer cell line; i.e. Eca109 cells. We found that YC-1 abolished the hypoxia-induced up-regulation of HIF-1 α . YC-1 arrested cell growth and inhibited cell migration activities in Eca109 cells. These results suggest that YC-1 may be a chemotherapy candidate against oesophageal squamous cancers.

Keywords: apoptosis; ESCC; hypoxia; proliferation; YC-1

1. Introduction

ESCC (oesophageal squamous cell carcinoma) occurs mostly commonly in eastern Asian countries, including China, with the highest mortality in the world. Only a small proportion of patients with ESCC receive surgical resection. Chemoradiotherapy is the major treatment alternative for patients with an advanced stage ESCC. However, a hypoxia microenvironment is involved in ESCC, which seems to result in photodynamic therapy resistance (Sohda et al., 2004; Ji et al., 2006). Therefore, the prognosis for patients with ESCC remains poor.

ESCC is one of the common solid tumours with low oxygen tension. Hypoxia in tumour tissues induces serial changes that promote tumour growth, invasion and metastasis, among which is the overexpression of HIF-1 (hypoxia-inducible factor 1), which is the most predominant regulator of oxygen homeostasis (Chan et al., 2007; Benizri et al., 2008; Semenza, 2008). Evidence has been provided that overexpression of HIF-1 is associated with a malignant phenotype (Gordan and Simon, 2007; Maxwell et al., 2007). HIF-1 is a heterodimer protein composed of HIF-1 α and HIF-1 β , and HIF-1 protein stabilization and transactivation depends on the HIF-1 α subunit. Under normoxic conditions, HIF-1 α always remains at low levels, and hypoxia can lead to a rapid increase in the level of HIF-1 α expression and activates transcription of more than 60 target genes by binding to the 5'-ACGTG-3' in the 3' flanking region of these genes (Ambrosini et al., 2002; Binley et al., 2003). Thus, HIF-1 α has various cancer biological activities, including angiogenesis, invasion, cell survival and apoptosis.

Recently many chemicals with anti-HIF-1 α effects have been developed, one of which is YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole]. YC-1 shows anticancer effects by down-regulation of hypoxia-induced genes via the HIF-1 α pathway (Chun et al., 2001; Yeo et al., 2003; Kim et al., 2006). However, little is known about the effects of YC-1 in ESCC. In addition, the possible mechanisms of

YC-1-mediated-HIF-1 α suppression remain to be explored (Chiang et al., 2005; Kim et al., 2006; Lau et al., 2006; Yeo et al., 2006; Sun et al., 2007; Li et al., 2008). In the present study, we investigated the effects of YC-1 in ESCC cell line Eca109 cells. We further investigated the anticancer mechanisms of YC-1 in Eca109 cells and possible mediating signalling pathways were evaluated.

2. Materials and methods

2.1. Materials

YC-1 (Cayman) was dissolved in DMSO to achieve the stock concentration 10 mmol/l and was stored at -20°C . The final concentration of DMSO in the culture medium was kept below 0.1%. A hypoxic incubator was used in our study (Thermo Scientific).

2.2. Cell line and cell culture

Human ESCC cell line Eca109 cells were purchased from the Shanghai Institute of Biochemistry. Cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Gibco, Invitrogen Corporation) with high glucose, supplemented with 10% FBS (fetal bovine serum, Gibco, Invitrogen Corporation), 1% penicillin and 50 mg/ml streptomycin. All cells were grown in a humidified 5% CO₂ atmosphere at 37°C in an incubator, in which oxygen tension was held at either 20% (normoxic condition) or below 1% (hypoxic condition).

2.3. Colony formation assay

Eca109 cells, 5000, were seeded on to 60-mm dishes. After 24 h, various concentrations (0, 0.1, 1, 10 and 100 μM) of YC-1 were added to the dishes. The drug in the DMEM medium with 10%

¹ To whom correspondence should be addressed (email ruihuashi@126.com).

Abbreviations: c-JNK, c-Jun-NH₂-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; HIF-1 α , hypoxia-inducible factor 1 alpha; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

FBS was changed twice a week and incubated for 2 weeks. Then colonies were fixed with 70% ethanol and stained by HE (Haematoxylin and Eosin).

2.4. Cell viability assay

Cell viability was determined by using a tetrazolium-based assay (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay). Eca109 cells, 2×10^3 , were seeded on to 96-well plates. After 24 h incubation to allow for attachment, different doses (0, 1, 5, 10, 20, 30 and 40 μ M) of YC-1 were added into the plates. Cells were then incubated for 24, 48 or 72 h. After incubation, MTT was added into each well (1 mg/ml), and cells were incubated for 4 h. Then, the formazan precipitate was dissolved in 200 μ l of DMSO, and absorbance was measured in a microplate reader at a wavelength of 570 nm.

2.5. Cell cycle assay

Eca109 cells, 1×10^6 , were seeded on to 60-mm dishes. After 24-h incubation to allow for attachment, cells were then treated with different doses of YC-1 for 24, 48 or 72 h. Cells were harvested and fixed with 70% ice-cold ethanol at -20°C after treatment. After fixation, cells were washed with PBS, then labelled with propidium iodide (0.05 mg/ml) in the presence of RNase A

(0.5 mg/ml) and incubated at room temperature in the dark for 30 min. DNA contents were analysed by a flow cytometer (FACS; Becton Dickinson), and cell cycle phase distributions were analysed by ModFit software.

2.6. Cytofluorometric apoptosis assay

Eca109 cells, 1×10^6 , were seeded on to 60-mm dishes. After 24-h incubation under normoxic conditions, YC-1 was added into the medium. The cells were incubated under either normoxic or hypoxic conditions for 48 h. The cells were then labelled with AnnexinV-FITC and propidium iodide (BD Biosciences Pharmingen) and detected by a flow cytometer (FACS; Becton Dickinson). Unstained cells were used as a negative control.

2.7. Cell migration assay

Eca109 cell migration assays were performed in Transwell chambers with 6.5-mm diameter filters containing 8- μ m pores (Corning Life Science). YC-1, 35 μ M, in DMEM containing 10% FBS as a chemoattractant was added into the lower chambers. Then, aliquots of 1×10^4 cells in 100 μ l of FBS-free DMEM were seeded into the upper chambers. After incubation under either normoxic or hypoxic conditions for 24 h, cells were fixed with ice-cold methanol and stained with HE staining. Cells on the upper side of the membrane were removed using a cotton swab. Then, the migrated cells were counted under a light microscope at $\times 200$ magnification.

2.8. Western blot analysis

Eca109 cells were incubated with YC-1 (with terminal concentration 0, 20 μ M) under both normoxic and hypoxic conditions for 48 h. Total proteins were then extracted. Protein samples, 40 μ g, were separated in 10–15% SDS/PAGE gels and then electrophoretically transferred to PVDF membranes (Millipore). The membrane was blotted with 5% non-fat milk, washed and then probed with antibodies of p53 (1:1000), Bcl2 (1:1000), Bax (1:1000) and survivin (1:1000), HIF-1 α (1:500), VEGF (1:500), MMP2 (1:2000) and α -tubulin (1:5000) overnight at 4°C . After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary

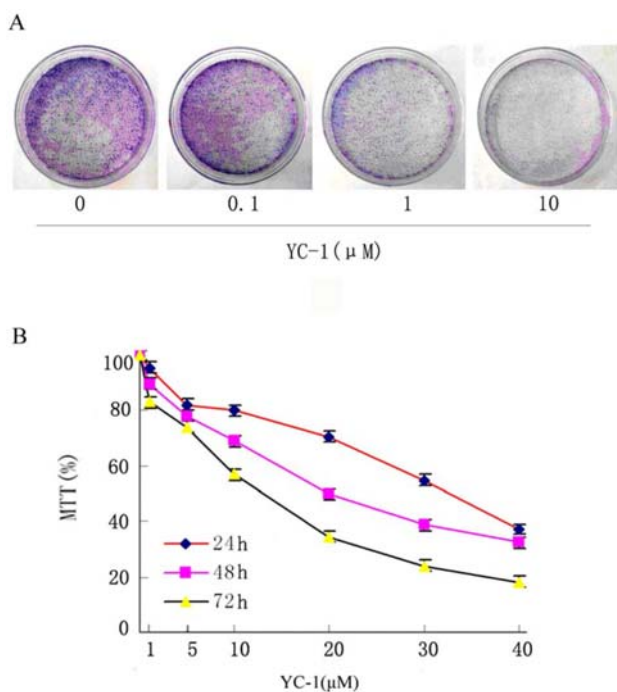


Figure 1 YC-1 exerted growth inhibition in Eca109 cells (A) YC-1 inhibited colony formation ability of Eca109 cells. Decreased numbers of colonies were detected with YC-1 administration. (B) Determination of cell viability by YC-1 in Eca109 cells (MTT assay). Cells were treated with YC-1 at 1, 5, 10, 20, 30, 40 μ M for 24, 48, 72 h. Each value is presented as mean \pm S.D. of three independent experiments. YC-1 inhibited Eca109 cell proliferation in a time- and dose-dependent manner. The IC₅₀ for 24, 48, 72 h were 35, 20 and 15 μ M respectively.

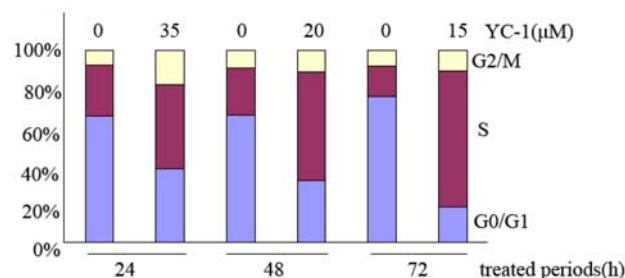


Figure 2 YC-1 induced S arrest in Eca109 cells Cells were treated with 35, 20, 15 μ M YC-1 for 24, 48, 72 h, respectively. The cell cycle populations were examined by flow cytometry after treatment. The data are presented as means \pm S.D. of three independent experiments.

antibodies (Dako Corporation), visualized by an enhanced chemiluminescence detection kit (Pierce, Thermo Scientific USA) and exposed to film.

2.9. Statistical analysis

Data were expressed as means \pm S.D. and analysed using SSPS13.0. The Mann–Whitney U test was used to assess the statistical significance of differences, with P values of <0.05 considered statistically significant.

3. Results

3.1. YC-1 suppressed cell proliferation in Eca109 cells

We investigated whether YC-1 treatment showed an antiproliferation effect against Eca109 cells. YC-1 treatment inhibited the colony formation ability of Eca109 cells. YC-1 treatment at a 10 μ M dose for 14 days resulted in almost 100% inhibition (Figure 1A). The YC-1 treatment-induced antiproliferation effect occurred in a time- and dose-dependent manner (Figure 1B).

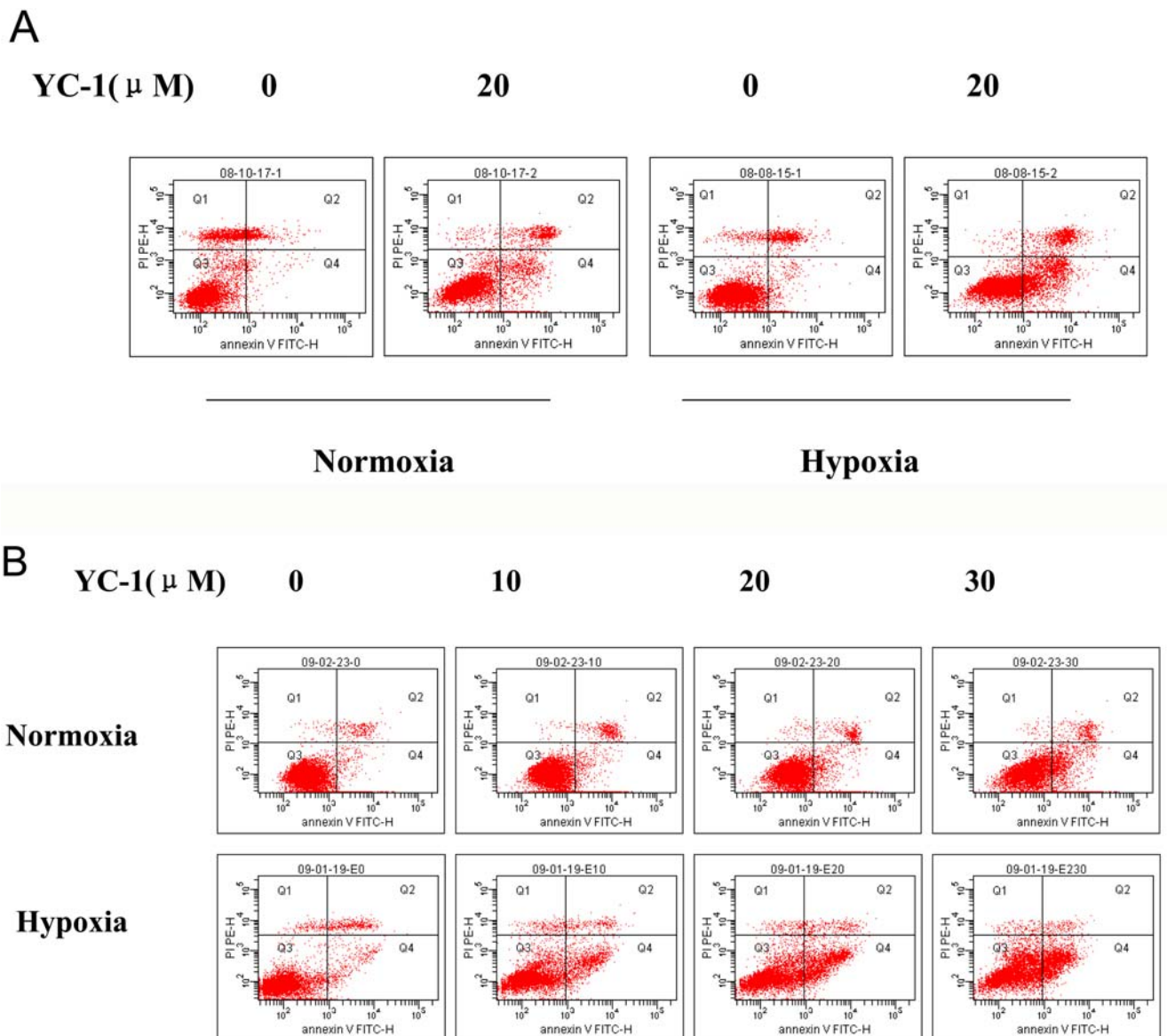


Figure 3 YC-1 caused apoptosis in Eca109 cells

(A) Effects of YC-1 on apoptosis under normoxia and hypoxia. YC-1, 20 μ M treatment for 48 h increased apoptosis both under normoxia and hypoxia. The data are presented as means \pm S.D. of three independent experiments. (B) YC-1 induced strong apoptosis in Eca109 cells. Compared with the control group, the apoptosis rate increased with increasing dose of YC-1. Representative of three independent experiments.

YC-1-induced cytotoxicity in Eca109 cells was assessed by measuring IC₅₀. The IC₅₀ values were approximately 35, 20 and 15 μ M at 24, 48 and 72 h, respectively. To clarify the mechanism of YC-1-induced inhibition, the effect of YC-1 on the cell cycle was analysed. YC-1 at 35, 20 and 15 μ M, was used to treat Eca109 cells for 24, 48 and 72 h, respectively. Cell populations in the G₀-G₁ and S-phases were 65% and 26%, 67% and 24%, 76% and 15%, respectively, in control Eca109 cells. After incubation with YC-1, the S phase population was noticeably enhanced (42%, 54% and 71%, respectively) while the G₀-G₁ population was decreased (32%, 36% and 18%, respectively; Figure 2).

3.2. Exposure to YC-1 induced apoptosis in Eca109 cells

To evaluate its biological significance, we assessed the effect of YC-1 on apoptotic death in Eca109 cells under normoxic and hypoxic conditions. Results of Western blots showed that the expression of HIF-1 α in Eca109 cells increased markedly for 48 h under hypoxia (data not shown). Therefore, YC-1 was added to the medium (20 μ M dose) and incubated for 48 h both under normoxia and hypoxia. YC-1 was absent in the control groups. Annexin V/PI staining of control groups showed a large viable cell population with very few cells staining as early apoptotic, late apoptotic or dead (Figure 3A). Treatment of cells with YC-1 at a 20 μ M dose for 48 h resulted in a strong shift from live cells to early apoptotic cell populations and late apoptotic cell populations both under normoxic and hypoxic conditions. Then, serial doses of

YC-1 were added into the medium with final concentrations of 0, 10, 20 and 30 μ M, and Eca109 cells were treated for 48 h under normoxic and hypoxia conditions. The apoptotic assay clearly showed dose-dependent apoptotic effects of YC-1 in Eca109 cells (Figure 3B).

3.3. YC-1 suppressed migration activity in Eca109 cells

Since HIF-1 α controls the expression of many metastasis-associated genes, such as MMP-2 (matrix metalloproteinase-2), we investigated whether YC-1 interrupts the metastasis activity in Eca109 cells by a migration assay. Cell migration was found to be markedly decreased by YC-1 under both normoxia and hypoxia. However, hypoxia showed no significant effects on cell migration (Figure 4).

3.4. Effects of YC-1 on HIF-1 α and HIF-1 α -mediated genes in Eca109 cells

VEGF, MMP-2 and Bcl-2 family proteins have been identified as targets of HIF-1 α . Therefore, HIF-1 α , VEGF, MMP-2, Bcl-2, Bax were assessed by Western blotting detection. YC-1 exerted an inhibitory effect on HIF-1 α and HIF-1 α transcriptionally driven genes. As shown Figure 5(A), 20 μ M YC-1 treatment for 48 h resulted in a down-regulation of HIF-1 α , VEGF, MMP-2 and Bcl-2, while Bax was up-regulated under both normoxia and hypoxia (Figure 5A). The major anti-apoptotic function of survivin, one of the inhibitors of apoptosis, results in blocking apoptotic cell death. In addition, p53 plays an

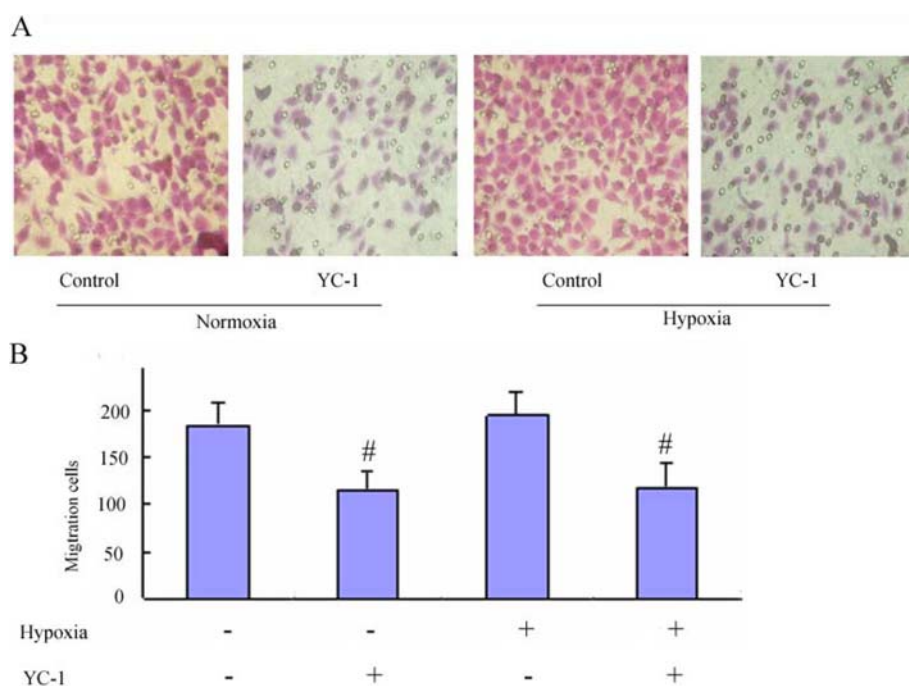


Figure 4 YC-1 inhibited cell migration in Eca109 cells

(A) Effect of YC-1 on cell migration. Cells were loaded into top chambers and treated with 0, 35 μ M YC-1 for 24 h. After incubation, migrated cells were stained and counted under a light scope at a magnification of $\times 200$. (B) Numbers of migrated cells and statistical analyses. The data are presented as means \pm S.D. of three independent experiments. [#] $P < 0.01$ compared with control (without YC-1).

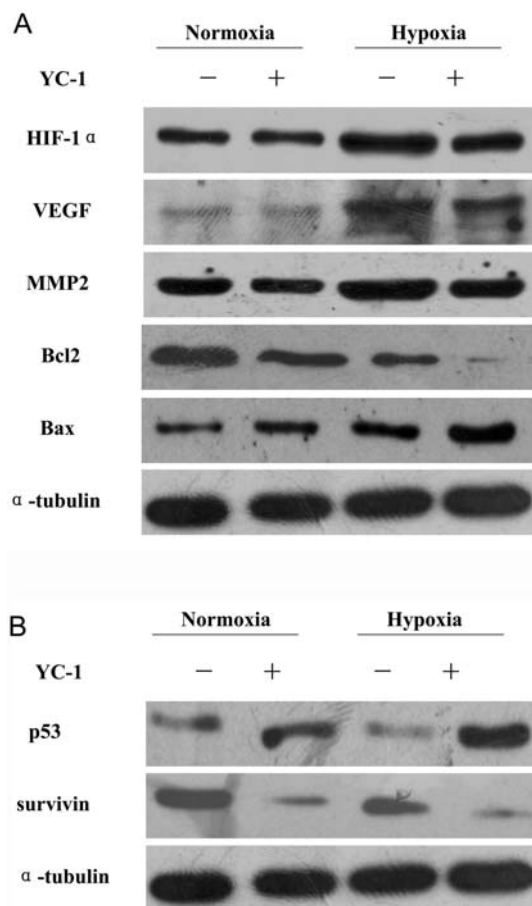


Figure 5 Effects of YC-1 on HIF-1 α and HIF-1 α -associated proteins in Eca109 cells. Eca109 cells were treated with 20 μ M YC-1 for 48 h both under normoxia and hypoxia. Then, cells were harvested and subjected to Western Blot. **(A)** HIF-1 α and HIF-1 α transcriptionally driven genes. Decreased levels of HIF-1 α , VEGF, Bcl2, MMP 2 were detected, while expression of Bax was increased. **(B)** p53 and survivin protein expression. YC-1 up-regulated protein expression of p53 and down-regulated expression of survivin.

important role in mediating apoptosis in the hypoxic regions of tumours. We therefore assessed whether YC-1 treatment resulted in a change in expression of p53 and survivin. YC-1 treatment of cells resulted in a strong to complete decrease in survivin protein levels and an increase in the expression of p53 (Figure 5B).

3.5. Effects of PD98059, SP600125, SB203580 and LY294002 on YC-1-mediated HIF-1 α expression inhibition

To determine the role of MAPKs and PI3K signalling cascades in the YC-1 effect, we used the ERK (extracellular signal-regulated kinase)-specific inhibitor (PD98059), c-JNK (c-Jun-NH₂-terminal kinase)-specific inhibitor (SP600125), p38 MAPK-specific inhibitor (SB203580) and PI3K-specific inhibitor (LY294002) on sustained activation of MAPKs and PI3K. Cells were pretreated with or without the aforementioned inhibitors for 1 h and then were incubated with or without YC-1 for 48 h under both normoxia and hypoxia. After treatment, expression of HIF-1 α was examined by Western blotting analysis. YC-1-induced HIF-1 α suppression was

reversed by PD98059, SP600125, SB20380, respectively, while LY294002 treatment showed a synergistic effect on YC-1-mediated HIF-1 α suppression (Figure 6).

4. Discussion

HIF-1 α is induced by hypoxia, and under non-hypoxic conditions, HIF-1 α is minimally expressed because of hydroxylation by a prolyl hydroxylase enzyme (Chan et al., 2002; To and Huang, 2005). However, it has been reported that normoxic expression of HIF-1 α can be stabilized by transition metals, nitric oxide and reactive oxygen species. In addition, in some tumours, normoxic HIF-1 α is overexpressed due to genetic alterations in oncogenes (Semenza, 2002). Moreover, loss-of-function mutations in tumour suppressor genes also contribute to high levels of HIF-1 α in normoxic conditions (Baba et al., 2003). In ESCC, overexpression of HIF-1 α has been reported (Kurokawa et al., 2003; Katsuta et al., 2005), so it is understandable that HIF-1 α can be detected in Eca109 cells under normoxia. Thus, the blockade of HIF-1 α appears to be a promising strategy for the treatment of ESCC.

In previous studies, YC-1 has been found to markedly reduce HIF-1 α and HIF-1 α -regulated genes expression, vascularization and tumour growth. YC-1 was identified as a substance with multiple pharmacological actions, among which the most important may be anti-HIF-1 α activity (Chun et al., 2001; Yeo et al., 2003; Kim et al., 2006). YC-1 is considered as a novel anticancer agent.

In the present study, we first demonstrated YC-1 exerted HIF-1 α suppression activity in ESCC cell line Eca109 cells. We found that YC-1 inhibited the proliferation of Eca109 cells in both a dosage- and time-dependent manner. To evaluate the mechanism of YC-1-induced inhibitory proliferation, the cell cycle was analysed. In our present study, YC-1 resulted in S-phase cycle arrest in Eca109 cells. This is consistent with the study of Yeo et al. (2006). However, it has been reported that YC-1 also induces G₀/G₁ cell cycle arrest (Wang et al., 2005). HIF-1 α plays a key role in anti-apoptosis in many malignant tumours. During hypoxia, anti-apoptotic proteins, such as IAP and Bcl2, are up-regulated by HIF-1 α ; meanwhile, HIF-1 α is found to initiate apoptosis by inducing expression of tumour suppressors such as p53, Bax and BNIP3 (Greijer and van der Wall, 2004; Zhou et al., 2006). Since these findings seem to be contradictory, there may be an intricate balance between factors that induce or counteract apoptosis (Piret et al., 2002). According to our results, YC-1 induced apoptosis by down-regulation of Bcl2 and survivin. Up-regulation of Bax by YC-1 was also observed in our study. Previous studies demonstrated that decreased levels of Bcl2, survivin and up-regulated Bax are involved in YC-1-induced apoptosis (Lau et al., 2007; Chen et al., 2008). Tumour invasion and metastasis are vital cancer characteristics regulated by HIF-1 α . We displayed YC-1-inhibited cell migration by down-regulation expression of MMP2 and VEGF. However, hypoxia showed no significant effects on migration in our study. As mentioned above, overexpression of basal HIF-1 α was detected in Eca109 cells, which might contribute to cell migration, even under normoxia. Many elegant studies showed that tumours always exert a positive selection pressure on the loss-of-function

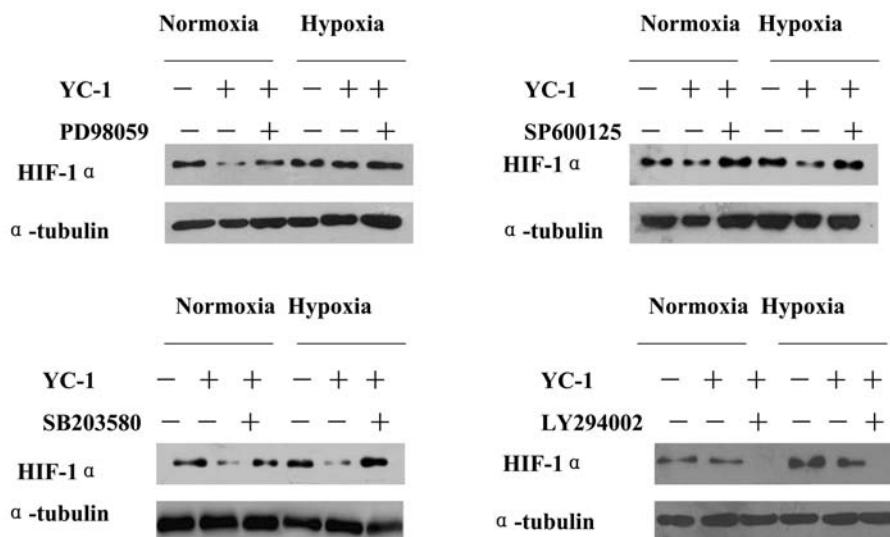


Figure 6 YC-1 induced HIF-1 α inhibition by the ERK, c-JNK and p38 cascades in Eca109 cells

Cells were pretreated with or without inhibitors (PD98059, SP600125, SB203580, LY294002) for 1 h and then treated with 20 μ M for 48 h both under normoxia and hypoxia. The concentrations of PD98059, SP600125, SB203580 and LY294002 were 20, 15, 10 and 20 μ M, respectively. After treatment, cells were harvested and subjected to Western blot. YC-1-induced HIF-1 α expression inhibition was partly abolished by PD98059, SP600125 and SB203580. However, LY294002s synergistically down-regulated YC-1-mediated HIF-1 α suppression.

mutation of p53 (Jackson et al., 2005; Dearth et al., 2007; McMurray et al., 2008). In our present study, p53 was minimally expressed, even under hypoxic conditions, which might contribute to basal overexpression of HIF-1 α . YC-1 treatment induced higher levels of p53, which might be involved in HIF-1 α suppression, proliferation inhibition and apoptosis.

It seems that some cytoskeleton proteins may be involved in YC-1-mediated antitumour activity. However, YC-1 does not alter the expression of some vital membrane receptors such as Fas, DR4, DR5 and VEGF receptors (Funasaka et al., 2005; Wu et al., 2008). Mechanistically, YC-1 inhibiting tumour growth action is due to anti-HIF-1 α activities. YC-1 has been found to accelerate HIF-1 α degradation and inhibits HIF-1 α synthesis. So, in the present study, we postulated that the inhibitory effects of YC-1 on excessive expression of HIF-1 α *de novo* synthesis are due to the overexpression of HIF-1 α in Eca109 cells, even in normoxia. It has been demonstrated that MAPKs and PI3K play important roles in HIF-1 α synthesis and transactivation (Comerford et al., 2004; Liu et al., 2005; Ryan et al., 2007; Liu et al., 2008). Recent studies have suggested that, in malignant tumours, YC-1-induced HIF-1 α expression suppression was partly or completely reversed by p38 and JNK inhibitors, respectively (Chiang et al., 2005; Wang et al., 2005; Sun et al., 2007). To investigate the possible mechanisms in Eca109 cells, we elucidated whether YC-1 had any effects on MAPKs–HIF-1 α and PI3K–HIF-1 α interactions. According to our results, YC-1-mediated HIF-1 α inhibition was abrogated by PD98059, SB203580 and SP600125, which suggested that ERK, p38 MAPK and c-JNK may be involved in YC-1-mediated HIF-1 α expression suppression. However, inhibition of PI3K did not modify the effect of YC-1-induced HIF-1 α suppression, suggesting that the PI3K pathway might not be involved in the YC-1 HIF-1 α inhibitory effect in Eca109 cells.

In summary, we find that YC-1 shows multiple anticancer effects in ESCC Eca109 cells. On the basis of our findings, YC-1 induces both

cell cycle arrest and apoptosis and inhibits proliferation and invasion ability *in vitro* by HIF-1 α suppression, which seems to occur via ERK, p38 and c-JNK/MAPK pathways. Consistent with other findings, our study may provide a strong rationale for intervention strategies for YC-1 in ESCC treatment.

Author contribution

Yadong Feng and Hong Zhu contributed equally to this work. Ruihua Shi designed this work. Bo Hao and Guoxin Zhang did the cell culture and proliferation, and the apoptosis measurements. Statistical analysis was carried out by Tingsheng Ling.

Acknowledgements

We thank Dr Hongjie Zhang and Jie Hua for their co-operation during the process of the experiments.

Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 30770991, 30800511].

References

- Ambrosini G, Nath AK, Sierra-Honigmann MR, Flores-Riveros J. Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. *J Biol Chem* 2002;277:34601–9.
- Baba M, Hirai S, Yamada-Okabe H, Hamada K, Tabuchi H, Kobayashi K et al. Loss of von Hippel-Lindau protein causes cell density dependent deregulation of CyclinD1 expression through hypoxia-inducible factor. *Oncogene* 2003;22:2728–38.

- Benizri E, Ginouves A, Berra E. The magic of the hypoxia-signaling cascade. *Cell Mol Life Sci* 2008;65:1133–49.
- Binley K, Askham Z, Martin L, Spearman H, Day D, Kingsman S et al. Hypoxia-mediated tumour targeting. *Gene Ther* 2003;10:540–9.
- Chan DA, Sutphin PD, Denko NC, Giaccia AJ. Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1 α . *J Biol Chem* 2002;277:40112–7.
- Chan DA, Krieg AJ, Turcotte S, Giaccia AJ. HIF gene expression in cancer therapy. *Methods Enzymol* 2007;435:323–45.
- Chen CJ, Hsu MH, Huang LJ, Yamori T, Chung JG, Lee FY et al. Anticancer mechanisms of YC-1 in human lung cancer cell line, NCI-H226. *Biochem Pharmacol* 2008;75:360–8.
- Chiang WC, Teng CM, Lin SL, Chen YM, Tsai TJ, Hsieh BS. YC-1-inhibited proliferation of rat mesangial cells through suppression of cyclin D1-independent of cGMP pathway and partially reversed by p38 MAPK inhibitor. *Eur J Pharmacol* 2005;517:1–10.
- Chun YS, Yeo EJ, Choi E, Teng CM, Bae JM, Kim MS et al. Inhibitory effect of YC-1 on the hypoxic induction of erythropoietin and vascular endothelial growth factor in Hep3B cells. *Biochem Pharmacol* 2001;61:947–54.
- Comerford KM, Cummins EP, Taylor CT. c-Jun NH2-terminal kinase activation contributes to hypoxia-inducible factor 1 α -dependent P-glycoprotein expression in hypoxia. *Cancer Res* 2004;64:9057–61.
- Dearth LR, Qian H, Wang T, Barnoi TE, Zeng J, Chen SW et al. Inactive full-length p53 mutants lacking dominant wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers. *Carcinogenesis* 2007;28:289–98.
- Funasaka T, Yanagawa T, Hogan V, Raz A. Regulation of phosphoglucose isomerase/autocrine motility factor expression by hypoxia. *FASEB J* 2005;19:1422–30.
- Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev* 2007;17:71–7.
- Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol* 2004;57:1009–14.
- Jackson EL, Olive KP, Tuveson DA, Bronson R, Crowley D, Brown M et al. The differential effects of mutant p53 alleles on advanced murine lung cancer. *Cancer Res* 2005;65:10280–8.
- Ji Z, Yang G, Shahzidi S, Tkacz-Stacowska K, Suo Z, Nesland JM et al. Induction of hypoxia-inducible factor-1 α overexpression by cobalt chloride enhances cellular resistance to photodynamic therapy. *Cancer Lett* 2006;244:182–9.
- Katsuta M, Miyashita M, Makino H, Nomura T, Shinji S, Yamashita K et al. Correlation of hypoxia inducible factor-1 α with lymphatic metastasis via vascular endothelial growth factor-C in human esophageal cancer. *Exp Mol Pathol* 2005;78:123–30.
- Kim HL, Yeo EJ, Chun YS, Park JW. A domain responsible for HIF-1 α degradation by YC-1, a novel anticancer agent. *Int J Oncol* 2006;29:255–60.
- Kurokawa T, Miyamoto M, Kato K, Cho Y, Kawarada Y, Hida Y et al. Overexpression of hypoxia-inducible-factor 1 α (HIF-1 α) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. *Br J Cancer* 2003;89:1042–7.
- Lau CK, Yang ZF, Lam CT, Tam KH, Poon RT, Fan ST. Suppression of hypoxia inducible factor-1 α (HIF-1 α) by YC-1 is dependent on murine double minute 2 (Mdm2). *Biochem Biophys Res Commun* 2006;348:1443–8.
- Lau CK, Yang ZF, Lam SP, Lam CT, Ngai P, Tam KH et al. Inhibition of Stat3 activity by YC-1 enhances chemo-sensitivity in hepatocellular carcinoma. *Cancer Biol Ther* 2007;6:1900–7.
- Li SH, Shin DH, Chun YS, Lee MK, Kim MS, Park JW. A novel mode of action of YC-1 in HIF inhibition: stimulation of FIH-dependent p300 dissociation from HIF-1 α . *Mol Cancer Ther* 2008;7:3729–38.
- Liu C, Shi Y, Du Y, Ning X, Liu N, Huang D et al. Dual-specificity phosphatase DUSP1 protects overactivation of hypoxia-inducible factor 1 through inactivating ERK MAPK. *Exp Cell Res* 2005;309:410–8.
- Liu L, Ning X, Han S, Zhang H, Sun L, Shi Y et al. Hypoxia induced HIF-1 accumulation and VEGF expression in gastric epithelial mucosa cell: involvement of ERK1/2 and PI3K/Akt. *Mol Biol* 2008;42:459–69.
- Maxwell PJ, Gallagher R, Seaton A, Wilson C, Scullin P, Pettigrew J et al. HIF-1 and NF- κ B-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells. *Oncogene* 2007;26:7333–45.
- McMurray HR, Sampson ER, Compitello G, Kinsey C, Newman L, Smith B et al. Synergistic response to oncogenic mutations defines gene class critical to cancer phenotype. *Nature* 2008;453:1112–6.
- Piret JP, Mottet D, Raes M, Michiels C. Is HIF-1 α a pro- or an anti-apoptotic protein? *Biochem Pharmacol* 2002;64:889–92.
- Ryan S, McNicholas WT, Taylor CT. A critical role for p38 map kinase in NF- κ B signaling during intermittent hypoxia/reoxygenation. *Biochem Biophys Res Commun* 2007;355:728–33.
- Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* 2002;8:S62–7.
- Semenza GL. Hypoxia-inducible factor 1 and cancer pathogenesis. *IUBMB Life* 2008;60:591–7.
- Sohda M, Ishikawa H, Masuda N, Kato H, Miyazaki T, Nakajima M et al. Pretreatment evaluation of combined HIF-1 α , p53 and p21 expression is a useful and sensitive indicator of response to radiation and chemotherapy in esophageal cancer. *Int J Cancer* 2004;110:838–44.
- Sun HL, Liu YN, Huang YT, Pan SL, Huang DY, Guh JH et al. YC-1 inhibits HIF-1 expression in prostate cancer cells: contribution of Akt/NF- κ B signaling to HIF-1 α accumulation during hypoxia. *Oncogene* 2007;26:3941–51.
- To KK, Huang LE. Suppression of hypoxia-inducible factor 1 α (HIF-1 α) transcriptional activity by the HIF prolyl hydroxylase EGLN1. *J Biol Chem* 2005;280:38102–7.
- Wang SW, Pan SL, Guh JH, Chen HL, Huang DM, Chang YL et al. YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] exhibits a novel antiproliferative effect and arrests the cell cycle in G0–G1 in human hepatocellular carcinoma cells. *J Pharmacol Exp Ther* 2005;312:917–25.
- Wu SY, Pan SL, Chen TH, Liao CH, Huang DY, Guh JH et al. YC-1 induces apoptosis of human renal carcinoma A498 cells *in vitro* and *in vivo* through activation of the JNK pathway. *Br J Pharmacol* 2008;155:505–13.
- Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS et al. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. *J Natl Cancer Inst* 2003;95:516–25.
- Yeo EJ, Ryu JH, Chun YS, Cho YS, Jang IJ, Cho H et al. YC-1 induces S cell cycle arrest and apoptosis by activating checkpoint kinases. *Cancer Res* 2006;66:6345–52.
- Zhou J, Schmid T, Schnitzer S, Brune B. Tumor hypoxia and cancer progression. *Cancer Lett* 2006;237:10–21.

Received 15 November 2009/8 September 2010; accepted 26 October 2010

Published as Immediate Publication 26 October 2010, doi 10.1042/CBI20090419