Hypoxic hepatocellular carcinoma cells acquire arsenic trioxide resistance through upregulating HIF-1 α expression

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Abstract

Background: Although arsenic trioxide (ATO) is used in treatment of advanced hepatocellular carcinoma (HCC) in clinical trials, it is not yet satisfied in improving HCC patients' overall survival. Intratumoral hypoxia and overexpression of hypoxia-inducible factor-1 α (HIF-1 α) may result in ATO-resistance and tumor progression. We investigated the mechanisms between HIF-1 α expression and acquired ATO-chemoresistance in HCC cells and in mice. **Methods:** Therapeutic effects of ATO in normoxic and hypoxic HCC cells were assessed using cell viability and apoptosis assays in vitro and xenografts model. mRNA and protein expression of HIF-1 α , P-glycoprotein, and VEGF were measured by qRT-PCR and western blotting. HIF-1 α inhibition was performed to investigate the mechanism of ATO-resistance. VEGF secretion was tested using ELISA and tube-formation assay. **Results:** Hypoxic HCC cells showed more resistance to ATO, with higher IC50 values and less apoptosis, than normoxic cells and upregulated HIF-1 α protein expression, accompanied with the enhancement of P-glycoprotein and VEGF synthesis after ATO treatment. VEGF secretion was elevated in ATO-treated supernatant to potentiate angiogenesis in vitro. HIF-1 α inhibition attenuated ATO-resistance and angiogenesis, and promoted the anticancer effects of ATO both in vitro and in vivo by downregulating therapy-induced P-glycoprotein and VEGF overexpression. **Conclusions:** Hypoxic HCC cells acquire ATO-resistance by upregulating HIF-1 α levels; thus ATO combined with targeting HIF-1 α levels may lead to the enhanced antitumor effects in HCC. **Keywords:** hepatocellular carcinoma, arsenic trioxide, drug resistance, HIF-1 α , targeted therapy

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and estimated over 1 million new cases annually soon [1, 2]. However, less than 50% of new HCC are presently amenable to the curative treatment [2, 3]. In addition, high tumor recurrence is the main obstacle for long-time survival in curative patients and majority of patients including early-stage HCC ultimately may develop an advanced stage [2]. Under these conditions, systemic therapies are recommended effective in prolonging overall survival (OS) of the advanced-stage disease [2-4]. However, the first first-line sorafenib prolonged OS in HCC patients with BCLC stage C by approximately 2.8 months, and the recently another first-line lenvatinib was noted to be non-inferior to sorafenib in terms of OS in the phase III trial [1, 2, 5-7]. Thus, there is an urgent need to develop a more effective systemic therapy in clinical practice for HCC patients with acquired resistance or intolerance to targeted therapies.

Arsenic trioxide (ATO) is a high-efficiency chemotherapeutic agent for acute promyelocytic leukemia (APL) in prolonging patients' remission and OS by directly targeting PML-RAR α fusion protein at low dose [8, 9]. Meanwhile, high-dose ATO exerts antitumor effects *via* various independent mechanisms, such as reactive oxygen species (ROS) production, JNK activation, and apoptosis of the vascular endothelium [10, 11]. Moreover, ATO has been recommended as a molecular targeted drug in the Standard for Diagnosis and Treatment of Primary HCC in China (2017), as it can target and in turn induce the degradation of PML-RAR α protein secreting by HCC cells [8, 12]. However, recent experimental research and clinical studies have shown that the single-agent ATO is not effective for HCC [12-15]. Among various factors

concerning the discrepant efficacy of ATO monotherapy for HCC, chemoresistance might be one of the most important reasons for this explanation [13, 14, 16, 17]. However, the molecular mechanism underlying the ATO resistance remains unclear.

Hypervascularized HCC is characterized as paradoxically intratumoral hypoxia and HIF-1 α overexpression resulting from the rapid tumor growth with aberrant blood vessels [16, 18, 19]. HIF-1 α is an oxygen-dependent protein. Under normoxic conditions, HIF-1 α is hydroxylated at Pro402 and Pro564 by prolyl hydroxylase (PHD) promoting its ubiquitylation by von Hippel-Lindau protein and degradation in proteasome. Hypoxia inhibits the PHD activation and leads to HIF-1 α stabilization. Furthermore, mechanistic studies have confirmed that the generation of intracellular ROS and nitric oxide (NO) by some therapies, such as some chemotherapeutic drugs, radiotherapy, and hyperthermia, can enhance HIF-1 α expression under hypoxia [16, 18, 20-23]. Stabilized HIF-1 α translocates to nucleus, heterodimerizes with HIF-1 β , and activates transcription of HIF-1 α -dependent genes. Accumulating evidence has demonstrated that higher levels of HIF-1 α are involved in chemoresistance and poorer prognosis in HCC [16, 18, 24, 25]. In addition, many studies have illuminated that the antiangiogenic treatments can negatively promote intratumoral hypoxia in solid tumors, resulting to an increased level of HIF-1 α [6, 17, 26, 27].

Together with the above findings, in the present study, we hypothesized that hypoxic HCC cells may acquire resistance to ATO through upregulating HIF-1 α expression. In this study, we aimed to investigate the alteration of HIF-1 α expression in HCC cells after ATO treatment and its role in acquired resistance to ATO *in vitro* and *in vivo*. The expression of vascular endothelial growth factor (VEGF) and P-glycoprotein, which are two key resistance-related and HIF-1 α -dependent biomarkers [15, 16, 25, 28], were also assessed.

Methods

Reagents and antibodies

Clinical grade ATO was purchased from ShuangLu Pharmaceuticals (Beijing, China). A stock solution of ATO (1 mM in phosphate-buffered saline (PBS)) was prepared and stored at 4°C. YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] was purchased from Cayman (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO). DMSO was added to culture at 0.1% (v/v) as a vehicle control for YC-1 administration. Primary antibodies for HIF-1 α , P-glycoprotein, VEGF, Ki67, CD31 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam (Cambridge, TX, USA).

Cell lines and cell culture

Three human HCC cell lines, HepG2 (Cat. No. SCSP-510), Huh7 (Cat. No. TCHu182), and Bel-7402 (Cat. No. TCHu 10), were kindly provided by Prof. Guangmei Yan, originally from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China), and immortalized human umbilical vein endothelial cells (HUVECs, Cat. No. pcs-100-010) were purchased from the American Type Culture Collection (Manassas,

VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin and streptomycin, supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5%CO₂ at 37.0°C as normoxic conditions. For hypoxic conditions, HCC cells were placed in a humidified anaerobic workstation 14500 COY DRIVE (Grass Lake, MI, USA) filled with 1%O₂, 5%CO₂, and 94%N₂ at 37.0°C for 24 hours prior to treatment.

Inhibition of HIF-1a expression

Small interfering RNA (siRNA) was used to knockdown HIF-1a mRNA expression at the post-transcriptional level. Three oligonucleotides of HIF-1a siRNA (siHIF-1a) and a negative control siRNA (siNC) were designed and synthesized by Ribobio Biotechnology (Guangzhou, China). The siHIF-1a sequences and the targeting sequences of human HIF-1a mRNA (GenBank Accession No.AB733094.1) are listed in Additional file Table 1. Cells were cultured under hypoxia overnight and then transfected with either siHIF-1a or siNC using Lipofectamine 2000 (Invitrogen). After 24 hours of transfection, cells were treated with or without ATO for an additional 24 hours and harvested for mRNA and protein assessment or used for the following assays.

YC-1 was used to inhibit HIF-1a activation at the post-translational level [29], resulting to HIF-1a degradation and decreased HIF-1 transcriptional activity. Cells were cultured under hypoxia overnight, pretreated with YC-1 or vehicle for 2 hours and then co-treated with or without ATO for 24 hours for the indicated assays.

Cell viability assays

Cell viability was examined using MTS assay with the CellTiter 96 AQueous (Promega) according to the manufacturer's instruction. 5×10³ cells per well were seeded onto 96-well plates in triplicate and cultured under normoxic or hypoxic conditions overnight. Cells were treated with ATO, or pretreated with siRNA or inhibitor for HIF-1a inhibition and then co-incubated with ATO. At the treatment time-point, MTS reagent was added to the wells and the absorbance was measured at 490 nm 2 hours later. Values of 50% inhibitory concentration (IC₅₀) and coefficient of drug interaction (CDI) [6] were based on three independent experiments.

Apoptosis assay

HCC cells, unpretreated or pretreated by HIF-1a inhibiting, were administered with ATO for 24 hours under normoxic or hypoxic conditions, then washed, harvested, suspended in PBS and stained using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN) following the manufacturer' protocol. The stained cells were analyzed using a flow cytometer (Beckman Coulter).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HCC cells using TRIzol Reagent (Life Technologies), and the RNA samples were used as templates for reverse transcription into complementary DNA (cDNA) using a PrimeScript RT

reagent kit (TaKaRa). For qRT-PCR analysis, aliquots of cDNA were amplified using a SYBR Premix Ex Taq II kit (TaKaRa) following the manufacturer's instructions, and the gene expression was normalized against GAPDH levels in the same sample. PCR reactions were assessed in triplicate and performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems), and all experiments were performed three times. The relative mRNA levels were calculated using the comparative Ct (cycle threshold) method as described by the manufacturer. The primer sets are shown in Additional file Table 2.

Western blot analysis

Western blot assays were performed to analyze protein expression with GAPDH as a control as previously described [30]. Briefly, protein samples were prepared using Mammalian Cell Lysis Reagent (Fermentas). Equal amounts of protein extracts were loaded and separated by electrophoresis on SDS-PAGE, and electrophoretically transferred to PVDF membranes (Roche) using a mini trans-blot apparatus (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat milk and then separately incubated with the primary antibodies on a shaker at 4°C overnight followed by appropriate HRP-conjugated secondary antibodies. Membranes were visualized on a ChemiDoc XRS+ System (Bio-Rad) using Immobilon Western Chemiluminescent HRP Substrate (Millipore) [31].

VEGF secretion and angiogenesis assessment *in vitro*

HepG2 cells (5×10^3), seeded onto 6-well plates and incubated under normoxia or hypoxia, were treated with ATO or co-treated with other agents for 24 hours. The culture medium was exchanged 2 ml new complete medium, and the cells were incubated for an additional 24 hours. The culture supernatants were collected, centrifuged and used to quantify the VEGF levels using the VEGF ELISA kit (R&D Systems), which was normalized to the cell numbers for each well.

Angiogenesis was assessed by the tube formation of HUVECs using an *in vitro* Angiogenesis assay kit (Millipore) according the manufacturer's protocol. The ATO-treated supernatant with the absolute highest level of VEGF was used for the ATO-treatment control, and all the collected supernatants were re-supplemented with 5% FBS. HUVECs (1×10^4 /well) in 150 μ l supernatant were seeded onto the surface of the pre-gelled ECMatrix 96-well plates and incubated under normoxic conditions for 4-12 hours. Tube formation was observed, imaged, and assessed according to the parameters presents in Additional file Table 3, recommended by the kit's manufacturer.

Xenograft HCC model assays

Animal care and experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals, and animal use protocols were approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (IACUC No.: DB-15-1215). Male BALB/c nu/nu mice (4–6 weeks old) were purchased from Vital River Laboratories (Beijing, China) and housed under specific-pathogen-free conditions at the Experimental Animal Center of Sun Yat-sen University. To establish HCC tumor xenografts, 5×10^6 HepG2 cells in 200 μ l PBS were subcutaneously injected into the right upper flank of

each mouse. When all of the tumors reached 100~150 mm³ in volume, the mice were randomized into four groups (8 mice per group): control, YC-1 (intraperitoneally, 10 mg/kg/day), ATO (intraperitoneally 4 mg/kg/day), and ATO+YC-1. In the ATO+YC-1 group, YC-1 was administrated 2 hours prior to ATO treatment. Mice in the control, YC-1, or ATO group were injected with an equivalent volume of fresh vehicle (DMSO) and PBS, PBS, or vehicle as a control.

The mouse weight and tumor volume were measured twice weekly starting with the first day of treatment during a 3-week period. Tumor volume was calculated using the equation as: length × width² × 0.5. At the end point of the treatments, all mice were sacrificed post deep-anesthesia with 3% v/v pentobarbital sodium (150 mg/kg) and tumors were dissected and weighted. Tumor tissues were either homogenized in lysis buffer for protein analysis or fixed in 4% paraformaldehyde and subsequently embedded in paraffin, sectioned into 6 µm slices for histological study. Immunohistochemical (IHC) staining of the tumors was done using a SP Rabbit & Mouse HRP kit (CWBioTech, China) with following human primary antibodies: anti-CD31 (1:200) and anti-Ki67 (1:200). To evaluate the apoptosis level, TUNEL staining was performed based on the protocol of an In Situ Cell Death Detection kit (Roche) [30].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (San Diego, CA, USA). All continuous variables were expressed as the mean with standard deviation (SD) and analyzed using 2-tailed Student's *t* tests. A *P* value less than 0.05 was considered statistically significant for all experiments.

Results

Hypoxic HCC cells are more resistant to ATO than normoxic cells.

To evaluate the effect of oxygen concentration on the chemoresistance of HCC cells to ATO, 3 cell lines were respectively treated with increasing concentrations of ATO for 24-72 hours under normoxic and hypoxic conditions using the MTS assay. As shown in **Figure 1a**, both normoxic and hypoxic cells exhibited sensitivity to ATO in a dose- and time-dependent manner. Among these lines at different treatment points, HepG2 cells demonstrated more resistance to ATO, with the highest IC₅₀ values, and Huh7 cells showed more sensitivity to ATO, with the lowest IC₅₀ values (**Figure 1b** and Additional file Table 4). And the IC₅₀ values were significantly higher under hypoxia than under normoxia at the same time-point, except for the IC₅₀ for Huh7 cells at 72 hours.

To further verify that hypoxic HCC cells were more resistance to ATO, apoptosis assay were performed. Consistent with the above MTS results, hypoxic HCC cells showed less apoptosis than normoxic cells (**Figure 1c**). The above data indicate that hypoxic HCC cells are more resistant to ATO than normoxic ones.

HIF-1a levels are associated with the sensitivity of HCC cells to ATO.

We then performed qRT-PCR and western blot analyses to compare the difference of HIF-1a expression among 3 HCC lines and to determine whether HIF-1a levels affected the sensitivity of HCC to ATO. HepG2 cells expressed the highest endogenous levels of HIF-1a mRNA and protein under both normoxic and hypoxic conditions, whereas Huh7 showed the lowest levels of HIF-1a (**Figure 1d**). Taken together, these findings suggest that HCC cells with higher levels of HIF-1a are more resistant to ATO.

Hypoxic HCC cells upregulate HIF-1a protein expression post ATO-treatment *in vitro*.

As hypothesized, ATO could increase HIF-1a expression in hypoxic HCC cells, we analyzed the change of HIF-1a mRNA and protein expression levels in HCC cells treated with ATO under normoxic and hypoxic conditions. qRT-PCR analysis revealed that HIF-1a mRNA levels did not change in the 3 HCC lines in the absent or present of ATO (**Figure 2a**). However, western blot analysis indicated that HIF-1a protein expression showed a significant increase in all the three hypoxic HCC lines post-ATO exposure under hypoxic conditions, whereas there was no significant difference in normoxic cells, which may be associated with the rapid oxygen-dependent degradation of HIF-1a protein under normoxic conditions (**Figure 2b**). These results demonstrate that hypoxic HCC cells upregulate HIF-1a protein expression at the post-transcriptional level in response to ATO.

HIF-1a accumulation increases VEGF and P-glycoprotein synthesis in hypoxic HCC cells.

Mechanistically, accumulating stabilized HIF-1a can transcriptionally activate its target genes, such as multidrug resistance gene 1 (MDR1) and VEGF. MDR1 encodes for P-glycoprotein, the overexpression of which decreases the concentration of intracellular drugs and is one of the most common reason for chemotherapeutic resistance, such as ATO [15, 16], and VEGF plays a key role in tumor angiogenesis and proliferation [28]. As shown in **Figure 2a**, ATO-induced HIF-1a accumulation stimulated a 2.2–2.8-fold and 4.2–5.8-fold increase of MDR1 and VEGF mRNA expression in hypoxic HCC cells compared with normoxic cells, whereas neither of them showed an increase in normoxic cells. Furthermore, MDR1 and VEGF mRNA levels were markedly higher post-ATO exposure than hypoxia-alone. Western blot analysis further confirmed the increased levels of P-glycoprotein and VEGF expression in the present of ATO under hypoxia (**Figure 2b**).

Both HIF-1a silencing and inhibition can enhance the sensitivity to ATO by downregulating P-glycoprotein and VEGF expression

To determine the role of HIF-1a upregulation in the acquired resistance to ATO, we tested whether inhibition of HIF-1a overexpression in hypoxic HCC cells can restore their sensitivity to ATO. Among the three duplexes of siRNA, siHIF-1a_3 at 50 nM concentration was confirmed to result in the highest inhibition of HIF-1a expression in hypoxic HepG2 cells, accompanied with complete P-glycoprotein and VEGF expression suppression (Additional file Figure S1a and **Figure 3a**). This siRNA was used in subsequent experiments to silence HIF-1a mRNA. Cell viability assay indicated that HIF-1a silencing could significantly enhance the chemosensitivity of the hypoxic HCC cells to ATO (Additional file Figure S1b).

Next, we conducted YC-1 to facilitate HIF-1a protein degradation. Western blot analysis indicated that YC-1 could inhibit ATO-induced HIF-1a accumulation dose dependently and effectively suppress the expression of HIF-1a, P-glycoprotein, and VEGF at 20 μ M (Additional file Figure S2a and **Figure 3b**). CDI studies showed that, in the presence of 20 μ M ATO under hypoxic conditions, YC-1 enhanced the cell proliferative inhibition of ATO in the three HCC lines, and all CDIs values were less than 1, suggesting the synergistically inhibitory effect due to their combination (Additional file Figure S2b). On the basis of these results, the optimal concentration of YC-1 on inhibiting HIF-1a accumulation was 20 μ M, which was used in the following combination assays.

To investigate HIF-1a inhibition on HCC cell apoptosis, the apoptosis assay was performed in HepG2 cells. Confirmed HIF-1a inhibition by western blot, YC-1 and siHIF-1a combined with ATO could significantly increase apoptosis compared with ATO-alone under hypoxia (**Figure 3c-d**).

Taken together, these findings strongly suggest that HIF-1a upregulation in hypoxic HCC cells post-ATO treatment is involved in acquired chemoresistance to ATO *in vitro*, and targeting HIF-1a could reverse the ATO-induced chemoresistance of HCC cells.

ATO-induced HIF-1a accumulation increases VEGF secretion and stimulates angiogenesis *in vitro*.

As shown in **Figure 2c**, secretory VEGF levels in the culture supernatants of HepG2 cells examined by ELISA were markedly increased, further confirming the results obtained qRT-PCR and western blot analysis. With 40 μ M ATO treatment, the surviving hypoxic HCC cells could maintain a relative high level of VEGF secretion. Importantly, the culture supernatant prepared from 10 μ M ATO-treated cells under hypoxic conditions induced a significant pro-angiogenesis compared with the control (**Figure 2d**). Consistent with these findings, the angiogenesis effect of the supernatant was inhibited by blocking HIF-1a expression using HIF-1a siRNA or inhibitor (**Figure 3e**).

ATO-induced HIF-1a upregulation attenuates the sensitivity of HCC tumors to ATO and contributes to the acquired ATO-resistance *in vivo*.

To confirm an alteration of HIF-1a expression and its role on ATO-resistance *in vivo*, we administered nu/nu mice bearing subcutaneous HepG2 tumors with YC-1, ATO or both in combination over a 3-week period. As shown in **Figure 4a**, YC-1 did not significantly increase ATO toxicity on weight loss in mice. Although tumors treated with ATO were decreased to 60.9% in volume as compared with the control tumors ($1905 \pm 172 \text{ mm}^3$ vs. $1160 \pm 171 \text{ mm}^3$, $p < 0.001$), ATO and YC-1 exerted a more significant antitumor effect than ATO-alone on subcutaneous HCC tumors in mice ($1160 \pm 171 \text{ mm}^3$ vs. $327 \pm 78 \text{ mm}^3$, $p < 0.001$) (**Figure 4b**). Tumor weight further confirmed the synergistic inhibitory effect of YC-1 combined with ATO (**Figure 4c**).

We further analyzed tumors by H&E staining and immunohistochemistry and western blot analyses. Tumors treated with ATO showed effective tumor-growth inhibition, foci of necrosis, and a decrease of microvessel density (MVD), whereas there was an significant increase of HIF-1a, P-glycoprotein, and

VEGF expression in ATO-therapy tumors compared with the control tested by western blotting (**Figure 5a-c**). These findings reveal that ATO at 4mg/kg/day is effective on antitumor growth and could simultaneously induce upregulation of HIF-1a, VEGF, and P-glycoprotein, as hypothesized.

However, ATO in combination with YC-1 produced enhanced antitumor effects with greater necrosis in tumors than ATO alone, which may result from, at least partially, lower MVD in the combinational tumors (**Figure 5a-b**). In line with our observations *in vitro*, the increased protein levels of P-glycoprotein and VEGF were remarkably suppressed with the inhibition of ATO-induced HIF-1a overexpression in tissue lysates prepared from tumours treated with YC-1 and the combination (**Figure 5c**). In addition, HIF-1a inhibition could also augment the antitumor effects of ATO *via* an increase of apoptosis and a decrease of proliferation in HCC tumors (**Figure 5a-b**). Taken together, these results confirmed that ATO-induced HIF-1a upregulation attenuates the sensitivity of HCC to ATO and contributes to the molecular mechanism of acquired resistance *in vivo*.

Discussion

The present study demonstrates that ATO exerts its cytotoxicity in HCC cells in a dose- and time-dependent manner under both normoxic and hypoxic conditions. However, hypoxic and higher HIF-1a level in HCC cells showed more resistance to ATO than normoxic cells. As expected, ATO treatment could further induce increased HIF-1a protein expression in hypoxic HCC cells and tumours, which has been proven to be an important mechanism mediating acquired chemoresistance and other malignant features in solid tumors including HCC [6, 18, 24, 25]. Moreover, we provided evidence that increased HIF-1a expression enhanced the transcriptional activation of HIF-1a-mediated signaling pathway, the promotion of VEGF and P-glycoprotein levels, resulting in acquired resistance to ATO and tumor progression.

As a multi-target drug, it has been confirmed that ATO increases intracellular ROS and NO levels while it drives antitumor effects [10, 32]. Thus, we found that HCC cells increased HIF-1a expression after ATO administration under hypoxic conditions *in vitro* (**Figure 2a-b**). Furthermore, we observed elevated HIF-1a levels and activation in ATO-treated tumors, resulting from HCC cells in response to ATO as describe *in vivo* and increased intratumoural hypoxia caused by targeting vascular endothelium (decreased MVD), it may be the mechanism that the therapeutic effect of some antiangiogenic agents is still poorly increased by insufficient efficacy, intrinsic refractoriness, and acquired resistance after initial response partially due to its regulation of HIF-1a overexpression(**Figure 4b-c** and **5a-c**).

On the basis of the results of this study, targeting HIF-1a using HIF-1a siRNA or an inhibitor could markedly decrease the levels of HIF-1a, P-glycoprotein, and VEGF, and simultaneously promoted the chemosensitivity of hypoxic HCC cells to ATO *in vitro* and *in vivo*. Our study also provided evidence that the surviving hypoxic HCC cells treated with ATO could secrete greater amount of VEGF, potentially resulting in a high risk of stimulating angiogenesis *in vitro* (**Figure 2c-d**), even though it receive high concentration of ATO. And *in vivo* studies, there were increased VEGF levels in sustained ATO-treated HCC

tumors. Consistent with a previous report showing that various dosages of ATO can induce increased VEGF expression in tumors [33], our study extends this finding and proposes a mechanism of ATO involving VEGF promotion *via* the HIF-1a-mediated pathway. Indeed, VEGF also mediates drug resistance and other key aspects in tumor progression [25, 28]. Therefore, HIF-1a inhibition significantly attenuated the angiogenesis in ATO-treated hypoxic cell-supernatant *in vitro* or tumors *in vivo* and enhanced the antitumor effects of ATO-alone *in vivo* (**Figure 3d-e** and **Figure 5a-c**). In addition, a large body of research has demonstrated that antiangiogenic therapies alone may induce acquired resistance and malignant progression [6, 17, 26, 27, 34]. Taken together, these data strongly suggest that the hypoxic HCC cells can acquire chemoresistance by upregulating HIF-1a expression in response to ATO.

Actually, ATO is a rigorous dose-limiting and dependent agent used in treatment of solid tumors, with tumor growth promotion at lower-dose and severe systemic toxicities at higher-dose [10, 13, 14, 32, 33, 35, 36]. Therefore, we consider that the dosage (4 mg/kg) at schedule use may be an appropriate and efficacious concentration for HCC treatment in mice and could exert an antiangiogenic effect due to the lower IC₅₀ values for HUVEC compared with HCC cells (**Figure 1b** and **5d**). The antiangiogenic effect of ATO at this dose has been reported previously, whereas the adverse effects of increased intratumoral hypoxia and HIF-1a overexpression were not studied [11, 15, 33]. *In vivo* studies have also shown that ATO combined with HIF-1a inhibitor produced a larger necrosis area and a further decreased MVD in HCC tumors than ATO treatment, which suggested that a component of microvessels in ATO-treated may be newly formed by the effect of increased HIF-1a-mediated VEGF levels (**Figure 4c** and **5a-c**). To overcome the acquired resistance and angiogenesis of ATO treatment, a combination of ATO and an HIF-1a inhibitor may be necessary. Even though these findings, several limitations in the *in vivo* study on ATO combined with HIF-1a inhibitor. Firstly, YC-1, not a mono-targeting HIF-1a agent, is a synthetic compound that has been demonstrated with various potent biological and pathological activities. It would play anticancer efficacy through multiple molecular pathways. Secondly, it was still unknown which the combination treatment of ATO and YC-1 affected the concentration of arsenic in tumor tissues.

Conclusions

In conclusion, the present study reveals that ATO can induce growth inhibition in HCC cells both *in vitro* and *in vivo*. However, it simultaneously drives post-transcriptional HIF-1a upregulation and HIF-1a-mediated transcriptional activity, leading to the acquired chemoresistance and tumor progression. To obtain maximal antitumor effects, ATO in combination with an HIF-1a inhibitor may be a promising system therapy for advanced HCC (**Figure 6**).

Abbreviations

HCC	hepatocellular carcinoma	ROS	reactive oxygen species
ATO	arsenic trioxide	PHD	prolyl hydroxylase
HIF-1 α	hypoxia-inducible factor-1 α	NO	nitric oxide
APL	promyelocytic leukemia	DMSO	dimethyl sulfoxide
VEGF	vascular endothelial growth factor	JNK	c-Jun NH2-terminal kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	PBS	phosphate-buffered saline
siRNA	small interfering RNA	CDI	coefficient of drug interaction
siNC	negative control siRNA	DMEM	Dulbecco's modified Eagle's medium
IC ₅₀	50% inhibitory concentration	qRT-PCR	quantitative real-time PCR
cDNA	complementary DNA	HUVECs	human umbilical vein endothelial cells
IHC	immunohistochemical	OS	overall survival
SD	standard deviation	TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
MVD	microvessel density	MDR1	multidrug resistance gene 1

Declarations

Ethics approval and consent to participate

Animal care and experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals, and animal use protocols were approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (IACUC No.: DB-15-1215)

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors have no conflict of interest to disclose.

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Authors' contributions

YC, DL and HS designed the experiments. YC, HL, DC, XJ and WW performed experiments and data analysis. YC, HL, and DC wrote the manuscript. YC, HL, DC, DL and SH reviewed and revised it critically. All authors read and approved the final version of the manuscript.

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