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

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Research article

Keywords: hepatocellular carcinoma, arsenic trioxide, drug resistance, HIF-1 α , targeted therapy

Posted Date: April 1st, 2020

DOI: <https://doi.org/10.21203/rs.2.18624/v3>

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Abstract

Background : Although arsenic trioxide (ATO) is used in the treatment of advanced hepatocellular carcinoma (HCC) in clinical trials, it is not satisfactory in terms of improving HCC patients' overall survival. Intratumoral hypoxia and overexpression of hypoxia-inducible-1 α (HIF-1 α) may result in ATO-resistance and tumor progression. We investigated the mechanisms involving HIF-1 α expression and acquired ATO chemoresistance in HCC cells and mice.

Methods: The therapeutic effects of ATO in normoxic and hypoxic HCC cells were assessed using cell viability and apoptosis assays in vitro and a xenograft model in vivo . 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 assays.

Results : Compared to normoxic cells, hypoxic HCC cells were more resistant to ATO, with higher IC 50 values and less apoptosis, 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 the supernatant of ATO-treated HCC cells, and this change can 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, combining ATO with a HIF-1 α -targeting agent may lead to enhanced antitumor effects in HCC.

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is projected to occur in over 1 million new cases annually [1, 2]. However, less than 50% of new HCC cases are presently amenable to curative treatment [2, 3]. In addition, high tumor recurrence is the main obstacle for long-term survival in curative patients, and the majority of patients, including those with early-stage HCC, ultimately develop an advanced-stage disease [2]. Under these conditions, systemic therapies are recommended to be effective in prolonging the overall survival (OS) of patients with advanced-stage disease [2-4]. However, first-line sorafenib only prolonged OS in HCC patients with Barcelona Clinic Liver Cancer stage C by approximately 2.8 months, and recently, another first-line lenvatinib was noted to be noninferior to sorafenib in terms of OS in a 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 leukaemia (APL) that prolongs patient remission and OS by directly targeting the PML-RAR α fusion protein at a 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 secreted by HCC cells [8, 12]. However, recent experimental research and clinical studies have shown that 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 ATO resistance remains unclear.

Hypervascularized HCC is characterized paradoxically as intratumoral hypoxia and HIF-1 α overexpression resulting from 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 by the proteasome. Hypoxia inhibits 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 hypoxic conditions [16, 18, 20-23]. Stabilized HIF-1 α translocates to the nucleus, heterodimerizes with HIF-1 β , and activates the transcription of HIF-1 α -dependent genes. Accumulating evidence has demonstrated that higher levels of HIF-1 α are involved in the chemoresistance and poorer prognosis of HCC [16, 18, 24, 25]. In addition, many studies have illuminated that antiangiogenic treatments can negatively promote intratumoral hypoxia in solid tumors, resulting in 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], was 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 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.

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, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin and streptomycin, supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs, Cat. No. CRL-1730™) were purchased from the American Type Culture Collection and propagated in F-12K Medium containing 2 mM L-glutamine, 1500 mg/L sodium bicarbonate and 10% FBS. All the cells were cultured in a humidified incubator of 5%CO₂ at 37.0°C as normoxic conditions. For hypoxic conditions, HCC cells were placed in a 14500 COY DRIVE humidified anaerobic workstation (COY Laboratory) 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. 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 hypoxic conditions overnight and then transfected with either siHIF-1a or siNC using Lipofectamine 2000 (Life Technologies). 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 in 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 the MTS assay with the CellTiter 96 AQueous reagent (Promega) according to the manufacturer's instructions. A total of 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 to inhibit HIF-1a 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 using an Infinite F200 multimode plate reader (Tecan). Values of 50% inhibitory concentration (IC₅₀) and coefficient of drug interaction (CDI) [6] were based on three independent experiments.

Apoptosis assay

HepG2 cells, unpretreated or pretreated with HIF-1a inhibitor, were administered ATO for 24 hours under normoxic or hypoxic conditions, washed, harvested, suspended in PBS and stained using the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN) following the manufacturer's protocol. The stained cells were analysed using an EXPO 32 ADC XL 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 the 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. PCRs 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 analyse 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). The membranes were blocked with 5% nonfat milk and then separately incubated with the primary antibodies on a shaker at 4°C overnight and then incubated with the appropriate HRP-conjugated secondary antibodies. Membranes were visualized on a ChemiDoc XRS+ System (iMark, 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 normoxic or hypoxic conditions, were treated with ATO or co-treated with other agents for 24 hours. The culture medium was exchanged with 2 ml of 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), and the results were normalized to the cell numbers for each well.

Angiogenesis was assessed by a tube-formation assay of HUVECs using an *in vitro* Angiogenesis assay kit (Millipore) according to 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 presented 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 administered 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 following equation: length $\times$ width² $\times$ 0.5. At the end point of the treatments, all mice were sacrificed post deep-anaesthesia with 3% v/v pentobarbital sodium (150 mg/kg), and tumors were dissected and weighed. Tumor tissues were either homogenized in lysis buffer for protein analysis or fixed in 4% paraformaldehyde and subsequently embedded in paraffin; and sectioned into 6 μ m slices for histological study. Immunohistochemical (IHC) staining of the tumors was performed using an SP Rabbit & Mouse HRP kit (CWBioTech) with the 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 analysed 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 treated with increasing concentrations of ATO for 24-72 hours under normoxic and hypoxic conditions and analysed 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 cell lines, HepG2 cells demonstrated higher resistance to ATO, with the highest IC₅₀ values, and Huh7 cells showed more sensitivity to ATO, with the lowest IC₅₀ values at each time point (Figure 1b and Additional file Table 4). The IC₅₀ values for the cell lines were significantly higher under hypoxic conditions than under normoxic conditions at the same time-point, except for the IC₅₀ for Huh7 cells at 72 hours.

To further verify that hypoxic HCC cells were more resistant to ATO, apoptosis assays were performed. Consistent with the above MTS results, hypoxic HepG2 cells showed lower apoptosis rates than normoxic cells (Figure 1c). The above data indicate that hypoxic HCC cells are more resistant to ATO than normoxic HCC cells.

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

We then performed qRT-PCR and western blot analyses to compare the difference in HIF-1a expression among the 3 HCC lines and to determine whether HIF-1a levels affected the sensitivity of HCC to ATO. As shown in Figure 1d-e, HepG2 expressed the highest endogenous levels of HIF-1a mRNA and protein under both normoxic and hypoxic conditions, whereas Huh7 expressed the lowest levels of HIF-1a. Taken together with above, these findings suggest that HCC cells with higher levels of HIF-1a are more resistant to ATO. These results are in agreement with a previous study by Tung et al. [32] and extend these findings.

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

We hypothesized that ATO could increase HIF-1a expression in hypoxic HCC cells, we analyzed the change in 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 absence or presence of ATO (Figure 2a). However, western blot analysis indicated that HIF-1a protein showed more expression in all the three hypoxic HCC lines post-ATO exposure under hypoxic conditions, whereas there was no upregulation in normoxic cells; this finding may be associated with the rapid oxygen-dependent degradation of HIF-1a protein under normoxic conditions (Figure 2b) [6, 25]. 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 P-glycoprotein, the overexpression of which decreases the concentration of intracellular drugs and is one of the most common reasons for chemotherapeutic resistance, such as ATO resistance [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 in MDR1 and VEGF mRNA expression, respectively, 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 with ATO exposure than with hypoxia alone. Western blot analysis further confirmed the increased levels of P-glycoprotein and VEGF expression in the presence of ATO under hypoxic conditions (Figure 2b).

Increased VEGF secretion by HIF-1a accumulation 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 maintained a relatively high level of VEGF secretion. Importantly, compared with the control supernatant, the culture supernatant obtained from 10 μ M ATO-treated cells under hypoxic conditions induced a significant pro-angiogenic compared with the control (Figure 2d).

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 by 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 assays indicated that HIF-1a silencing could significantly enhance the chemosensitivity of hypoxic HCC cells to ATO (Additional file Figure S1b).

Next, we treated cells with 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 CDI values were less than 1, suggesting the synergistic inhibitory effect due to their combination (Additional file Figure S2b). On the basis of these results, the optimal concentration of YC-1 for inhibiting HIF-1a accumulation was 20 μ M, which was used in the following combination assays.

To investigate HIF-1a inhibition of HCC cell apoptosis, an apoptosis assay was performed in HepG2 cells. Confirming HIF-1a inhibition by western blotting, YC-1 or siHIF-1a combined to that with ATO could significantly increase apoptosis of HepG2 cells compared with ATO alone under hypoxic conditions (Figure 3c-d). Furthermore, the angiogenic effect of the supernatant of HepG2 cells was significantly inhibited by blocking HIF-1a expression using HIF-1a siRNA or inhibitor (Figure 3e).

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 that targeting HIF-1a could reverse the ATO-induced chemoresistance of HCC cells.

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

To confirm an alteration of HIF-1a expression and its role in 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 or weight loss in mice. Although tumors treated with ATO were decreased to 60.9% in volume 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 analysed tumors by H&E staining and immunohistochemistry and western blot analyses. Tumors treated with ATO showed effective tumor growth inhibition, foci of necrosis, and decreased microvessel density (MVD), whereas there was a significant increase in HIF-1a, P-glycoprotein, and VEGF expression in ATO treated tumors compared with control, as tested by western blotting (Figure 5a-c). These findings reveal that ATO at 4 mg/kg/day is effective for inhibiting tumor 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 partially result from, lower MVD in the combination-treated 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 tumors treated with YC-1 and the combination (Figure 5c). In addition, HIF-1a inhibition could also augment the antitumor effects of ATO via promoting the apoptosis and inhibiting the proliferation of 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, our data and a previous study by Tung et al. [32] revealed that normoxic or hypoxic HCC cells with higher HIF-1a levels were more resistance to ATO than HCC cells with lower HIF-1a levels. And as expected, ATO treatment could further increase HIF-1a protein expression in hypoxic HCC cells and tumors, 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, 32]. Moreover, we provided evidence that increased HIF-1a expression enhanced the transcriptional activation of the HIF-1a-mediated signalling pathway; and increased the VEGF and P-glycoprotein levels, resulting in acquired resistance to ATO and tumor progression.

As a multitarget drug, ATO increases intracellular ROS and NO levels while it drives antitumor effects [10, 33]. Thus, we found that HCC cells had increased HIF-1a expression after ATO administration under hypoxic conditions *in vitro* (Figure 2a-b). Furthermore, we observed elevated HIF-1a levels and increased activation in ATO-treated tumors, resulting from the response of HCC cells to ATO, as described *in vivo*, and increased intratumoral hypoxia caused by targeting of the vascular endothelium (decreased MVD);

this may be the mechanism that explains why the therapeutic effect of some antiangiogenic agents is unsatisfactory with insufficient efficacy, intrinsic refractoriness, and acquired resistance after the initial response partially due to their 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 promote 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 amounts of VEGF, which is likely to stimulate angiogenesis *in vitro* (Figure 2c-d), even when they are treated with high concentrations of ATO. In addition, *in vivo* studies showed 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 [34], 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 of tumor progression [25, 28]. Therefore, HIF-1a inhibition significantly attenuated angiogenesis in ATO-treated hypoxic cell supernatants *in vitro* and 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, 35]. Taken together, these data strongly suggest that hypoxic HCC cells can acquire chemoresistance by upregulating HIF-1a expression in response to ATO.

ATO is a rigorous dose-limiting and dependent agent used in the treatment of solid tumors that promotes tumor growth at low doses and elicits severe systemic toxicities at high doses [10, 13-14, 33-34, 36-38]. Therefore, we consider that a regular dose of 4 mg/kg 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 HUVECs 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 have not been studied [11, 15, 34]. *In vivo* studies have also shown that ATO combined with HIF-1a inhibitor produced a larger necrotic area and a further decrease in MVD in HCC tumors than ATO treatment, which suggested that few new microvessels during ATO administration may be formed by the HIF-1a-mediated increase in VEGF levels (Figure 4c and 5). To overcome the acquired resistance and potential proangiogenic effect of ATO treatment alone, a combination of ATO and HIF-1a inhibitor may be necessary and exert its most effective antitumor effect.

Despite these findings, however, several limitations to the *in vivo* study on ATO combined with HIF-1a inhibitor. First, YC-1, which does not exclusively target HIF-1a, is a synthetic compound that has been demonstrated to have various potent biological and pathological activities. It would have anticancer efficacy through multiple molecular pathways. Second, it is still unknown whether combination treatment with ATO and YC-1 affects 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 acquired chemoresistance and tumor progression. To obtain maximal antitumor effects, ATO in combination with a HIF-1a inhibitor may be a promising systemic therapy for advanced HCC (Figure 6).

List Of Abbreviations

HCC	hepatocellular carcinoma	ROS	reactive oxygen species
ATO	arsenic trioxide	PHD	prolyl hydroxylase
HIF-1α	hypoxia-inducible factor-1α	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
FCM	flow cytometer		

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 conflicts of interest to disclose.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81620108017 to HS, No. 81771879 to DL), the Natural Science Foundation of Guangdong Province (No. 2017A030313807 to YC). The funding bodies were not involved in the design of this study, in the collection, analysis, and interpretation of data and in writing of the manuscript.

Authors' contributions

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

Acknowledgements

We are grateful to Prof. Xintao Shuai and Prof. Guangmei Yan at North Campus of Sun Yat-sen University for providing key technical assistance.

References

1. Rimassa L, Assenat E, Peck-Radosavljevic M, Pracht M, Zagonel V, Mathurin P, Rota Caremoli E, Porta C, Daniele B, Bolondi L *et al*: Tivantinib for second-line treatment of MET-high, advanced

- hepatocellular carcinoma (METIV-HCC): a final analysis of a phase 3, randomised, placebo-controlled study. *The Lancet Oncology* 2018, 19(5):682-693.
2. Llovet JM, Montal R, Sia D, Finn RS: Molecular therapies and precision medicine for hepatocellular carcinoma. *Nature reviews Clinical oncology* 2018, 15(10):599-616.
 3. Bruix J, Reig M, Sherman M: Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016, 150(4):835-853.
 4. Bruix J, Takayama T, Mazzaferro V, Chau GY, Yang J, Kudo M, Cai J, Poon RT, Han KH, Tak WY *et al*: Adjuvant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): a phase 3, randomised, double-blind, placebo-controlled trial. *The Lancet Oncology* 2015, 16(13):1344-1354.
 5. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A *et al*: Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine* 2008, 359(4):378-390.
 6. Liang Y, Zheng T, Song R, Wang J, Yin D, Wang L, Liu H, Tian L, Fang X, Meng X *et al*: Hypoxia-mediated sorafenib resistance can be overcome by EF24 through Von Hippel-Lindau tumor suppressor-dependent HIF-1 α inhibition in hepatocellular carcinoma. *Hepatology* 2013, 57(5):1847-1857.
 7. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J *et al*: Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018, 391(10126):1163-1173.
 8. Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M *et al*: Arsenic trioxide controls the fate of the PML-RAR α oncoprotein by directly binding PML. *Science* 2010, 328(5975):240-243.
 9. Valenzuela M, Glorieux C, Stockis J, Sid B, Sandoval JM, Felipe KB, Kwiecinski MR, Verrax J, Buc Calderon P: Retinoic acid synergizes ATO-mediated cytotoxicity by precluding Nrf2 activity in AML cells. *British journal of cancer* 2014, 111(5):874-882.
 10. Plataniias LC: Biological responses to arsenic compounds. *The Journal of biological chemistry* 2009, 284(28):18583-18587.
 11. Roboz GJ, Dias S, Lam G, Lane WJ, Soignet SL, Warrell RP, Jr., Rafii S: Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. *Blood* 2000, 96(4):1525-1530.
 12. Zhang X, Yang XR, Sun C, Hu B, Sun YF, Huang XW, Wang Z, He YF, Zeng HY, Qiu SJ *et al*: Promyelocytic leukemia protein induces arsenic trioxide resistance through regulation of aldehyde dehydrogenase 3 family member A1 in hepatocellular carcinoma. *Cancer letters* 2015, 366(1):112-122.
 13. Lin CC, Hsu C, Hsu CH, Hsu WL, Cheng AL, Yang CH: Arsenic trioxide in patients with hepatocellular carcinoma: a phase II trial. *Investigational new drugs* 2007, 25(1):77-84.
 14. Subbarayan PR, Ardalan B: In the war against solid tumors arsenic trioxide needs partners. *Journal of gastrointestinal cancer* 2014, 45(3):363-371.

15. Zheng T, Yin D, Lu Z, Wang J, Li Y, Chen X, Liang Y, Song X, Qi S, Sun B *et al*: Nutlin-3 overcomes arsenic trioxide resistance and tumor metastasis mediated by mutant p53 in Hepatocellular Carcinoma. *Molecular cancer* 2014, 13:133.
16. Tredan O, Galmarini CM, Patel K, Tannock IF: Drug resistance and the solid tumor microenvironment. *Journal of the National Cancer Institute* 2007, 99(19):1441-1454.
17. Worns MA, Galle PR: HCC therapies—lessons learned. *Nature reviews Gastroenterology & hepatology* 2014, 11(7):447-452.
18. Wilson GK, Tennant DA, McKeating JA: Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *Journal of hepatology* 2014, 61(6):1397-1406.
19. Liu LP, Hu BG, Ye C, Ho RL, Chen GG, Lai PB: HBx mutants differentially affect the activation of hypoxia-inducible factor-1alpha in hepatocellular carcinoma. *British journal of cancer* 2014, 110(4):1066-1073.
20. Moeller BJ, Cao Y, Li CY, Dewhirst MW: Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer cell* 2004, 5(5):429-441.
21. Berchner-Pfannschmidt U, Tug S, Kirsch M, Fandrey J: Oxygen-sensing under the influence of nitric oxide. *Cellular signalling* 2010, 22(3):349-356.
22. Moon EJ, Sonveaux P, Porporato PE, Danhier P, Gallez B, Batinic-Haberle I, Nien YC, Schroeder T, Dewhirst MW: NADPH oxidase-mediated reactive oxygen species production activates hypoxia-inducible factor-1 (HIF-1) via the ERK pathway after hyperthermia treatment. *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107(47):20477-20482.
23. Sandau KB, Fandrey J, Brune B: Accumulation of HIF-1alpha under the influence of nitric oxide. *Blood* 2001, 97(4):1009-1015.
24. Cao S, Yang S, Wu C, Wang Y, Jiang J, Lu Z: Protein expression of hypoxia-inducible factor-1 alpha and hepatocellular carcinoma: a systematic review with meta-analysis. *Clinics and research in hepatology and gastroenterology* 2014, 38(5):598-603.
25. Semenza GL: Targeting HIF-1 for cancer therapy. *Nature reviews Cancer* 2003, 3(10):721-732.
26. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O: Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer cell* 2009, 15(3):220-231.
27. Welte J, Loges S, Dimmeler S, Carmeliet P: Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *The Journal of clinical investigation* 2013, 123(8):3190-3200.
28. Goel HL, Mercurio AM: VEGF targets the tumor cell. *Nature reviews Cancer* 2013, 13(12):871-882.
29. Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, Park JW: YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. *Journal of the National Cancer Institute* 2003, 95(7):516-525.
30. Wu C, Gong F, Pang P, Shen M, Zhu K, Cheng D, Liu Z, Shan H: An RGD-modified MRI-visible polymeric vector for targeted siRNA delivery to hepatocellular carcinoma in nude mice. *PloS one* 2013,

8(6):e66416.

31. Lin Y, Zhang H, Liang J, Li K, Zhu W, Fu L, Wang F, Zheng X, Shi H, Wu S *et al*: Identification and characterization of alphavirus M1 as a selective oncolytic virus targeting ZAP-defective human cancers. *Proceedings of the National Academy of Sciences of the United States of America* 2014, 111(42):E4504-4512.
32. Tung JN, Cheng YW, Hsu CH, Liu TZ, Hsieh PY, Ting LL, Ko HL, Chang YJ, Chiou JF, Wu AT: Normoxically overexpressed hypoxia inducible factor 1-alpha is involved in arsenic trioxide resistance acquisition in hepatocellular carcinoma. *Annals of surgical oncology* 2011, 18(5):1492-1500.
33. Emadi A, Gore SD: Arsenic trioxide - An old drug rediscovered. *Blood reviews* 2010, 24(4-5):191-199.
34. Liu B, Pan S, Dong X, Qiao H, Jiang H, Krissansen GW, Sun X: Opposing effects of arsenic trioxide on hepatocellular carcinomas in mice. *Cancer science* 2006, 97(7):675-681.
35. Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, Hicklin DJ, Chaplin D, Foster FS, Benezra R *et al*: Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 2006, 313(5794):1785-1787.
36. Maeda H, Hori S, Nishitoh H, Ichijo H, Ogawa O, Kakehi Y, Kakizuka A: Tumor growth inhibition by arsenic trioxide (As₂O₃) in the orthotopic metastasis model of androgen-independent prostate cancer. *Cancer research* 2001, 61(14):5432-5440.
37. Ahn RW, Chen F, Chen H, Stern ST, Clogston JD, Patri AK, Raja MR, Swindell EP, Parimi V, Cryns VL *et al*: A novel nanoparticulate formulation of arsenic trioxide with enhanced therapeutic efficacy in a murine model of breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2010, 16(14):3607-3617.
38. Yu G, Chen X, Chen S, Ye W, Hou K, Liang M: Arsenic trioxide reduces chemo-resistance to 5-fluorouracil and cisplatin in HBx-HepG2 cells via complex mechanisms. *Cancer cell international* 2015, 15:116.

Figures

Figure 1

HIF-1α levels are involved in ATO chemoresistance under both normoxic and hypoxic conditions. (a) HCC cells cultured in normoxic and hypoxic conditions were treated with increasing concentrations of ATO for the indicated periods of time, and MTS cytotoxicity assays were used to compare chemoresistance. (b) The IC₅₀ of each cell line was calculated from dose-response curves by cell viability assay. Data are presented from three independent experiments in triplicate. (c) Apoptosis was performed to compare ATO-sensitivity in HepG2 cells treated with ATO for 24 hours under normoxic and hypoxic conditions, and detected using annexin V/PI staining by FCM analysis. Each concentration was analysed in triplicate. (d) qRT-PCR analysis showed relative HIF-1α mRNA, and (e) western blots and quantification of HIF-1α in three HCC cell lines under normoxic and hypoxic conditions for 24 hours. GAPDH was applied as a loading control. For b and c, bars represent the mean ± SD (n=3). *P < 0.05, **P < 0.01, as assessed with a two-tailed Student's t test for unpaired data.

Figure 2

Hypoxic HCC cells upregulate HIF-1α, VEGF and P-glycoprotein expression, and induce angiogenesis after ATO treatment. (a-b) Relative mRNA and protein expression of HIF-1α, VEGF and P-glycoprotein was detected by qRT-PCR and western blotting in three HCC cell lines treated with increasing ATO concentrations for 24 hours. GAPDH was applied as a loading control. Data are the mean ± SD (n=3). **P < 0.01 and ***P < 0.001 versus absence of ATO under 21% O₂. #P < 0.05 and ##P < 0.01 versus absence of ATO under 1% O₂. (c) Levels of VEGF secreted in the culture supernatants of HepG2 cells were assessed by ELISA. ** indicates P < 0.01 and *** indicates P < 0.001 versus control. (d) Representative tube-formation images and corresponding values were shown to illustrate the angiogenesis of HUVECs cultured with the conditioned supernatants for 8 hours, as detected by an in vitro angiogenesis assay. Bar = 200 μm.

Figure 3

HIF-1α inhibition decreases VEGF and P-glycoprotein expression, enhances ATO cytotoxicity in hypoxic HCC cells and attenuates angiogenesis in HUVEC. HepG2 cells were pretreated with HIF-1α siRNA (50 nM) or inhibitor (20 μM) with or without ATO (10 μM). (a-b) The protein expression of VEGF and P-glycoprotein was analysed after HIF-1α inhibition by siHIF-1α (a) or YC-1 (b) using western blotting, and GAPDH was used as a loading control. (c) Before the next two assays (c and d), HIF-1α protein inhibition detected by western blotting was confirmed in cells following the indicated treatments in normoxic or hypoxic conditions. (d) Apoptosis was analysed to compare the sensitivity of HCC cells following indicated treatments using annexin V/PI staining. Bars are the mean ± SD (n=3). *P < 0.05, **P < 0.01 versus the control (Student's t test). (e) Tube-formation was detected and evaluated by an in vitro angiogenesis assay of HUVECs cultured with the conditioned supernatants for 6 hours. Bar = 200 μm.

Figure 4

HIF-1 α inhibition enhances the antitumor effect of ATO on HCC in vivo. HepG2 cells were injected subcutaneously into the right upper flanks of nu/nu mice, and the indicated treatments started 14 days postimplantation. Mouse weight (a) and tumor volume (b) were measured biweekly during the treatment period and analysed at the beginning and the end of treatment; and were analysed for significant difference. (c) The representative tumor masses of the 4 groups were presented, and the dissected tumor weights were compared to confirm the antitumor effects of various treatments. Data are presented as the mean $\pm$ SD (n=8). ***P < 0.001 versus the indicated control (Student's t test).

Figure 5

Enhanced antitumor effects of ATO and YC-1 in vivo are associated with antiangiogenesis and HIF-1α expression inhibition. (a) Representative images of HCC sections were evaluated by the indicated histochemical stain. The scale bars represent 200 μm (H&E) and 20 μm (CD31, Ki67, and TUNEL). (b) Statistical analysis showed corresponding changes in the indicated staining of the groups. Bars are the mean ± SD (n = 9 randomized fields for H&E and CD31, n = 5 randomized fields with 500 cells per field for Ki67 and TUNEL). *P < 0.05, **P < 0.01 versus control (Student's t test). (c) HCC tumor tissues were subjected to protein extraction to determine the expression of HIF-1α, VEGF, and P-glycoprotein by western blotting. GAPDH was used as a loading control. (d) IC50 values were shown to indicate the sensitivity of HUVECs to ATO for 24 hours, 48 hours, and 72 hours. Bars are the mean ± SD (n = 3).

Figure 6

A schematic diagram shows a summary of the present study. The molecular mechanisms of ATO-induced HIF-1 α upregulation include ROS and NO generation, and antiangiogenesis. Targeting HIF-1 α synthesis or activity may be a novel therapeutic strategy to enhance the antitumor effects of ATO in HCC.

Supplementary Files

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