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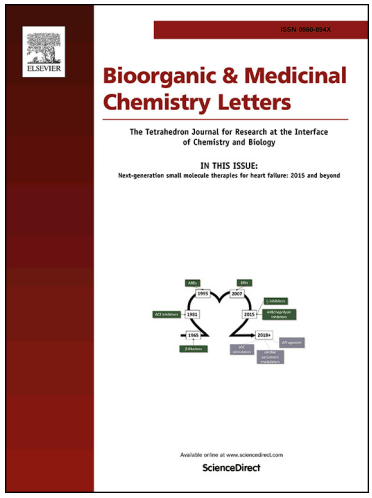

Design, synthesis, and screening of novel ursolic acid derivatives as potential anti-cancer agents that target the HIF-1α pathway

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Design, synthesis, and screening of novel ursolic acid derivatives as potential anti-cancer agents that target the HIF-1α pathway

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Abstract

The transcription factor hypoxia-inducible factor-1α (HIF-1α) plays an important role in tumor angiogenesis, growth, and metastasis and is recognized as an important potential therapeutic target for cancer. Here, we designed and synthesized three novel series of ursolic acid derivatives containing an aminoguanidine moiety and evaluated them as HIF-1α inhibitors and anti-cancer agents using human cancer cell lines. Most of the compounds exhibited significant inhibition of HIF-1α transcriptional activity, as measured using a Hep3B cell-based luciferase reporter assay. Among these compounds, **7b** was the most potent inhibitor of HIF-1α expression under hypoxic conditions (IC_{50} 4.0 μM) and did not display significant cytotoxicity against any cell lines tested. The mechanism of action of **7b** was investigated, we found that **7b** downregulated HIF-1α protein expression, possibly by suppressing its synthesis, reduced production of vascular endothelial growth factor, and inhibited the proliferation of cancer cells.

Keywords: Ursolic acid; Aminoguanidine; Anticancer activity; HIF-1α inhibitor; Cytotoxicity

Cancer is the second leading cause of death worldwide after cardiovascular disease. The World Health Organization estimates that cancer will account for approximately 9.6 million deaths globally in 2018,[1] and its incidence is steadily increasing. The major treatments for cancer are surgery, radiation, and chemotherapy.[2,3] However, traditional chemotherapeutic agents have a number of critical drawbacks, including harmful side-effects, non-specific biodistribution, short circulation times, and poor solubility, which result in poor therapeutic efficiency.[4,5] Thus, there is a tremendous need to develop new compounds for the prevention and treatment of cancer.[6]

Hypoxia is a common feature of many solid tumors and is generally caused by the rapid proliferation of tumor cells, which leads to formation of solid masses and obstruction and compression of the blood vessels surrounding them.[7] Hypoxia-inducible factor 1α (HIF-1α) is a transcription factor that regulates the expression of numerous genes involved in nutrient uptake, cell survival, angiogenesis, invasion, and metastasis, and thus plays an important role in cancer development.[8-10] In addition to hypoxia, exposure to certain hormones, cytokines, and growth factors can also upregulate HIF-1α expression.[11] Consequently, HIF-1α has gained attention as a potential target for the development of anti-cancer agents.[12] Inhibition of the HIF-1α pathway may be a particularly useful therapy for specific types of cancers, especially those commonly associated with hypoxia.[13,14] Due to the importance of HIF-1α in tumor development and progression, a considerable amount of effort has been made to identify HIF-1α inhibitors for treatment of cancer.[15-18]

Ursolic acid (UA) is a pentacyclic triterpenoid found in most plant species,[19] and it is known to possess a number of bioactive properties[20] such as anti-inflammatory,[21] anti-microbial,[22] anti-oxidant,[23] immunomodulatory,[24] and anti-cancer activities.[25] Indeed, Japanese researchers have ranked UA as one of the most promising potential

therapeutic compounds for tumor prevention.[26] Over the past decade, many attempts have been made to develop bioactive UA derivatives that more potently inhibit cancer cell growth.[27] These studies have indicated that the configuration at C-3 is a critical factor for the anti-proliferative activity of UA, whereas a free hydroxyl at C-3 decreases its anti-cancer activity.[27] Lin *et al.* showed that incorporation of an isopropyl ester at C-28 significantly improves the anti-proliferative activity of UA,[28] whereas introduction of a methyl at the same position together with an amino moiety at C-3 significantly enhanced its activity against HeLa cells[29] (**Fig. 1A**). In contrast, Dar *et al.* showed that incorporation of various substituted benzene rings at the C-2 position and retention of the carboxyl group at C-28 in UA (**Fig. 1B**) also improved its anti-cancer activity, as reflected by induction of cell cycle arrest in G1 and apoptosis of HCT-116 cells.[30]

The medicinal properties of guanidine derivatives are also of great interest due to their diverse anti-microbial,[31] anti-inflammatory,[32] anti-viral,[33] and anti-cancer[34,35] activities (**Fig. 1C** and **1D**). Guanidine-containing drugs, such as *m*-iodobenzylguanidine and methylglyoxal bis(guanylhydrazone), were shown several decades ago to have anti-tumor properties and have since been subjected to intense preclinical and clinical evaluation.[36] In our previous work, we reported the design and synthesis of a series of UA derivatives containing heterocyclic moieties at the C-28 position of UA, and one compound (**Fig. 1E**) was shown to potently inhibit HIF-1α transcriptional activity under hypoxic conditions.[37] Building on this, our ongoing research seeks to identify HIF-1α inhibitors with the potential to be developed as anti-cancer agents.

Here, we report on the activities of three series of UA derivatives in which we changed the hydroxyl moiety of UA to an aminoguanidine group at C-3 and simultaneously (i) introduced smaller moieties, such as benzene and alkyl groups, at the C-28 position; (ii) retained the carboxyl group at C-28; or (iii) introduced different substituted benzene rings or heterocyclic groups at the C-2 position. These novel series, totaling 28 compounds, were evaluated for their ability to inhibit hypoxia-induced HIF-1α transcriptional activity. The biological mechanism of action

of one selected compound, **7b**, was investigated in detail.

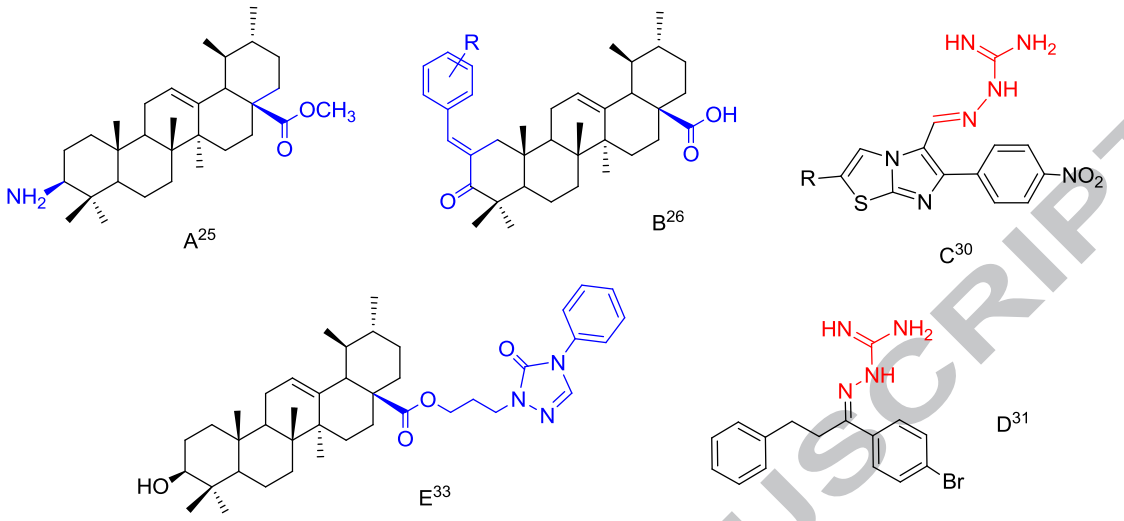
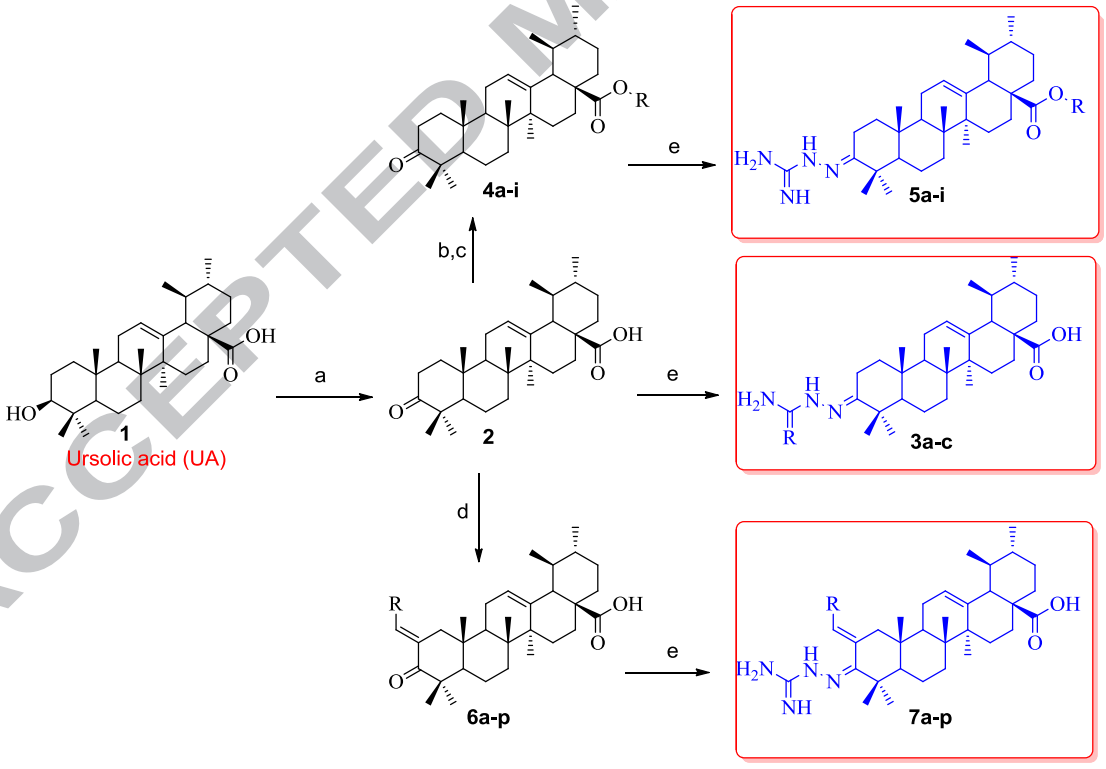


Figure 1. Structures of previously reported compounds as anticancer agents. (blue: previous work on UA derivatives; red: structure of aminoguanidine- active group).

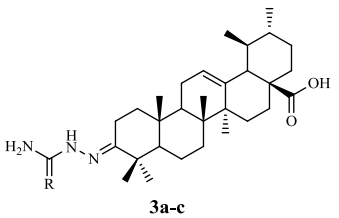


Scheme 1. Synthetic scheme for the synthesis of compounds **3a-c**, **5a-i**, **7a-p**. Reagents and conditions: (a) Jones reagent, acetone, 0 °C, 5h, 90%; (b) Methyl iodide, bromoethane or bromopentane, K_2CO_3, DMF, r.t., 6h, 80%-82%; (c) Benzyl chlorides, K_2CO_3, acetone, reflux, 8h, 80%-90%; (d) Aldehydes, 5 % NaOH, absolute ethanol, r.t 2h, 30%-75%; (e) Concentrated HCl, absolute ethanol, reflux, 8h, 34%-65%.

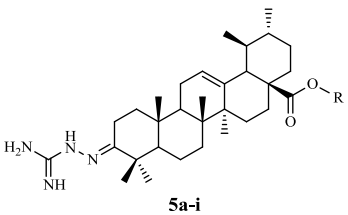
The synthetic pathway of the target compounds **3a**–**c**, **5a**–**i,** and **7a**–**p** is presented in Scheme 1. The intermediate **2** was synthesized by reacting UA with Jones reagent in acetone at 0°C.[38] Compounds **3a**, **3b**, and **3c** were prepared by reacting intermediate **2** with aminoguanidine bicarbonate, semicarbazide hydrochloride, and thiosemicarbazide, respectively, in refluxing ethanol. Compounds **4a**, **4b**, and **4c** were prepared by reacting intermediate **2** with methyl iodide, ethyl bromide, and amyl bromide, respectively. Intermediate **2** was reacted with different substituents of benzyl chloride to provide compounds **4d**–**i**. Compounds **6a**–**p** were prepared by Claisen–Schmidt condensation of intermediate **2** with different aldehydes.[30] Compounds **4a**–**i** and **6a**–**p** were reacted with aminoguanidine bicarbonate in refluxing ethanol to yield the target compounds **5a**–**i** and **7a**–**p**. The structures of the desired compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry.

The *in vitro* activities of the UA derivatives are summarized in **Table 1**. To examine the effects on hypoxia-induced HIF-1α transcriptional activity, Hep3B cells were transfected with a luciferase reporter construct driven by six hypoxia-response elements and then exposed to hypoxia (1% O_2). After 24 h, luciferase activity in the supernatants was measured. UA was used as a positive control. We found that most of the tested compounds inhibited HIF-1α transcriptional activity. For the substituted guanidine derivatives 3a (IC_{50} > 100 μM), 3b (IC_{50} 88.8μM), and 3c (IC_{50} 13.3μM), the inhibitory activity was in the order O > S > NH. It indicated that the semicarbazide derivatives have good HIF-1α inhibitory activities and merit further investigation as potential anti-cancer agents.

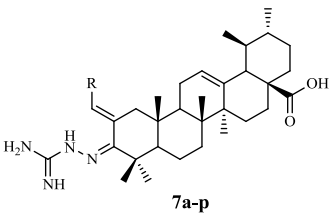
Table 1. *In vitro* inhibition of HIF-1α transcriptional activity in cell-based HRE reporter assay under hypoxia conditions and cytotoxic activity.



3a-c



5a-i



7a-p

Compound	R	IC_{50} (μM)	
		HRE [a]	Cytotoxic activity [b]
UA	—	>100	23.8
3a	NH	>100	>100
3b	S	88.8	>100
3c	O	13.3	69.8
5a	Methyl	6.7	>100
5b	Ethyl	>100	6.0
5c	Amyl	>100	22.0
5d	Phenyl(3-Cl)	30.3	34.1
5e	Phenyl(4-Cl)	21.1	32.1
5f	Phenyl(2,4-2Cl)	40.5	>100
5g	Phenyl(2,6-2Cl)	13.3	22.3
5h	Phenyl(4-F)	13.2	12.9
5i	Phenyl(4-CH_3)	10.3	18.0
7a	Phenyl	37.8	>100
7b	Phenyl(4-Cl)	4.0	>100
7c	Phenyl(2-Cl)	>100	>100
7d	Phenyl(3-Cl)	>100	>100
7e	Phenyl(4-F)	>100	>100
7f	Phenyl(2-F)	>100	>100
7g	Phenyl(3-F)	>100	10.5
7h	Phenyl(2-Br)	>100	>100
7i	Phenyl(3-Br)	75.4	>100
7j	Phenyl(2,4-2Cl)	90.4	>100
7k	Phenyl(2,6-2Cl)	>100	>100
7l	Phenyl(4-CH_3)	>100	>100
7m	Phenyl(4-NO_2)	>100	>100
7n	Naphthyl	>100	>100
7o	Furyl	>100	>100
7p	Thienyl	22.8	>100

[a] The inhibitory effects of all the derivatives on HIF-1α transcriptional activity were tested by HRE luciferase reporter assay after 24 h treatment of HCT116 cells under hypoxic conditions. Values were shown as mean, n=3.

[b] The cytotoxic activity was evaluated by MTT assay after 24 h treatment of compounds under normoxic conditions. Values were shown as mean, n=3.

For the compounds in series **5**, alkyl groups of different carbon chain lengths were introduced at C-28 of UA. Of these, only compound **5a** showed potent anti-cancer activity (IC_{50} 6.7 μM), indicating that a methyl group at C-28 improves the anti-cancer activity UA, whereas extending the carbon chain may have the reverse

effect. Compounds **5d**–**i** which contained different substituents on the phenyl ring, exhibited significant HIF-1α inhibition, with IC_{50} values between 10.3 and 40.5 μM. For the chlorinated compounds **5d** (IC_{50} 30.3μM), **5e** (IC_{50} 21.1μM), **5f** (IC_{50} 40.5μM), and **5g** (IC_{50} 13.3μM), the inhibitory activity was in the order 2,6-Cl_2 > 4-Cl > 3-Cl > 2,4-Cl_2. For compounds **5e** (IC_{50} 21.1μM), **5h** (IC_{50} 13.2μM), and **5i** (IC_{50} 10.3μM), electron-donating groups showed beneficial effects compared with electron-withdrawing groups (4-CH_3 > 4-F > 4-Cl). No clear structure-activity relationship could be found in series **7**. Compounds **7a** (H), **7b** (4-Cl), **7i** (3-Br), and **7j** (2,4-Cl_2) exhibited potent anti-cancer activities when a benzene ring was introduced at the C-2 position of UA. Among these compounds, **7b** was the most potent inhibitor of HIF-1α transcriptional activity (IC_{50} 4.0 μM). Compound **7p** also potently inhibited HIF-1α, suggesting that the thiophene moiety is more favorable than furan for inhibition.

The cytotoxicity of these compounds against Hep3B cells was assessed using the MTT assay. As shown in **Table 1**, compounds in series **3** and **7** (except **7g**), which retained the carboxyl at C-17, showed no appreciable cytotoxic activity (IC_{50} >100 μM), whereas compounds in series **5** (except **5a**,**f**) were modestly cytotoxic, indicating that introduction of a phenyl ring or long carbon chain (C > 2) into the carboxyl group could increase the cytotoxicity. Interestingly, we previously showed that esterification of the carboxyl group of UA greatly decreased its cytotoxicity and enhanced its HIF-1α inhibitory activity. However, introduction of an aminoguanidine group at C-3 but retention of the free carboxyl group also reduced the cytotoxicity and increased the inhibitory activity of UA, indicating that a free carboxyl group is not critical for the compounds' cytotoxicity or HIF-1α inhibitory activity. Importantly, the cytotoxicity of UA derivatives were weaker than their HIF-1α transcription inhibitory activities, suggesting that ursolic acid derivatives suppressed HIF-1α transcriptional activity without cytotoxicity.

Compound **7b**, with the best HIF-1α inhibitory effect (IC_{50} 4.0μM), was selected for further biological evaluation. As shown in Figure 2, **7b** dose-dependently inhibited the luciferase activity in Hep3B cells (**Fig. 2A**) and concentrations up to 30 μM did

not adversely affect cell viability (**Fig. 2B**).

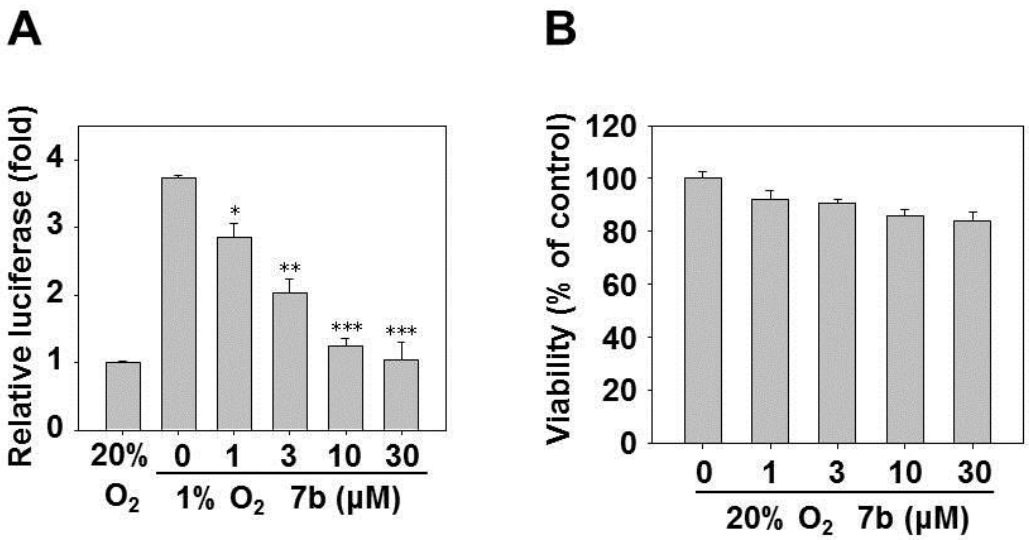


Figure 2. Effect of compound 7b on HRE-mediated reporter gene expression.

(A) Hep3B cells were transiently co-transfected with a pGL3-HRE-Luciferase and pRL-CMV vectors. Following 24 h incubation, the cells were incubated under hypoxia in the absence or presence of the indicated concentrations of compound **7b**. Luciferase activities were determined as described in "Materials and Methods". Data are represented as the mean ± standard deviation of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant with respect to the control. (B) Hep3B cells were treated with the indicated concentrations of compound **7b**. After 24 h incubation, cell viability was determined by MTT assays.

To understand the mechanism underlying the ability of **7b** to suppress HIF-1α transcriptional activity, we first measured HIF-1α protein levels by western blot analysis. Under normoxic conditions, HIF-1α protein is normally rapidly turned over and is virtually undetectable in cells. In contrast, hypoxic conditions or exposure to $CoCl_2$ leads to stabilization of HIF-1α, and its expression becomes readily detectable. Notably, HCT116, Hep3B, A549, and HeLa cells treated with **7b** under hypoxic conditions for 12 h showed a dose-dependent inhibition of HIF-1α protein levels compared with untreated cells (**Fig. 3A**), whereas **7b** had virtually no effect on levels of the control protein topoisomerase-I. To confirm these results, we performed

immunofluorescence staining of HIF-1α protein in Hep3B cells. As expected, exposure of the cells to **7b** (10 μM) under hypoxic conditions for 12 h virtually abolished the expression of HIF-1α protein in the nuclei (**Fig. 3B**).

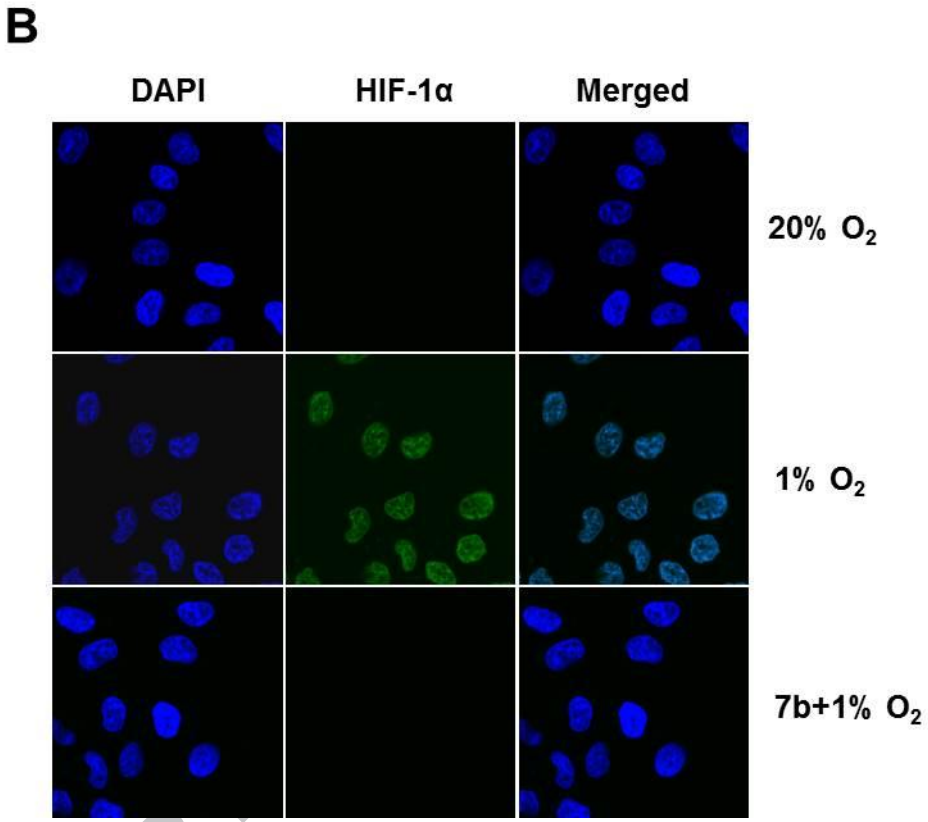


Figure 3. Compound **7b** inhibits hypoxia-induced the expression of HIF-1α protein.
(A) Cancer cells lines (HCT116, A549, Hep3B, and HeLa) were incubated under normoxia or hypoxia for 12 h, in the absence or presence of indicated concentration of **7b**, the nuclear extract for HIF-1α was analyzed by Western bloting. The same blot was reprobed with an anti-Topo-I antibody as a loading control. (B) Hep3B cells were incubated with 10 μM **7b** for 12 h under hypoxic conditions. After fixation, the cells were stained with anti-HIF-1α (1:100) antibody and Alexa fluor® 488 goat anti-mouse lgG (H+L) and examined by fluorescence microscopy. DAPI staining shows the location and size of nuclei. Scale bars: 20 μm. Images were acquired for each fluorescence channel, using suitable filters with 40× objective. The green and blue images were merged using J software.

To understand the potential mechanism underlying HIF-1α inhibition by **7b**, we explored the effects of **7b** on HIF-1α translational regulation. To examine whether the down-regulation of HIF-1α protein was caused by proteasomal degradation, we

blocked proteolytic activity of the 26S proteasome using the proteasome inhibitor MG132. Even in the presence of MG132, **7b** treatment decreased HIF-1α protein levels (**Fig. 4A**), suggesting that HIF-1α protein synthesis in Hep-3B cells is markedly impaired in the presence of **7b**. To address the effect of **7b** on HIF-1α protein stability, the protein translation inhibitor cycloheximide (CHX) was used to prevent *de novo* HIF-1α protein synthesis, and the half-life ($t_{1/2}$) of HIF-1α in the presence of **7b** was then calculated. We found that **7b** did not significantly modify the degradation rate of HIF-1α (**Fig. 4B**). To determine whether **7b** inhibited HIF-1α expression at the transcriptional level, the mRNA levels of HIF-1α were evaluated using RT-PCR. No significant effects of **7b** treatment on HIF-1α mRNA expression were observed under either normoxic or hypoxic conditions (**Fig. 5A**). Taken together, these findings suggest that **7b** does not facilitate or speed up the degradation of HIF-1α.

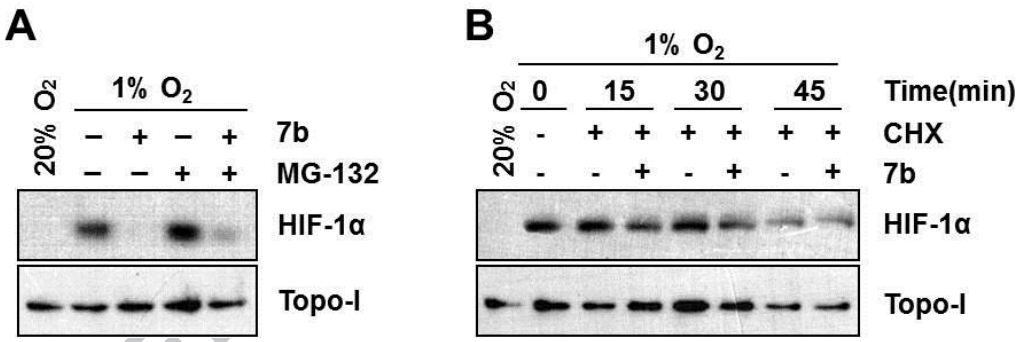


Figure 4. Compound **7b** inhibits the protein synthesis of HIF-1α but not by enhancing its degradation.

(A) Proteasome inhibitor MG-132 (10 μM) was added to Hep3B cells for 30 min prior to the treatment of compound **7b** (10 μM) and then the cells were incubated under hypoxia conditions for 12 h. The nuclear extract for HIF-1α was detected by western blotting. (B) Hep3B cells were incubated under normoxic or hypoxic conditions for 4 h. Cycloheximide (CHX) (10 μM) and compound **7b** (10 μM) were then mixed with culture media. After 0, 15, 30, or 45 min following the addition of cycloheximide, HIF-1α protein level was detected by western blotting.

Expression of VEGF, a crucial growth factor involved in tumor cell proliferation, angiogenesis, invasion, and metastasis, is known to be regulated by HIF-1α.[39,40] To determine whether **7b** suppresses VEGF gene expression, we performed RT-PCR

analysis in Hep3B cells. Indeed, treatment with **7b** resulted in a dose-dependent decrease in VEGF mRNA (**Fig. 5B**), and the effective concentrations were comparable to those inhibiting HIF-1α protein expression.

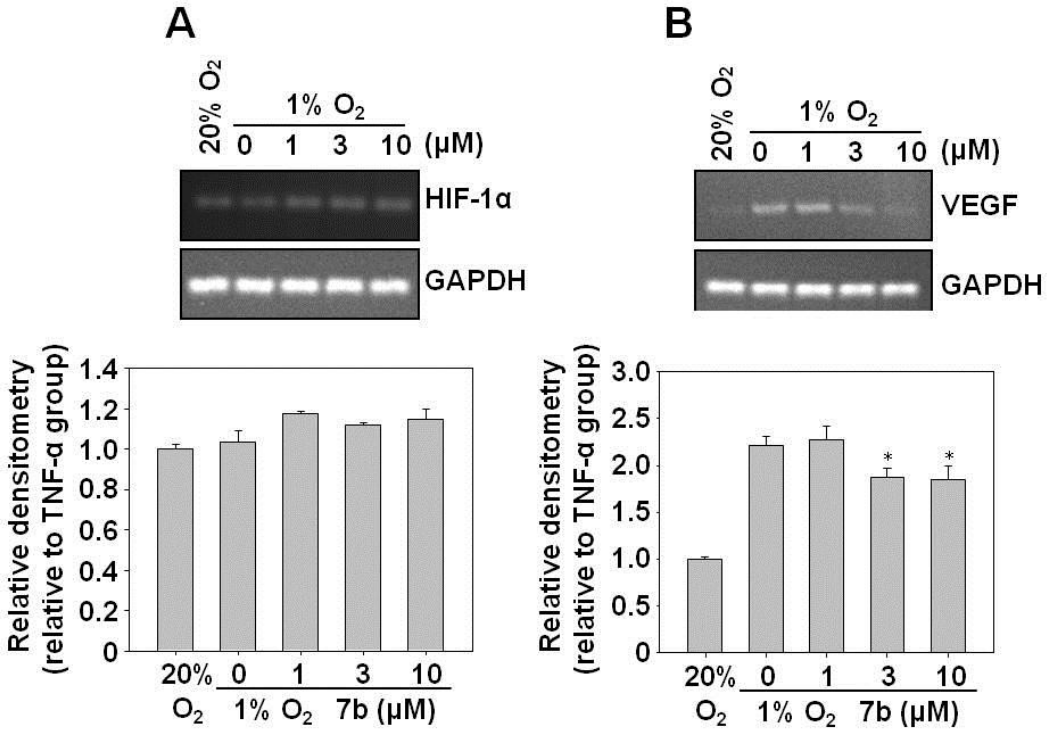


Figure 5. Effect of compound **7b** on the expression of HIF-1α target genes.
Hep3B cells were incubated under normoxic or hypoxic conditions for 12 h in the absence or presence of indicated concentration of compound **7b**. RNA was isolated from cells, reverse-transcribed, and analyzed by RT-PCR for HIF-1α (A) and VEGF (B). GAPDH was used to show equal loading of total RNA. Data are respresented as the mean ± standard deviation of three independent experiments. *$p < 0.05$, significant with respect to the control.

EdU is a thymidine analog that is incorporated into replicated chromosomal DNA during the S phase of the cell cycle.[41] DAPI is a fluorescent dye that binds strongly to A-T-rich regions in DNA and is commonly used for cell nucleus staining. We used the EdU incorporation assay, which is a highly sensitive and specific method, to investigate the inhibitory effects of 7b. As shown in Figure 6, treatment of Hep3B cells with **7b** (10 μM) for 12 h reduced the percentage of EdU-positive cells compared with the vehicle-treated cells (**Fig. 6A**), indicating that **7b** inhibits Hep3B

cell proliferation *in vitro*. We also performed clonogenic assays to determine the long-term anti-proliferative activity of **7b**. We observed a dose-dependent reduction in colony formation by **7b**-treated compared with control Hep3B cells (**Fig. 6B**).

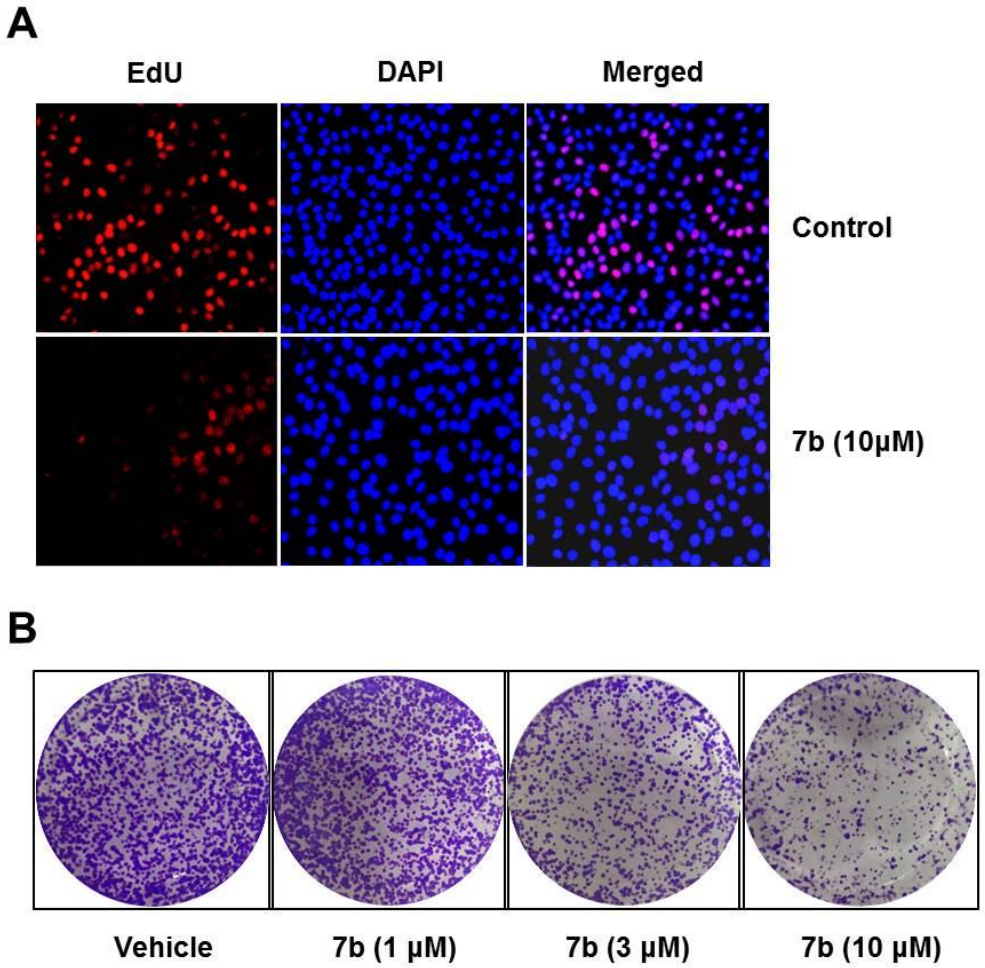


Figure 6. Effect of compound **7b** on the tumor cell proliferation.

(A) Hep3B cells were incubated for 12 h in the presence or absence of compound **7b** (10 μM). The EdU-labeled replicating cells were examined under a fluorescence microscope. DNA was stained using DAPI (blue). (B) Colony formation capability assay with treatment of compound **7b** (0, 1, 3 and 10 μM) for 12 h in Hep3B cells. Then the medium was replaced by fresh medium. Cells were allowed to grow for 10 d. The images were captured with a Nikon camera (Japan).

A series of UA derivatives containing an aminoguanidine moiety were designed, synthesized, and evaluated for their biological activity as HIF-1α inhibitors. Among the compounds tested, **7b** was the most active inhibitor of HIF-1α (IC_{50} 4.0 μM) and did not show significant cytotoxic activity against any of the tested cell lines. We

investigated the mechanism of action of compound **7b** and found that it reduced HIF-1α protein levels without affecting its transcription or degradation. Moreover, **7b** inhibited hypoxia-induced expression of VEGF at both the mRNA and protein levels. Compound **7b** was confirmed to inhibit the proliferation of cancer cells *in vitro*. These results provide initial support for the development of **7b** as a potential anti-cancer agent.

Acknowledgement

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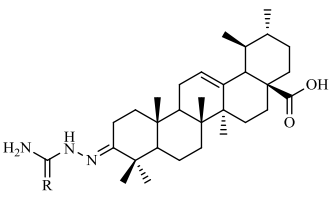
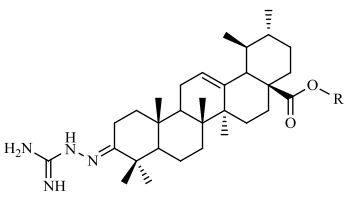
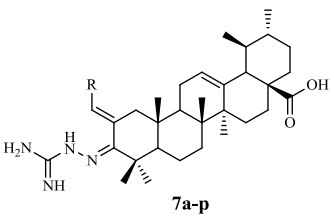
Table 1. *In vitro* inhibition of HIF-1α transcriptional activity in cell-based HRE reporter assay under hypoxia conditions and cytotoxic activity.



3a-c



5a-i



7a-p

	IC_{50} (μM)

Compound	R	HRE [a]	Cytotoxic activity [b]
UA	—	>100	23.8
3a	NH	>100	>100
3b	S	88.8	>100
3c	O	13.3	69.8
5a	Methyl	6.7	>100
5b	Ethyl	>100	6.0
5c	Amyl	>100	22.0
5d	Phenyl(3-Cl)	30.3	34.1
5e	Phenyl(4-Cl)	21.1	32.1
5f	Phenyl(2,4-2Cl)	40.5	>100
5g	Phenyl(2,6-2Cl)	13.3	22.3
5h	Phenyl(4-F)	13.2	12.9
5i	Phenyl(4-CH_3)	10.3	18.0
7a	Phenyl	37.8	>100
7b	Phenyl(4-Cl)	4.0	>100
7c	Phenyl(2-Cl)	>100	>100
7d	Phenyl(3-Cl)	>100	>100
7e	Phenyl(4-F)	>100	>100
7f	Phenyl(2-F)	>100	>100
7g	Phenyl(3-F)	>100	10.5
7h	Phenyl(2-Br)	>100	>100
7i	Phenyl(3-Br)	75.4	>100
7j	Phenyl(2,4-2Cl)	90.4	>100
7k	Phenyl(2,6-2Cl)	>100	>100
7l	Phenyl(4-CH_3)	>100	>100
7m	Phenyl(4-NO_2)	>100	>100
7n	Naphthyl	>100	>100
7o	Furyl	>100	>100
7p	Thienyl	22.8	>100

[a] The inhibitory effects of all the derivatives on HIF-1α transcriptional activity were tested by HRE luciferase reporter assay after 24 h treatment of HCT116 cells under hypoxic conditions. Values were shown as mean, n=3.

[b] The cytotoxic activity was evaluated by MTT assay after 24 h treatment of compounds under normoxic conditions. Values were shown as mean, n=3.

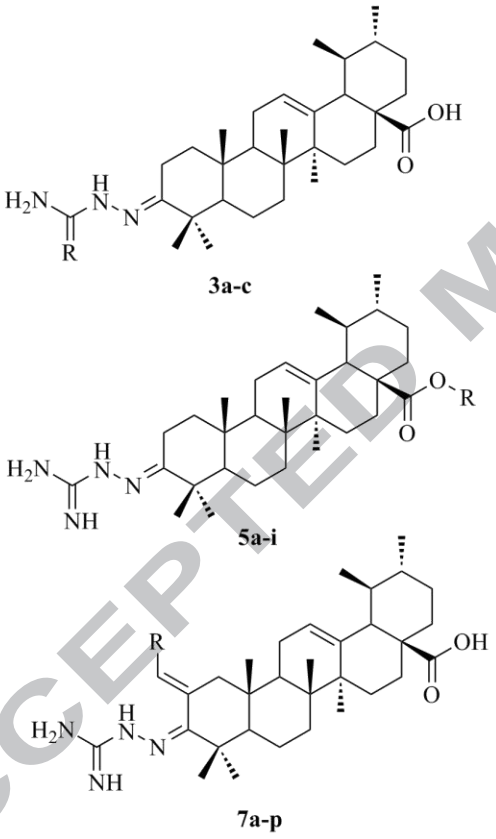
Highlights:

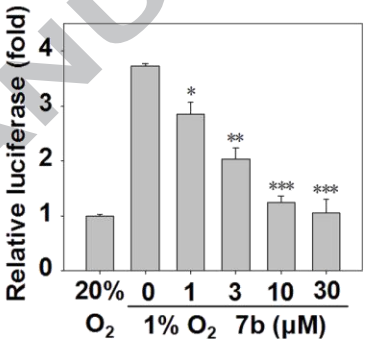
- Three series of ursolic acid derivatives were designed and synthesized.
- The inhibitory effects on HIF-1α transcriptional activity and cytotoxicity of compounds were evaluated.
- Compound **7b** is a promising HIF-1α inhibitory hit as anticancer agent**.**

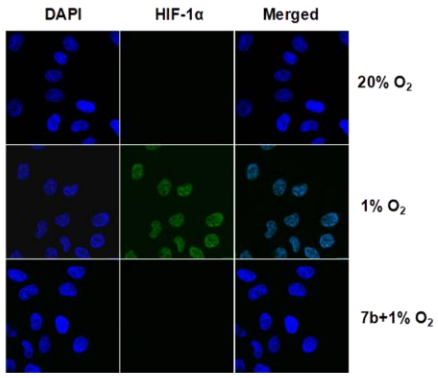
- The investigation of the mechanism on the mode of action of **7b** was performed.

Design, synthesis, and screening of novel ursolic acid derivatives as potential anti-cancer agents that target the HIF-1α pathway

Jie Wu [a,1], Zhi-Hong Zhang [a,1], Lin-Hao Zhang [a], Xue-Jun Jin [a], Juan Ma [a,*], and Hu-Ri Piao [a,*]







Three novel series of ursolic acid derivatives containing an aminoguanidine moietiy were designed, synthesized, and evaluated for anti-tumor activity as HIF-1α inhibitors.