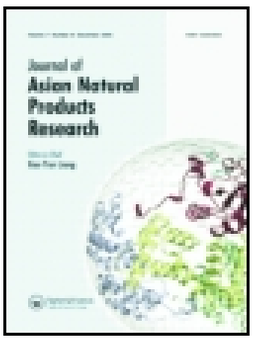

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Design, synthesis of a novel 4-*O*-methylsaucerneol analogue LXY7824 as potent HIF-1 inhibitor and anti-cancer agent

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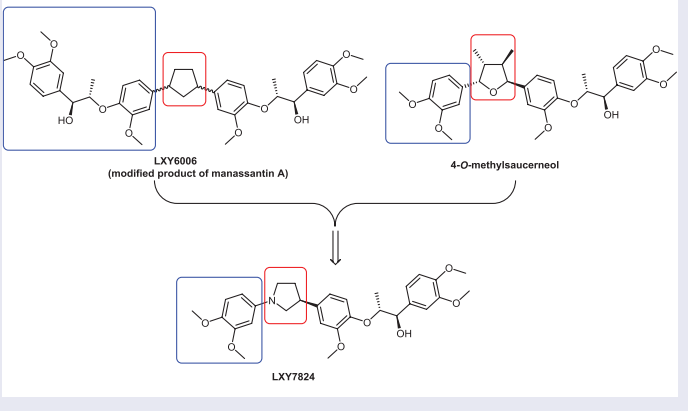
ABSTRACT

Hypoxia-inducible factor-1 (HIF-1), an important transcription factor for tumor survival, is an attractive target for anti-cancer treatment. Herein, we present the design and synthesis of LXY7824, a simplified analogue of 4-*O*-methylsaucerneol. In addition, its significant HIF-1 inhibitory activity and potent anti-cancer activity *in vivo* and *in vitro* were also reported.



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LXY7824; HIF-1; analogue; inhibition

1. Introduction

Cancer has the characteristic features of high morbidity and high mortality and becomes a serious threat to public health and life. However, the toxicity of the traditional chemotherapeutic drugs restricted them to be used. Therefore, more and more attention has been paid to low-toxicity and high-selectivity therapy.

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In human solid tumors, there are hypoxic regions and the tumor cells within these regions show resistance to both radiotherapy and chemotherapy [1,2]. Hypoxia-inducible factor-1 (HIF-1), which regulates cellular oxygen homeostasis, is an important transcription factor for tumor survival. It is a heterodimeric (α/β) transcriptional factor and its activity relies on HIF-1α because this protein is degraded in O_2-dependent manner [3–6]. The levels of HIF-1α are low under normal oxygen conditions (normoxia), but increase in response to hypoxia in solid tumor. HIF-1 is always over-expressed in various cancers as a result of this hypoxia and genetic alterations affecting key oncogenes (VEGF, HER2, FRAP, H-RAS, and c-SRC) and tumor suppressor genes. Therefore, HIF-1 is regarded as an attractive target for anti-cancer treatment [7–10].

Due to the importance of HIF-1 in tumor development and progression, a considerable amount of effort has been made to identify HIF-1 inhibitors for treatment of cancer [11]. As shown in Figure 1, various HIF-1 inhibitors have been reported, such as Topotecan [12], PX-478 [13,14], AC1-001 [15,16] and GN6767 [17,18].

In our previous studies, LXY6006, a simplified compound of manassantin A, has shown potential HIF-1 inhibitory and anti-cancer activity [19]. But in our further research we find LXY6006 possesses some toxicities such as red eyes of mice, death of mice as administration up to 60 mg/kg. 4-*O*-methylsaucerneol (IC_{50} 20 nmol), a simplified compound of manassantin A from *Saururus chinensis*, was reported as a sensitive inhibitor of HIF-1 [20]. This illustrated that the single side derivative could keep the HIF-1 inhibitory activity. In order to find novel, efficient, and low-toxicity HIF-1 inhibitor, a 4-*O*-methylsaucerneol analogue LXY7824, replacing the tetrahydrofuran moiety of 4-*O*-methylsaucerneol with a pyrrolidine ring, was designed and synthesized (Figure 2). Such a molecular design not only improves the chemical accessibility but also achieves structural novelty. The antitumor activity of LXY7824 was investigated both *in vitro* and *in vivo*.

2. Results and discussion

2.1. Chemistry

The preparation of target compound LXY7824 was described in Scheme 1.

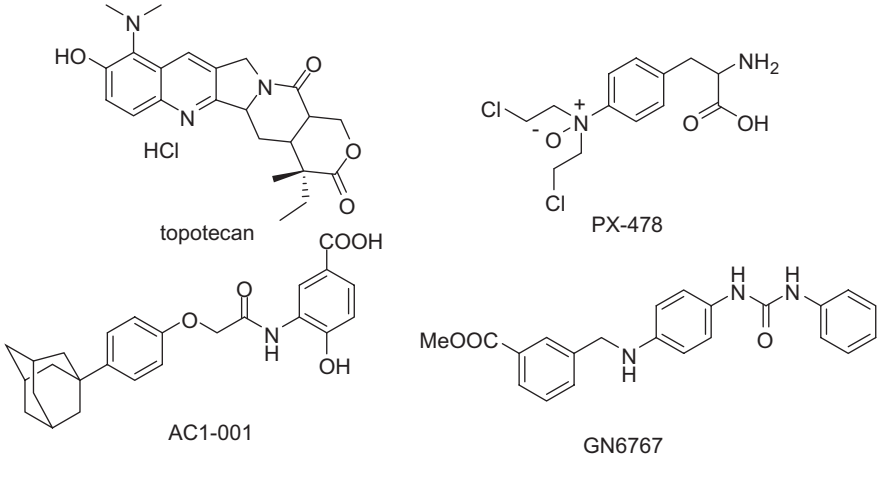


Figure 1. Structures of HIF-1 inhibitors.

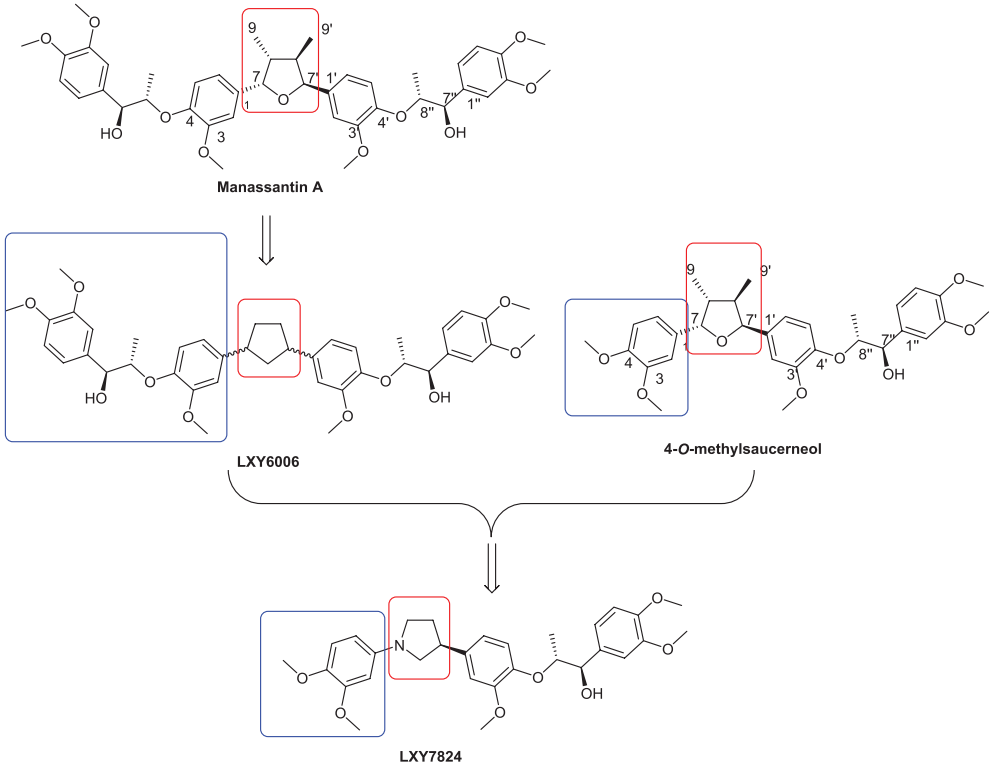


Figure 2. The structures of manassantin A, LXY6006, 4-*O*-methylsaucerneol and LXY7824.

Our synthesis commenced with 4-benzyloxy-3-methoxyphenylacetic acid using Evans oxazolidinone methodology to get compound **3** in high yield [21]. Hydrolysis of Evans product **3** with $LiBH_4$ gave **4** with 99% yield. Treatment of **4** with methanesulfonyl chloride and NEt_3 followed by NaN_3 in DMF gave azide product **6**. Staudinger reduction of azide **6** with triphenylphosphine (PPh_3) yielded primary amine **7** in 97% yield. After hydrolysis of amine **7** with trifluoroacetic acid, an intramolecular amidation produced compound **9** in great yield. With chiral lactam **9** in hand, we next embarked to introduce aromatic moiety. Buchwald–Hartwig [22] N-arylation of **9** gave **10** in moderate yield. After reduction of lactam to pyrrolidine with $LiAlH_4$, followed by catalytic hydrogenation reduction, we got key intermediate **12** in 71.4% yield for two steps. Treatment of **12** with **13** in acetonitrile (Cs_2CO_3 as a base) gave **14** in 85% yield. At last, compound **14** with 2, 6-di-tert-butyl-4-methylphenol and diisobutyl aluminum hydride provided the target compound LXY7824.

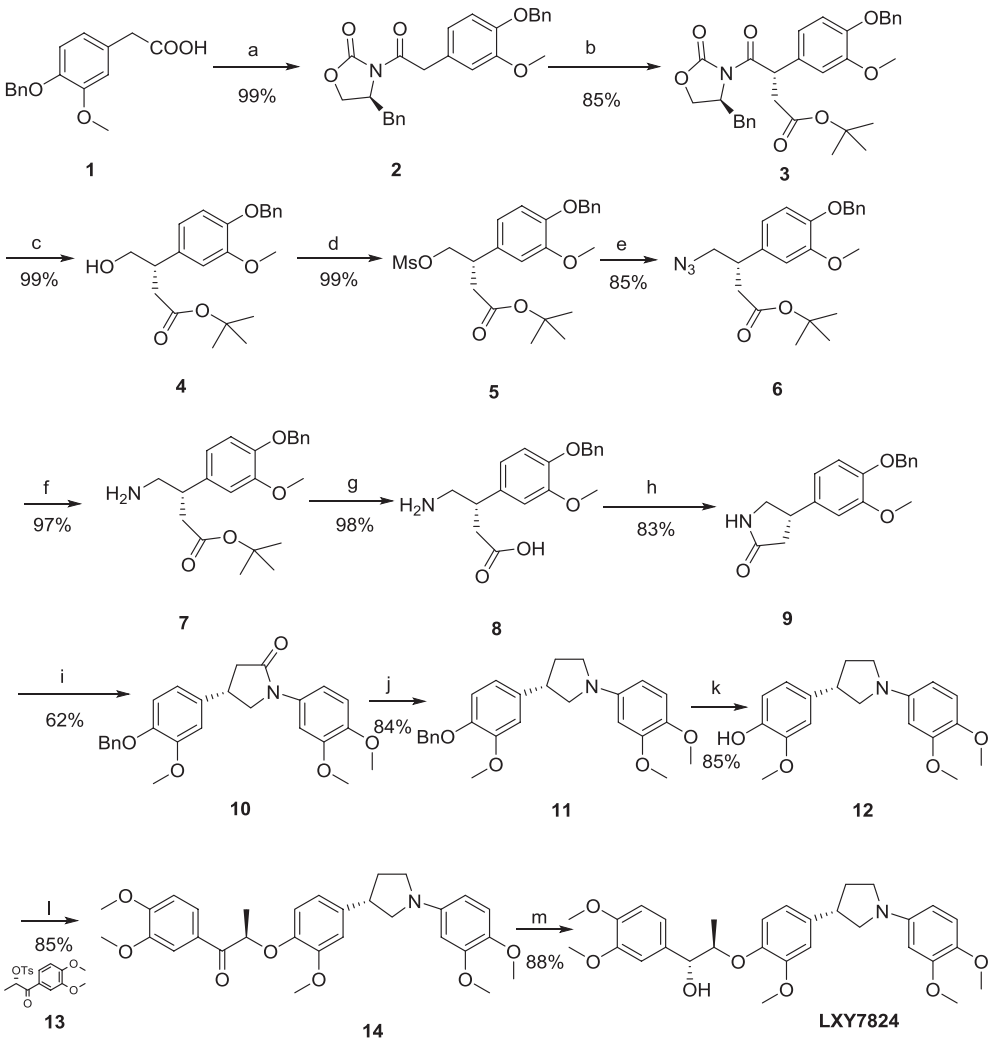
2.2. Biological evaluation

2.2.1. HIF-1 reporter activity assays

LXY7824 was initially evaluated using a T47D human breast tumor cell-based luciferase reporter assay for HIF-1 inhibitory activity. LXY7824 dose-dependently inhibited the HIF activity with the IC_{50} values of 22.8 nmol/L, the same level compared with 4-*O*-methylsaucerneol (Table 1).

Table 1. HIF-1-inhibitory activity of LXY7824.

	Inhibition (%)			
Compound	0.5	5	50	IC_{50} (nmol/L)
LXY7824	3.1	30.2	60.9	22.8



Scheme 1. Synthesis of target compound LXY7824. Reagents and conditions: (a) $SOCl_2$, followed by *n*-BuLi, (S)-4-benzyl-2-oxazolidinone, THF; (b) $BrCH_2COOtBu$, NaHMDS, THF; (c) $LiBH_4$, THF/Et_2O; (d) MsCl, NEt_3, CH_2Cl_2; (e) NaN_3, DMF; (f) PPh_3, THF, then H_2O; (g) CF_3COOH, CH_2Cl_2; (h) N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI), 1-Hydroxybenzotriazole (HOBT), N,N-Diisopropylethylamine (DIPEA), CH_2Cl_2; (i) $Pd(OAc)_2$, Xantphos, Cs_2CO_3, 1,4-Dioxane; (j) $LiAlH_4$, THF; (k) Pd-C, CH_2Cl_2/MeOH; (l) Cs_2CO_3, **13**, CH_3CN; (m) 2,6-di-tert-butyl-4-methylphenol, diisobutyl aluminum hydride, Et_2O.

2.2.2. Western blotting analysis

LXY7824 was next evaluated for the inhibition of the expression of HIF by western blotting analysis. In Daoy cells, hypoxic exposure (1% O_2, 6 h) leads to the accumulation of HIF-1α protein. LXY7284 could inhibit the expression of HIF with dose-dependent manner (Figure 3).

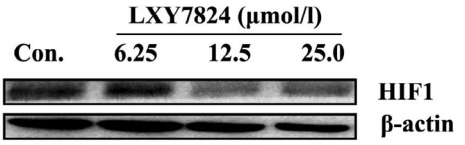


Figure 3. LXY7824 inhibits HIF-1 expression. After hypoxic exposure (1% O_2, 6 h) in the presence of test compound, levels of HIF-1 protein in Daoy cells were determined by western blot.

Table 2. The inhibition of LXY7824, GDC0449, Iressa on the proliferation of various cancer cells.

Comp.	IC_{50} (μmol/L)							
	U-87MG	Hs683	U251	T98G	Daoy	SH-SY5Y	SK-N-SH	SVGP12
LXY7824	16.37	34.59	29.98	48.43	12.25	55.97	41.00	307.31
GDC0449	11.46	245.00	>500	304.84	>500	243.37	303.04	474.43
Iressa	38.71	18.53	29.08	13.76	24.6	53.92	19.63	44.67

2.2.3. In vitro cytotoxicity evaluation of LXY7824

In order to determinate the anti-cancer activity of LXY7824, it was tested the inhibition activity against eight tumor cell lines and a human normal brain cell SVGP12 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method with Iressa and GDC0449 as the positive control. The eight tumor cell lines included human glioma cells U-87 MG, Hs683, U251, T98G, medulloblastoma cell line Daoy, human neuroblastoma cell lines SH-SY5Y and SK-N-SH. The results indicated that U-87 MG and Daoy were more sensitivity, and the IC_{50} values were 16.37 and 12.25 μmol/L respectively, suggesting LXY7824 was sensitive to brain tumor (Table 2, Figure 4).

2.2.4. In vivo studies

The results of the cytotoxicity evaluation of LXY7824 showed that it was sensitive to brain tumor. Therefore, *in vivo* studies of LXY7824 were further evaluated using Daoy xenografts model. The results showed that tumor growth was significantly suppressed in mice administrated with LXY7824 (100 mg/kg), with relative tumor volume (RTV, T/C) to 40.57% at the end of the treatment, and inhibition of tumor weight was 55.14% (Table 3, Figure 5).

3. Conclusions

Starting from 4-benzyloxy-3-methoxyphenylacetic acid, we get a simplified product LXY7824 of 4-*O*-methylsaucerneol in 13 steps with 18.3% overall yield. In this study, we found that LXY7284 could inhibit the expression of HIF with dose-dependent manner. Moreover, LXY7824 is sensitive to brain tumor and significantly inhibited growth of brain tumor in nude mice. It can be concluded that chemical modification of 4-*O*-methylsaucerneol certainly holds great promise, and that further exploration in this field may lead to potent anticancer agents.

Table 3. The antitumor activity of compounds on the medulloblastoma Daoy xenograft model.

			Tumor volume								
			Body weight (g) $\bar{X}$SD		$\bar{X}$SD (mm^3)			RTV		Tumor weight	
Comp.	Dose mg/kg	Animal No. Begin/End	Begin	End	Begin	End	Inhibition (%)	$\bar{X}$SD	T/C	$\bar{X}$SD (g)	Inhibition (%)
Control		6/6	17.8 ± 0.5	16.6 ± 1.4	211.2 ± 30.9	931.4 ± 140.0		4.45 ± 0.68		0.97 ± 0.36	
LXY7824	100	6/6	17.0 ± 0.6	17.0 ± 1.1	223.7 ± 61.4	393.9 ± 237.2***	57.70	1.81 ± 1.30**	40.57	0.44 ± 0.27*	55.14

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with control.

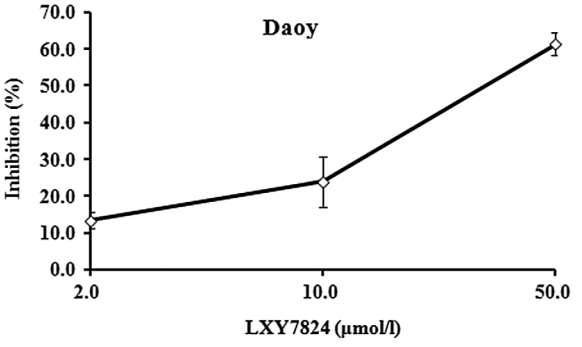


Figure 4. The dose-effect relationship of LXY7824 on proliferation in Daoy cell line.

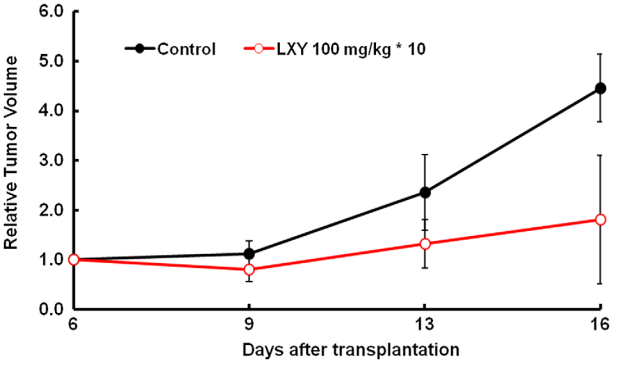


Figure 5. The inhibition effect of LXY7824 on the growth in medulloblastoma Daoy xenograft. Animals were randomly divided into two groups, when tumors grew to an average volume of 200 mm^3. One group received p.o (oral gavage) of Cremophor EL/ethanol/water and served as a vehicle control; the other group received an oral dose of 100 mg/kg LXY7824 every day for 10 days. Mice were euthanized at the end of the treatment period. Tumors were removed and weighed. Tumor growth curves were plotted for Daoy xenografts with administration of LXY7824 100 mg/kg at given time-points. Mean RTV at given-points for Daoy xenograft.

4. Experimental

4.1. General experimental procedures

^{1}H NMR and ^{13}C NMR were recorded on Mercury 400, Bruker AV500, AV600, JEOL ECZ400S spectrometer. Coupling constants are given in Hz and chemical shifts are expressed as δ values in ppm. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet. ESI-HRMS data were measured on Thermo Exactive Orbitrap plus spectrometer. All the chemicals were purchased from commercial sources: Sigma-Aldrich Chemical Co., Arcos Chemical Co., or J&K Chemical Co. with the purity of more than 95%. Solvents were dried according to standard procedures when needed. Flash column chromatography was performed on Biotage Isolera one or carried out on silica gel (200–300 mesh).

4.2. (S)-4-Benzyl-3-(2-(4-(benzyloxy)-3-methoxyphenyl)acetyl)oxazolidin-2-one (2)

To a solution of (S)-4-benzyl-2-oxazolidinone (6.43 g, 36.4 mmol) in 100-ml THF, *n*-BuLi (2.5 M, 14.5 ml, 36.4 mmol) was added at −78 °C, and then stirred for 30 min. After that, acyl chloride of **1** (10.5 g, 36.4 mmol) which was prepared from **1** with $SOCl_2$ was added to the reaction mixture and allowed to stir for 3 h at room temperature. Then the reaction was quenched by sat. NH_4Cl aq solution, diluted with ethyl acetate (300 ml), and washed with brine, dried, and evaporated to afford the crude product, which was purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:5) to give **2** (15.7 g, 99%) as a white solid;

$[\alpha]_D^{20}$ + 60.5 (c 0.5, $CHCl_3$); R_f 0.28 (3:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.83–7.45 (m, 13H), 5.16 (s, 2H), 4.66–4.69 (m, 1H), 4.26–4.29 (m, 1H), 3.89–4.22 (m, 3H), 3.89 (s, 3H), 3.25 (d, J = 12.8 Hz, 1H), 2.73–2.78 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 153.3, 149.5, 147.3, 137.1, 135.0, 129.4, 128.9, 128.5, 127.7, 127.3, 127.2, 126.4, 121.9, 113.8, 113.4, 70.9, 66.0, 55.9, 55.2, 40.9, 37.6; HRESIMS: *m/z* 432.1796 $[M + H]^+$ (calcd for $C_{26}H_{26}O_5$ N, 432.1806).

4.3. tert-Butyl (S)-4-((S)-4-benzyl-2-oxooxazolidin-3-yl)-3-(4-(benzyloxy)-3-methoxyphenyl)-4-oxobutanoate (3)

A solution of NaHMDS in THF (2.0 M, 3 ml, 6 mmol) was added to a solution of **2** (2.23 g, 5 mmol) in 50-ml THF at −78 °C. After 1.5 h, tert-Butyl bromoacetate (0.9 ml, 6 mmol) was added and the reaction was stirred for 2 h. The reaction was quenched by sat. NH_4Cl aq solution, diluted with ethyl acetate (200 ml), and washed with brine, dried, and evaporated to afford the crude product, which was purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:15) to give **3** (2.3 g, 85%) as a white solid.

$[\alpha]_D^{20}$ + 104.5 (c 1.0, $CHCl_3$); R_f 0.48 (3:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.79–7.43 (m, 13H), 5.42 (dd, J = 4.4, 11.2 Hz, 1H), 5.12 (s, 2H), 4.55–4.60 (m, 1H), 4.02–4.11 (m, 2H), 3.88 (s, 3H), 3.24–3.39 (m, 2H), 2.78–2.81 (m, 1H), 2.60 (dd, J = 4.4, 16.8 Hz, 1H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 171.0, 152.8, 149.6, 147.7, 137.1, 135.7, 129.9, 129.5, 128.9, 128.6, 127.8, 127.3, 120.7, 113.9, 112.2, 80.9, 71.0, 65.7, 56.0, 55.8, 44.2, 40.2, 37.6, 28.1; HRESIMS: *m/z* 568.2296 $[M + Na]^+$ (calcd for $C_{32}H_{35}O_7NNa$, 568.2306).

4.4. tert-Butyl (S)-4-((S)-4-benzyl-2-oxooxazolidin-3-yl)-3-(4-(benzyloxy)-3-methoxyphenyl)-4-oxobutanoate (4)

To a stirred solution of **3** (2.4 g, 4.4 mmol) in 5-ml THF and 50-ml Et_2O was added $LiBH_4$ (4 M, 2.42 ml, 9.7 mmol) at 0 °C. The mixture was allowed to warm to room temperature and stirred for 2 h. The reaction was quenched by addition of 10-ml 1 N NaOH and extracted with 100-ml Et_2O. Organic layers were washed with H_2O (20 ml), followed by brine (20 ml), and then dried ($MgSO_4$). The solution was concentrated *in vacuo* to give the crude product, which was purified by column chromatography on silica gel (EtOAc/ petroleum ether, 1:2) to give **4** (1.6 g, 99%) as an oil.

$[\alpha]_D^{20}$ + 11.3 (c 0.3, $CHCl_3$); R_f 0.14 (3:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69–7.43 (m, 8H), 5.13 (s, 2H), 3.88 (s, 3H), 3.72 (m, 2H), 3.21–3.25 (m, 1H), 2.67 (dd, J = 7.2, 15.2 Hz, 1H), 2.52 (dd, J = 7.2, 15.2 Hz, 1H), 1.34 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.8, 149.7, 147.1, 137.2, 134.2, 128.5, 127.8, 127.2, 119.6, 114.2, 111.7, 80.6, 77.2, 77.0, 76.8, 71.0, 67.0, 56.0, 44.3, 38.8, 27.9; HRESIMS: m/z 395.1817 $[M + Na]^+$ (calcd for $C_{22}H_{28}O_5Na$, 395.1829).

4.5. tert-Butyl (S)-3-(4-(benzyloxy)-3-methoxyphenyl)-4-((methylsulfonyl)oxy) butanoate (5)

To a stirred solution of **4** (1.64 g, 4.41 mmol) in 40-ml CH_2Cl_2, NEt_3 (0.9 ml, 6.62 mmol) was added dropwise at 0 °C, followed by MsCl (0.4 ml, 4.85 mmol). The reaction mixture allowed to warm to 25 °C. After stirring at 25 °C for 1 h, the reaction was diluted by 50-ml CH_2Cl_2. The organic layers were washed with 20-ml H_2O, followed by 20-ml brine, then dried, and evaporated to afford the crude product to give **5** (2 g, 99%) as an oil.

4.6. tert-Butyl (S)-4-azido-3-(4-(benzyloxy)-3-methoxyphenyl)butanoate (6)

To a solution of **5** (0.6 g, 1.33 mmol) in DMF (5 ml), NaN_3 (0.26 g, 4 mmol) was added. The reaction was stirred at 65 °C for 5 h. And then, H_2O (50 ml) and EtOAc (50 ml) were added. The organic layers were washed with brine (50 ml) and then dried ($MgSO_4$). The mixture was filtered, and the filtrate was concentrated under reduced pressure to give the crude product, which was purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:20) to give **6** (0.45 g, 85%) as an oil.

$[\alpha]_D^{20}$ -6.5 (c 0.3, $CHCl_3$); R_f 0.25 (10:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69–7.43 (m, 8H), 5.13 (s, 2H), 3.89 (s, 3H), 3.41–3.53 (m, 2H), 3.25–3.29 (m, 1H), 2.66 (dd, J = 6.8, 15.2 Hz, 1H), 2.51 (dd, J = 6.8, 15.2 Hz, 1H), 1.33 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.9, 149.6, 147.3, 137.1, 133.8, 128.5, 127.8, 127.2, 119.5, 114.1, 111.53, 80.8, 71.0, 56.5, 56.0, 41.8, 39.3, 27.9; HRESIMS: m/z 420.1879 $[M + Na]^+$ (calcd for $C_{22}H_{27}O_4N_3Na$, 420.1894).

4.7. tert-Butyl (S)-4-amino-3-(4-(benzyloxy)-3-methoxyphenyl)butanoate (7)

Compound **6** (1 g, 2.5 mmol) was dissolved in THF (25 ml) and PPh_3 (860 mg, 3.3 mmol) was added at 0 °C. The solution was stirred at 25 °C for 24 h, and then water (10 ml) was added. The mixture was concentrated after 6 h and extracted with EtOAc (100 ml). The combined organics were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (EtOAc/ petroleum ether, 1:1 to DCM/ MeOH, 20:1) to afford **7** (0.9 g, 96.8%) as an oil.

$[\alpha]_D^{20}$ + 13.8 (c 0.3, $CHCl_3$); R_f 0. 5 (10:1 DCM–MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67–7.44 (m, 8H), 5.13 (s, 2H), 3.88 (s, 3H), 3.02–3.05 (m, 1H), 2.80–2.93 (m, 2H), 2.59 (dd, J = 7.2, 14.8 Hz, 1H), 2.51 (dd, J = 7.2, 14.8 Hz, 1H), 1.31 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.6, 149.7, 147.0, 137.3, 135.2, 128.6, 128.5, 127.8, 127.3, 119.8, 114.3, 111.7, 80.4, 77.2, 77.0, 76.8, 71.1, 56.0, 47.7, 45.7, 40.2, 28.0; HRESIMS: m/z 372.2169 $[M + H]^+$ (calcd for $C_{22}H_{30}O_4$ N, 372.2169).

4.8. (S)-4-Amino-3-(4-(benzyloxy)-3-methoxyphenyl)butanoic acid (8)

A solution of CF_3COOH (0.5 ml, 6.7 mmol) in CH_2Cl_2 (3 ml) was added to a solution of **7** (215 mg, 0.58 mmol) in CH_2Cl_2 (5 ml) at 0 °C. The reaction was stirred at room temperature for 2 h. The reaction was concentrated under reduced pressure to give **8** (180 mg).

4.9. (S)-4-(4-(Benzyloxy)-3-methoxyphenyl)pyrrolidin-2-one (9)

Without further purification, **8** (180 mg, 0.57 mmol), EDCI (142 mg, 0.74 mmol), and HOBT (154 mg, 1.14 mmol) were dissolved in CH_2Cl_2 (30 ml), and then DIPEA (0.25 ml, 1.43 mmol) was added to the mixture at 0 °C. The reaction was stirred for 16 h. Water (15 ml) was added to the reaction and extracted with CH_2Cl_2 (30 ml). The combined organics were washed with 5% $NaHCO_3$, brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (DCM/MeOH, 50:1) to afford **9** (140 mg, 82.5%) as a white solid.

$[\alpha]_D^{20}$ + 28.1 (c 0.5, $CHCl_3$); R_f 0.25 (2 ml: 4 drops DCM–MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.71–7.36 (m, 8H), 6.42 (s, 1H), 5.13 (s, 2H), 3.89 (s, 3H), 3.72–3.76 (m, 1H), 3.60–3.64 (m, 1H), 3.36–3.40 (m, 1H), 2.67–2.73 (m, 1H), 2.44–2.50 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.8, 149.8, 147.2, 137.1, 135.2, 128.6, 127.9, 127.2, 118.7, 114.2, 110.6, 71.1, 56.1, 49.7, 40.1, 38.1; HRESIMS: *m/z* 298.1440 $[M + H]^+$ (calcd for $C_{18}H_{20}O_3N$, 298.1438).

4.10. (S)-4-(4-(Benzyloxy)-3-methoxyphenyl)-1-(3,4-dimethoxyphenyl)pyrrolidin-2-one (10)

Compound **9** (500 mg, 1.68 mmol), 4-bromoveratrole (493 mg, 2.27 mmol), $Pd(OAc)_2$ (20 mg, 5% mmol), Xantphos (75 mg, 7.5% mol), and Cs_2CO_3 (766 mg, 2.35 mmol) were dissolved in 1,4-dioxane (15 ml). The mixture was stirred at 100 °C for 25 h under N_2. EtOAc (50 ml) was added, the organic layers were washed with H_2O (50 ml), brine (50 ml), and then dried (Na_2SO_4). The mixture was filtered, and the filtrate was concentrated *in vacuo* to give the crude product, which was purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:1) to give **10** (0.45 g, 62%) as an oil.

$[\alpha]_D^{20}$ -10.4 (c 0.4, $CHCl_3$); R_f 0.13 (1:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30–7.51 (m, 6H), 6.77–6.87 (m, 5H), 5.15 (s, 2H), 4.11–4.14 (m, 1H), 3.89 (s, 6H), 3.87 (s, 3H), 3.81–3.85 (m, 1H), 3.63–3.65 (m, 1H), 2.95–2.97 (m, 1H), 2.78–2.80 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.7, 149.9, 148.9, 147.3, 146.2, 137.0, 134.7, 132.8, 128.6, 127.9, 127.2, 118.7, 111.7, 110.9, 110.5, 105.1, 71.1, 56.4, 56.1, 56.0, 56.0, 40.4, 36.9; HRESIMS: *m/z* 434.1954 $[M + H]^+$ (calcd for $C_{26}H_{28}O_5N$, 434.1962).

4.11. (S)-3-(4-(Benzyloxy)-3-methoxyphenyl)-1-(3,4-dimethoxyphenyl)pyrrolidine (11)

To a solution of $LiAlH_4$ (18 mg, 0.46 mmol) in 5-ml THF, **10** (80 mg, 0.18 mmol) in THF was added at 0 °C, and then stirred for 3 h at 60 °C. After cooling the mixture to 0 °C, the reaction was quenched by sat. Na_2SO_4 aq solution, diluted with dichloromethane (100 ml), and washed with brine, dried, and evaporated to afford the crude product, which was

purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:5) to give **11** (65 mg, 84%) as a white solid.

$[\alpha]_D^{20}$ -17.8 (c 0.15, $CHCl_3$); R_f 0.5 (2:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30–7.45 (m, 5H), 6.75–6.85 (m, 4H), 6.08–6.21 (m, 2H), 5.14 (s, 2H), 3.88 (s, 6H), 3.82 (s, 3H), 3.64–3.68 (m, 1H), 3.40–3.49 (m, 3H), 3.28–3.32 (m, 1H), 2.36 (m, 1H), 2.05–2.14 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.1, 149.7, 146.9, 143.4, 140.3, 137.2, 128.5, 127.8, 127.2, 119.0, 114.1, 113.8, 111.0, 102.5, 97.1, 71.1, 56.9, 56.1, 55.8, 55.4, 48.2, 43.9, 33.5; HRESIMS: *m/z* 420.2163 $[M + H]^+$ (calcd for $C_{26}H_{30}O_4N$, 420.2169).

4.12. (S)-4-(1-(3,4-Dimethoxyphenyl)pyrrolidin-3-yl)-2-methoxyphenol (12)

Compound **11** (60 mg, 0.14 mmol) was dissolved in DCM/MeOH (1 ml/4 ml). Pd-C (80 mg) was added and the solution was stirred at 25 °C for 4 h under hydrogen. The mixture was filtered and concentrated. The residue was purified by flash chromatography (EtOAc/petroleum ether, 1:5) to afford **12** (40 mg, 85%) as an oil.

R_f 0.4 (2:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.79–6.89 (m, 4H), 6.09–6.21 (m, 2H), 3.88 (s, 6H), 3.82 (s, 3H), 3.64–3.68 (m, 1H), 3.40–3.49 (m, 3H), 3.27–3.31 (m, 1H), 2.37 (m, 1H), 2.08–2.14 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.1, 146.9, 144.4, 119.8, 114.3, 113.9, 119.6, 102.5, 97.1, 57.0, 56.6, 55.9, 55.8, 48.2, 44.1, 33. 6, 29.7; HRESIMS: *m/z* 330.1689 $[M + H]^+$ (calcd for $C_{19}H_{24}O_4N$, 330.1700).

4.13. (R)-1-(3,4-Dimethoxyphenyl)-2-(4-((S)-1-(3,4-dimethoxyphenyl)pyrrolidin-3-yl)-2-methoxyphenoxy)propan-1-one (14)

Compound **12** (30 mg, 0.09 mmol) and Cs_2CO_3 (36 mg, 0.11 mmol) were mixed in acetonitrile (3 ml). **13** (40 mg, 0.11 mmol) in 1-ml acetonitrile was added to the mixture at 0 °C. The reaction was stirred at room temperature for 3 h. The reaction mixture was filtered and concentrated. The residue was purified by flash chromatography (EtOAc/petroleum ether, 1:2) to afford **14** (40 mg, 85%) as a solid.

$[\alpha]_D^{20}$-33 (c 0.13, $CHCl_3$); R_f 0.4 (1:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, J = 8.0 Hz, 1H), 7.68 (s, 1H), 6.89–6.90 (m, 5H), 6.19 (s, 1H), 6.08 (d, J = 8.0 Hz, 1H), 5.40–5.43 (m, 1H), 3.81–3.94 (m, 15H), 3.62 (t, J = 8.0 Hz, 1H), 3.37–3.45 (m, 3H), 3.25 (t, J = 8.0 Hz, 1H), 2.34–2.35 (m, 1H), 2.02–2.12 (m, 1H), 1.72 (d, J = 8.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.7, 153.6, 150.1, 149.8, 149.0, 145.6, 143.4, 140.3, 136.8, 127.3, 123.6, 119.2, 115.7, 113.8, 111.4, 111.2, 110.1, 102.4, 97.0, 78.1, 57.0, 56.1, 56.0, 56.0, 55.8, 55.4, 48.2, 43.9, 33.5, 29.7, 19.4; HRESIMS: *m/z* 522.2472 $[M + H]^+$ (calcd for $C_{30}H_{36}O_7N$, 522.2486).

4.14. (1R,2R)-1-(3,4-Dimethoxyphenyl)-2-(4-((S)-1-(3,4-dimethoxyphenyl) pyrrolidin-3-yl)-2-methoxyphenoxy)propan-1-ol (LXY7824)

To a stirred solution of 2,6-di-tert-butyl-4-methylphenol (3.23 g, 14.66 mmol) in 40-ml toluene, was added DIBALH (1.2 M, 12.2 ml, 14.66 mmol) at 0 °C. The mixture was stirred at this temperature for 1.5 h. And then compound **14** (430 mg, 0.8 mmol) was added dropwise at −78 °C and stirred for 3 h. The reaction was quenched by addition of 1 N HCl (25 ml) and extracted with 100-ml EtOAc. Organic layers were washed with H_2O (20 ml), followed

by brine (20 ml), and then dried (Na_2SO_4). The solution was concentrated *in vacuo* to give the crude product, which was purified by column chromatography on silica gel (EtOAc/petroleum ether, 1:2) to give **4** (380 mg, 88%) as a white solid.

$[\alpha]_D^{20}$ -62.5 (c 0.4, $CHCl_3$); R_f 0.25 (1:1 petroleum ether–ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.83–6.98 (m, 9H), 6.22 (s, 1H), 6.11 (d, J = 8.4 Hz, 1H), 4.64 (d, J = 8.4 Hz, 1H), 3.90–3.93 (m, 12H), 3.83 (s, 3H), 3.68 (t, J = 8.4 Hz, 1H), 3.41–3.49 (m, 3H), 3.35 (t, J = 8.0 Hz, 1H), 2.36–2.41 (m, 1H), 2.12–2.15 (m, 1H), 1.17 (d, J = 6.4 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.9, 150.2, 149.0, 148.9, 146.5, 143.4, 140.5, 138.0, 132.6, 120.0, 119.5, 119.1, 114.0, 111.1, 110.9, 110.1, 102.6, 97.2, 84.2, 78.4, 57.0, 55.9, 55.8, 55.5, 48.2, 44.1, 33.5, 17.1; HRESIMS: m/z 524.2634 $[M + H]^+$ (calcd for $C_{30}H_{38}O_7$ N, 524.2643).

4.15. HIF-1 reporter activity assays

The T47D cells (American Type Culture Collection) were incubated in RPIM 1640 Medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (PAA), 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C. Exponentially grown T47D cells were plated at 3×10^4 cells into 96-well plates. After 24 h, T47D cells were co-transfected with the pGL2-TK-3HRE luc reporter [23] and the internal control construct pRL-CMV(Promega) by lipofectamine 2000 (Invitrogen) following manufacturer's instructions, 0.2 µg pGL2-TK-3HRE luc reporter and 0.01 µg pRL-CMV used per well. After 24 h, test compounds were added in a volume of 100-µl RPIM 1640 medium per well, and the incubation continued for another 30 min at 37 °C. The cells were exposed to hypoxic (1% O_2/5% CO_2/94% N_2) or normoxic (5% CO_2/95% air) conditions at 37 °C for 20 h. A dual luciferase assay system (Promega, Madison, USA) was employed to determine luciferase activities following manufacturer's instructions. Firefly luciferase activity was normalized with that of the Renilla luciferase [19,24].

4.16. Western blotting analysis

Exponentially growing cells were treated with chemicals before exposed to normoxia (21% O_2), 1,10-phenanthroline (10 mM), or hypoxia (1% O_2) at 37 °C. Nuclear extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents (CWBio Co., Beijing, China). Cells were lysed using RIPA buffer containing 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktails (Amresco, Washington, USA). Equal amounts of proteins were resolved by SDS-PAGE gels and transferred to PVDF membranes (Millipore, Burlington, USA). Membranes were blocked with 5% non-fat dry milk followed by incubation with primary antibodies and HRP conjugated secondary antibodies for protein visualization. The HIF-1 antibody was purchased from CST (Shanghai) Biological Reagents Co., Ltd., China and Santa Cruz (USA), respectively.

4.17. In vitro cytotoxicity evaluation of LXY7824

Cell lines were seeded in a 96-well plate. After incubation for 24 h, cells were treated with varied concentrations of unnatural ginsenosides or dimethyl sulfoxide as vehicle. After incubation for 96 h, 50-µl MTT stock solution (2 mg/ml, Sigma Chemical) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. The solution was

then removed from each well, and 150-μl dimethyl sulfoxide was added. Following gentle agitation, the absorbance was measured using an ELISA reader (Bio-Rad, USA) at 570 nm. Three parallel samples were measured each time. Absorbance values were normalized to the values obtained for vehicle-treated cells to determine the percentage of surviving cells. The median inhibitory concentration (IC_{50}) was assessed from the dose response curve.

4.18. In vivo studies

xenografts. All animal studies were performed in compliance with the policies of the Institute of Materia Medica Animal Care and Use Committee. Six-week-old female BALB/c/nu nude mice were used in the present study. They were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in the controlled environment at 25 °C on a 12-h light/dark cycle (5 mice per group). When tumors grew to an average volume of 200 mm^3, tumor-bearing mice were randomly separated into two groups of six animals. One group received p.o (oral gavage) of Cremophor EL/ethanol/waterand served as a vehicle control; the other group received an oral dose of 100 mg/kg LXY7824 every day for 10 days. Mice were euthanized at the end of the treatment period. Tumors were removed and weighed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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