ORIGINAL ARTICLE

Inhibitory Activity of the Hypoxia-inducible Factor-1 Pathway by Tartrolone C

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Abstract Hypoxia-inducible factor-1 (HIF-1) is a central mediator of cellular responses to low oxygen and has recently become an important therapeutic target for solid tumor therapy. To identify small molecule inhibitors of the HIF-1 transcriptional activation, we have established a high through-put assay system using a stable transformant of mammalian cells that express a luciferase reporter gene construct containing a HIF-1 binding site. Using this system, we screened 5,000 cultured broths of microorganisms, and we found that fermentation broth produced by *Streptomyces* strain 1759-27 showed significant inhibition of the reporter activity induced by hypoxic conditions. The active substance NBR1759-27 was purified and determined to be tartrolone C by several methods including X-ray crystallography. In the reporter gene assay, tartrolone C inhibited the HIF-1 transcriptional activity under hypoxic conditions with an IC_{50} value of $0.17 \, \mu g/ml$.

Keywords hypoxia, hypoxia-inducible factor-1, tartrolone C, X-ray crystallography, reporter gene assay

Introduction

Angiogenesis is a key step in the tumor progression, invasion and metastasis, and vascular endothelial growth factor (VEGF) plays an important role during this step [1]. The expression of VEGF is regulated by hypoxia. Hypoxia is a common feature of many solid tumors and contributes to their progression. Hypoxic tumor cells are resistant to conventional chemotherapy and radiotherapy and hypoxia promotes a more malignant phenotype [2]. The transcription of VEGF is enhanced under hypoxic conditions and controlled primarily by hypoxia inducible factor-1 (HIF-1). HIF-1 is a central mediator of cellular responses to low oxygen and has recently become an important therapeutic target for solid tumor therapy. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β. HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator, is constitutively present in normoxic cells. In contrast, the HIF-1α protein levels are primarily dependent on the intracellular oxygen concentration [3]. Under normoxic conditions, the HIF-1α protein is hydroxylated by prolyl hydroxylases which require oxygen for their activity, and once hydroxylated, HIF-1α rapidly binds to pVHL, which is the recognition component of an E3 ubiquitin-protein ligase activity [4, 5], and then rapidly and continuously degraded by ubiquitination and proteasomal degradation [6, 7].

Recently several small-molecule inhibitors of HIF-1 pathway such as an Hsp90 inhibitor geldanamycin, a topoisomerase I inhibitor topotecan and an antimicrotubule agent vincristine [8~11] have been identified and chetomin, a disrupter of HIF-1 binding to p300, attenuated tumor growth through the disruption of HIF-1 pathway *in vivo* [12].

We previously established a high through-put assay system using a stable transformant of mammalian cells that expressed the luciferase gene under the control of the

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hypoxia-response element [13]. To identify novel HIF-1 pathway inhibitors, we screened the broths of microorganisms using this system, and found that NBR1759-27 inhibited the HIF-1 transcriptional activity under hypoxic conditions. In this paper, we describe the isolation, crystal structure and biological activity of NBR1759-27.

Materials and Methods

Fermentation

The slant culture of strain was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of the seed medium composed of galactose 1.0%, dextrin 1.0%, soypeptone 0.5%, corn steep liquor 0.25%, glycerol 0.5%, $(NH_4)_2SO_4$ 0.1%, $CaCO_3$ 220 mg/flask, pH 7.4, and cultured for 8 days at 27°C. One ml of the seed culture was then inoculated into 500-ml Erlenmeyer flasks containing 200 ml of the production medium composed of sucrose 2.0%, corn steep liquor 1.0%, $CaCO_3$ 220 mg/flask, pH 7.2, and cultured for 12 days at 27°C on a rotary shaker.

X-Ray Structure Analysis

A colorless needle crystal of NBR1759-27 ($0.14 \times 0.03 \times 0.03$ mm) was mounted in a loop. All measurements were made on a Bruker SMART APEX II ULTRA diffractometer with monochromated intense Mo-$K\alpha$ radiation. The Mo radiation was produced by a rotating anode generator (TXS) combined with a multilayer confocal mirror (Helios). Crystal data: Empirical formula; $C_{46}H_{68}BNaO_{18}$, Formula weight; 942.80, Crystal system; orthorhombic, Space group; P2(1)2(1)2(1), Lattice parameters; $a = 10.659(5)$ Å, $b = 17.404(8)$ Å, $c = 27.229(13)$ Å, Volume; 5051(4) Å^3, Z value; 4, D_{calc}; 1.240 Mg/m^3, Absorption coefficient; 0.101 mm^{-1}, The reflection data were collected at 90 K using ω scans. The structure was solved by a direct method (SHELXS-97).

Cell Lines and Reagents.

A stable transformant of CHO cells was established by the transfection of the HIF-1-dependent luciferase ($5 \times$HRE/pGL3/VEGF/E1b) and neomycin resistant genes as previously described [8]. Stably transfected cells were maintained in α-MEM supplemented with 10% fetal bovine serum (ICN Biomedicals, Costa Mesa, CA, USA) and kanamycin (50 μg/ml). HCT-8 human colorectal adenocarcinoma cells and MCF-7 human breast cancer cells were maintained in RPMI1640 with 10% fetal bovine serum and kanamycin (50 μg/ml). Cells were cultured at 37°C in a humidified incubator containing 5% CO_2 (normoxia). Hypoxic treatment was performed by incubating cells at 37°C in an air-tight chamber (Forma Scientific Inc, Marietta, OH, USA) filled with a mixture of 90% N_2, 5% CO_2 and 5% H_2.

Reporter Gene Assay

Stably transfected cells were seeded at 1×10^4 cells/well in 96-well microplates one day before the following treatment. They were treated with drugs for 15 hours under hypoxic conditions, and then harvested for determination of the luciferase activity. The assay was carried out using a Luciferase Assay System from Promega.

Western Blotting

HCT-8 and MCF-7 cells were seeded at 2×10^5 cells/well in 6-well plates one day before the following treatment. They were treated with NBR1759-27 for 15 hours under hypoxic conditions. Treated cells were washed with ice-cold PBS containing 0.1 mM Na_3VO_4 and then lysed in a lysis buffer (20 mM HEPES, 150 mM NaCl, 1.0% Triton X-100, 10% glycerol, 1.0 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 1.0 mM Na_3VO_4 and 25 μg/ml each of antipain, leupeptin, and pepstatin). The protein concentration of the lysates was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal protein extracts (50 μg) were separated by SDS-PAGE and transferred onto an Immobilon polyvinylidene fluoride (PVDF) membrane filter (Milipore, Bedford, MA, USA). The membranes were blocked for 1 hour at room temperature with blocking buffer and then incubated with primary antibodies specific for HIF-1α (1:200 dilution, BD Bioscience) or β-actin (1:5,000 dilution, Sigma Aldrich Japan). The membranes were then washed and chemiluminescence detection was performed using peroxidase-conjugated secondary antibodies.

Results and Discussion

Isolation of NBR1759-27

The cultured broth (5 liters) was centrifuged at 3000 rpm for 20 minutes. The broth was extracted with 4 liters of ethyl acetate and concentrated under reduced pressure to yield oily material. The material was dissolved in $CHCl_3$ and applied on a silica gel column (150 g), and eluted with $CHCl_3$. The active fractions (67.9 mg) were applied on a LH-20 column and eluted with 1:1 (v/v) of $CHCl_3$-MeOH. The fractions containing NBR1759-27 (57.8 mg) were further purified by HPLC (column; Capcell Pak C_{18}, SHISEIDO, 20×250 mm; solvent, 45% CH_3CN; detection, UV at 230 nm; flow rate, 6.0 ml/minute). Under these
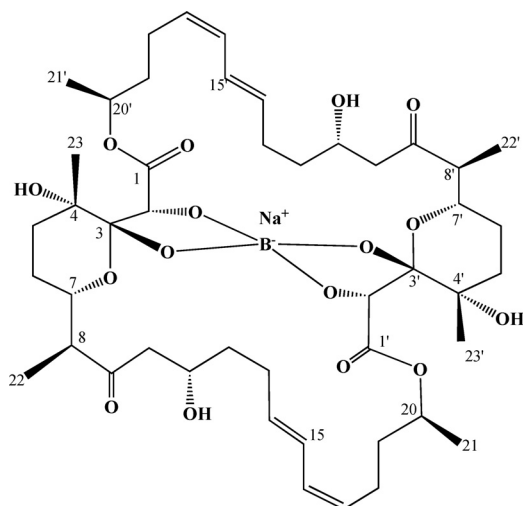
Fig. 1 Relative structure of NBR1759-27.

Fig. 2 Crystal structure of NBR1759-27.

conditions, NBR1759-27 was eluted as a peak with retention time of 50 minutes. The pooled fractions were concentrated under reduced pressure to give pure NBR1759-27 (46.1 mg) as white powders. It was crystallized from $CHCl_3$ - MeOH to give colorless needles. mp. 207~210°C (dec); UV (MeOH) λ_{max} nm (ε) 232 (30200); $[\alpha]_D^{23}$ +26.9 (c 0.13, MeOH) ; HRESI-MS (m/z) 933.4379 (M+2Na)$^+$ (calcd for $C_{46}H_{68}BNa_2O_{16}$, 933.4396).

Identification of the Active Substance as Tartrolone C
The planar structure of NBR1759-27 was identified as tartrolone C [14] by comparing their spectral data including ^1H and ^{13}C NMR. The relative structure shown in Fig. 1 was confirmed by the X-ray crystallography of NBR1759-27 as described bellow.

The Crystal Structure of NBR1759-27
The stereochemistry of tartrolone B, a closely related compound of tartrolone C, was determined by the X-ray crystallography. On the other hand, the relative stereochemistry of tartrolone C was reported to be same as that for tartrolone B based on the positive sign of the optical rotation and NOESY experiments [14]. To confirm the stereochemistry of NBR1759-27, we carried out X-ray crystallography of NBR175-27 using a single crystal grown from $CHCl_3$ - MeOH solution. Fig. 2 shows the ORTEP drawing of NBR1759-27.

Biological Activities
To identify novel small molecules targeting the HIF-1 pathway, we transfected Chinese hamster ovary (CHO) cells with a luciferase reporter gene construct containing 5
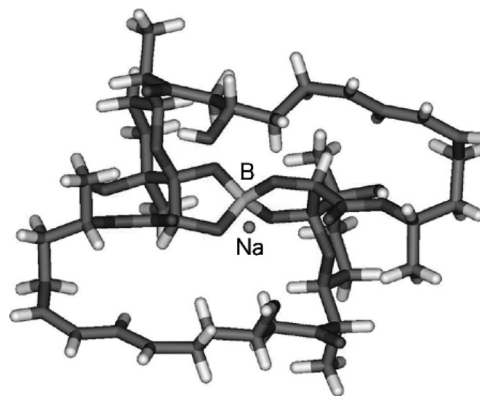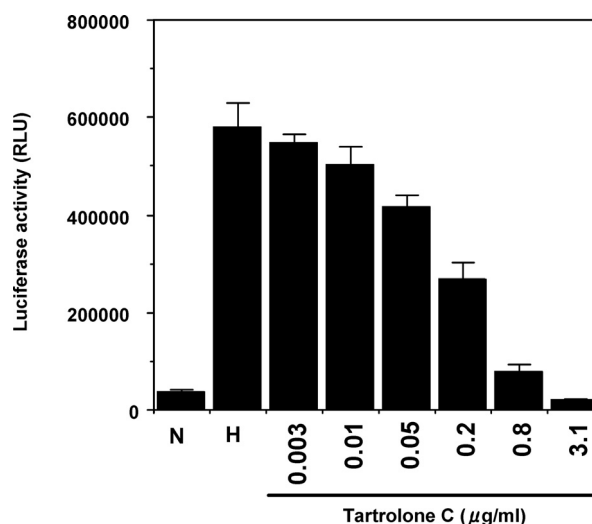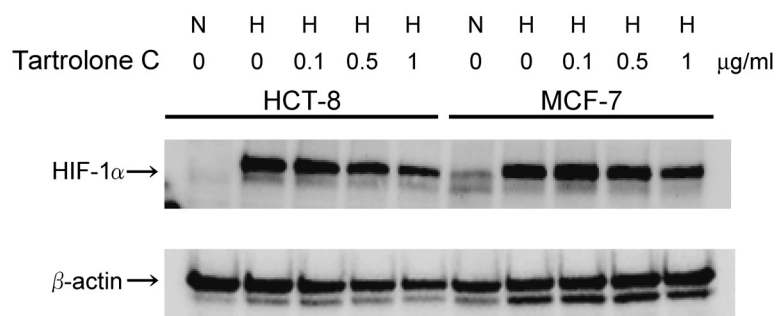

Fig. 3 Effect of tartrolone C on the expression of the VEGF promoter reporter gene.

N: normoxic condition, H: hypoxic condition.

copies of a HIF-1 binding site derived from the human VEGF promoter and established a stable clone. In this assay system, the HIF-1 transcriptional activity was significantly inhibited by tartrolone C in a dose-dependent manner under hypoxic conditions with an IC_{50} value of 0.17 μg/ml (Fig. 3). Tartrolone C did not show any cytotoxic effects at the concentrations used in this experiment.

We then examined the effect of tartrolone C on the HIF-1α protein expression by Western blot. As shown in Figs. 4A and 4B, tartrolone C decreased the hypoxic induction of the HIF-1α protein in HCT-8 human colorectal adenocarcinoma cells and MCF-7 human breast cancer cells. This decrease of HIF-1α protein is one of factors that cause the inhibition of HIF-1 transcriptional activity.
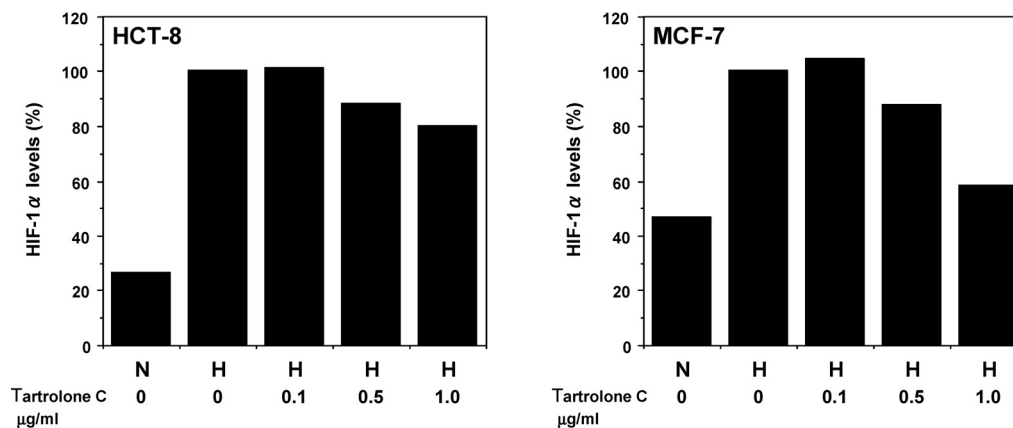
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A

	N	H	H	H	H	N	H	H	H	H	
Tartrolone C	0	0	0.1	0.5	1	0	0	0.1	0.5	1	µg/ml

HCT-8   MCF-7

HIF-1α →

β-actin →

B

Fig. 4 Effect of tartrolone C on the expression of HIF-1α protein.

A: The HIF-1α protein levels were measured by Western blot. B: The quantification of the HIF-1α protein levels by densitometry. Values were normalized to the β-actin levels. N: normoxic condition, H: hypoxic condition.

In summary, NBR1759-27 was isolated from the fermentaion broth of *Streptomyces* sp. by the screening system using a reporter under the control of the hypoxia-response element during hypoxia and then identified as tartrolone C through MS and NMR analysis. In the reporter gene assay, tartrolone C was found to inhibit the HIF-1 transcriptional activity under hypoxic conditions. Furthermore we have shown that tartrolone C decreased HIF-1α protein levels under hypoxic conditions. HIF-1 is recognized as an attractive target for development of novel cancer therapeutics and there is a possibility that tartrolone C acts as an inhibitor of angiogenesis.

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