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Menadione and ethacrynic acid inhibit the hypoxia-inducible factor (HIF) pathway by disrupting HIF-1α interaction with p300

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ABSTRACT

Hypoxia is a general characteristic of most solid malignancies and intimately related to neoplastic diseases and cancer progression. Homeostatic response to hypoxia is primarily mediated by hypoxia inducible factor (HIF)-1α that elicits transcriptional activity through recruitment of the CREB binding protein (CBP)/p300 coactivator. Targeted blockade of HIF-1α binding to CBP/p300 would thus constitute a novel approach for cancer treatment by suppressing tumor angiogenesis and metastasis. Here, we identified inhibitors against the interaction between HIF-1α and p300 by a fluorescence polarization-based assay employing a fluorescently-labeled peptide containing the C-terminal activation domain of HIF-1α. Two small molecule inhibitors, menadione (MD) and ethacrynic acid (EA), were found to decrease expression of luciferase under the control of hypoxia-responsive elements in hypoxic cells as well as to efficiently block the interaction between the full-length HIF-1α and p300. While these compounds did not alter the expression level of HIF-1α, they down-regulated expression of a HIF-1α target vascular endothelial growth factor (VEGF) gene. Considering hypoxia-induced VEGF expression leading to highly aggressive tumor growth, MD and EA may provide new scaffolds for development of tumor therapeutic reagents as well as tools for a better understanding of HIF-1α-mediated hypoxic regulation.

1. Introduction

Low oxygen tension is prevalent in most solid tumor development [1,2]. Adaptation of cancer cells to hypoxia is critical for survival and growth and is mediated through the transcriptional activation of genes involved in tumor angiogenesis, metastasis, progression and glucose homeostasis [3–5]. Such cellular response is mainly orchestrated by the master switch hypoxia inducible factor-1α (HIF-1α). Under normoxic conditions, HIF-1α is hydroxylated on proline residues 402 and 564 by O_2-dependent prolyl hydroxylases (PHDs), followed by specific recognition by von Hippel-Lindau (VHL) and subsequent proteosomal degradation [6,7]. Under hypoxia, HIF-1α cannot be hydroxylated and thus rapidly accumulates. The stabilized HIF-1α is translocated to the nucleus, where it heterodimerizes with the β subunit [8,9]. Upon binding to the cognate DNA sequence, hypoxia response element (HRE), the heterodimer recruits the transcriptional coactivator CREB binding protein (CBP)/p300 to initiate transcription of hypoxia related genes such as vascular endothelial growth factor (VEGF), matrix metalloproteinase and lysyl oxidase [10–13].

Given the centrality of the HIF pathway in the cellular adaptation to hypoxia, inhibition of HIF function using novel inhibitors is an attractive therapeutic strategy for cancer treatment. Previous studies have validated that inhibition of the HIF pathway resulted in tumor regression [14,15], and several small molecule inhibitors have been discovered to disrupt the HIF pathway via a variety of molecular mechanisms including the inhibition of HIF-1α protein synthesis, stabilization, nuclear translocation and transactivation [16–19]. In addition, many anti-cancer agents under preclinical evaluation or clinical development were found to interfere with the HIF pathway indirectly [20–22].

The transcriptional activation of genes by HIF-1α is well characterized in structural studies [23,24]. HIF-1α recruits CBP/p300 through the direct interaction between the C-terminal activation domain (C-TAD) of HIF-1α and the cysteine/histidine rich domain 1 (CH1) of p300, which is critical for transcriptional activity of HIF-1α [23,24]. Furthermore, it has been reported that blockade of the HIF-1α-p300 interaction significantly attenuated HIF activity [19,25,26]. Hence, targeting the C-TAD-p300/CH1 interaction would provide an ideal strategy for blocking the HIF pathway. In the present report, we describe the discovery of new small molecule inhibitors against the HIF-1α-p300 interaction. Employing a fluorescence polarization (FP)-based assay and a cell-based HRE-dependent luciferase reporter assay, we screened a chemical library. As a result, menadione (MD) and ethacrynic acid (EA) were identified as effective inhibitors for the interaction of HIF-1α with p300 in vitro and in cellular levels, and were further found to re-

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duce the mRNA expression level of VEGF, one of the downstream target genes of HIF-1α.

2. Materials and methods

2.1. Materials

Human HeLa cervical epithelium cells (CCL-2) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA). The cells were made hypoxic by incubating in a hypoxic incubator (Thermo Scientific, Waltham, MA, USA) at 37 °C. Chetomin (CTM), MD, EA and desferrioxamine (DFO) mesylate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-β-actin was purchased from Sigma–Aldrich, and anti-HIF-1α from BD Bioscience (San Jose, CA, USA). A fluorescein-labeled peptide containing amino acids 786–826 of HIF-1α CTAD denoted as F-786–826 was synthesized by conjugating fluorescein with the N-terminal insertion of an aminocaproic acid linker (AnyGen, KwangJu, Korea). All other chemicals were of the highest grade of purity commercially available.

2.2. Inhibitor screening

National Institute of Neurological Disorders and Stroke (NINDS) Custom Collection II (MicroSource Discovery Systems, Gaylordsville, CT, USA) was used to screen potential inhibitors against the interaction between HIF-1α and p300. An FP-based interaction assay was performed using GST-p300/CH1 and the F-786–826 peptide as described previously [22]. 100 nM of F-786–826 was incubated with 1.5 μM of GST-p300 in EBC buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.25% Nonidet P-40, and 1 mM DTT) containing 40 μM chemicals at 25 °C. FP was then measured by an Appliskan multimode microplate reader (Thermo Scientific, Waltham, MA, USA) with an excitation/emission filter set of 485/535 nm.

2.3. Luciferase reporter assay

Luciferase reporter assays were performed as described previously [23]. Briefly, cells were plated in 24-well plates at a density of 5×10^4 cells/well. After attachment, cells were transfected with 200 ng of hypoxia reporter plasmid $p(HRE)_4$-Luc containing four copies of erythropoietin HRE and 50 ng of plasmid pCHO110 for β-galactosidase using Lipofectamine (Life Technologies) according to the manufacturer's instruction. Cells were treated with compounds at a final concentration of 20 μM, and vehicle samples were treated with culture media containing 0.1% DMSO. After incubation for 16 h, hypoxia was induced by incubating cells in a hypoxic incubator for 16 h. Cell extracts were prepared and analyzed using the Luciferase Assay System (Promega, Fitchburg, WI, USA). Measured luciferase activities were normalized by total protein concentrations which were determined by the Bradford assay (BioRad Laboratories, Hercules, CA, USA).

2.4. Determination of inhibitory activity of compounds

190 μL of p300/CH1 at a final concentration of 250 nM were incubated with varying concentrations of compounds in EBC buffer for 2 h at 25 °C, followed by addition of 10 μL of F-786–826 at a final concentration of 100 nM. FP values were then measured at 25 °C, and IC_{50} values for compounds were determined by nonlinear regression with sigmoidal dose–response curves using Sigmaplot software. All assays were performed in triplicate.

2.5. Recombinant protein expression and protein–protein interaction assay

DNA encoding CH1 of p300 (amino acids 300–520) were subcloned into pGEX-4T-1 (GE Healthcare, Little Chalfont, UK) or pET-28a (EMD Millipore, Billerica, MA, USA). His-p300/CH1 and GST-p300/CH1 were overexpressed and purified using Ni–NTA Sepharose and glutathione-Sepharose resins (Qiagen, Venlo, NED), respectively. Concentrations of the recombinant proteins were determined by the Bradford assay. Nuclear extracts were prepared from HeLa cells grown to high density in 100 mm dish, followed by purification using the nuclear extraction kit (EMD Millipore). To mimic hypoxia, HeLa cells were treated with 150 μM of DFO for 18 h. The protein concentrations of the nuclear extracts were determined by the Bradford assay. For the protein–protein interaction assay, 5 μg of His-p300/CH1 was incubated with 20 μL of Ni–NTA Sepharose in binding buffer (150 mM Tris–HCl pH 8.0, 360 mM NaCl, and 20 mM imidazole) at 4 °C for 2 h. Immobilized His-tagged proteins were incubated with varying concentrations of compounds at 4 °C for 2 h, followed by incubation with 50 μg of nuclear extracts at 4 °C for 2 h. After washing with the same buffer, the complexes were separated by SDS–PAGE and visualized by immunoblotting with monoclonal anti-HIF-1α (BD Bioscience, Franklin Lakes, NJ, USA). His-p300/CH1 proteins were visualized by Coomassie Brilliant Blue staining.

2.6. cell viability assay

HeLa cells were plated in 96-well plates at a density of 1×10^4 cells/well. After overnight incubation, cells were treated without (vehicle sample) or with varying concentrations of compounds for 16 h. All samples contained a final concentration of 0.5% DMSO (v/v). Cell viability was determined using an MTT cell viability reagent (Sigma–Aldrich). GI_{50} values were calculated by fitting the averages of triplicate measurements to sigmoid dose–response curves using Sigmaplot software.

2.7. Immunoblotting

HeLa cells were seeded in 6-well culture dishes at a density of 2×10^6 cells/well. After attachment, cells were treated with 0.1% DMSO, CTM (200 nM), MD (20 μM) or EA (50 μM) in media for 4 h, followed by incubation for an additional 16 h under normoxic or hypoxic (1% O_2) conditions. Cells were then lysed and the protein concentrations were determined by the Bradford assay. For immunoblotting, whole cell lysates were resuspended in SDS sample buffer, boiled for 5 min and run on SDS–PAGE gels, followed by transfer of the proteins to nitrocellulose membranes by semi-dry transfer (Trans-Blot SD, Bio-Rad). Proteins were reacted with anti-human HIF-1α antibody and/or with anti-β-actin antibody, and visualized by enhanced chemiluminescence according to the manufacturer's instruction (Pierce), with anti-mouse IgG conjugated with horseradish peroxidase as a secondary antibody.

2.8. Quantitative real-time PCR

HeLa cells (2×10^5 cells/well in 6-well plates) were treated with compounds for 16 h under normoxic or hypoxic (1% O_2) conditions. Total mRNA was extracted from HeLa cells using RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA by using Maxime RT-PCR premix (Intron biotechnology, Seongnam, Korea). The primers for human VEGF and α-tubulin were designed as reported previously [24,25]: human VEGF (GenBank accession No.M32977) (forward: 5′-GCCTTGCTGCTCTACCTC-3′, reverse: 5′-GGCACACAGGATGGCTTG-3′); human α-tubulin (GenBank accession No. BC009314) (forward: 5′-AGCGTGCCTTTGTTCACTG-3′, re-