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A natural compound, aristoyagonine, is identified as a potent bromodomain inhibitor by mid-throughput screening

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ABSTRACT

Bromodomain-containing protein 4 (Brd4) is known to play a key role in tumorigenesis. It binds acetylated histones to regulate the expression of numerous genes. Because of the importance of brd4 in tumorigenesis, much research has been undertaken to develop brd4 inhibitors with therapeutic potential. As a result, various scaffolds for bromodomain inhibitors have been identified. To discover new scaffolds, we performed mid-throughput screening using two different enzyme assays, alpha-screen and ELISA. We found a novel bromodomain inhibitor with a unique scaffold, aristoyagonine. This natural compound showed inhibitory activity *in vitro* and tumor growth inhibition in a Ty82-xenograft mouse model. In addition, we tested Brd4 inhibitors in gastric cancer cell lines, and found that aristoyagonine exerted cytotoxicity not only in I-BET-762-sensitive cancer cells, but also in I-BET-762-resistant cancer cells. This is the first paper to describe a natural compound as a Brd4 bromodomain inhibitor.

1. Introduction

At present, 46 proteins have been reported to contain bromodomains; they can be divided into eight major families based on sequence similarity [1]. Among the bromodomain-containing proteins, BET (bromodomain and extraterminal domain) proteins, which have two bromodomains and a unique extra-terminal domain, including Brd2, Brd3, Brd4, and Brdt, are known to play a key role in chromatin biology. They modulate gene transcription through recruitment of the transcriptional machinery to the histone-chromatin complex via bromodomains, which bind to acetylated lysine residues of histone tails [2]. Brd4 is the most commonly studied member of BET proteins.

There are three isoforms of Brd4 in humans. One isoform has 1362 amino acid residues, and the others have 722 and 796 residues, respectively. Brd4 is recruited to lineage-specific enhancers and promoters by binding to acetylated histones. It recruits several proteins, such as NSD3/CHD8, and p-TEFb, which promote transcriptional activation, to enhancers/promoters through direct physical interaction [3].

Brd4 has been considered a therapeutic target for cancer since the discovery of the fusion protein Brd4-Nut in midline carcinoma [4]. More than 90% of midline carcinomas contain the Brd4-Nut fusion gene. An *in vitro* analysis revealed that knockdown of Brd4 markedly decreased Brd4-Nut positive cell proliferation [5]. In addition, Zuber et al. reported that Brd4 knockdown down-regulated c-Myc expression to induce cell death in AML cell line [6]. c-Myc is known as a key player in cancer cell proliferation. However, it has been regarded as a non-druggable target due to the absence of enzymatic activity or any deep pockets for small molecule inhibitors [7]. Zuber et al. indicated that Brd4 ablation can be a

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good strategy for cancer treatment because of its ability to down-regulate c-Myc expression. For this reason, there have been many efforts to develop potent Brd4 inhibitors [8]. At present, more than 30 papers relevant to the development of BET bromodomain inhibitors have been published [9–16], and 16 compounds are undergoing clinical trial [17]. No natural product, however, has been reported as a bromodomain inhibitor.

Here, we performed mid-throughput screening to identify novel Brd4 inhibitors with new scaffolds, and identified a natural product as a bromodomain inhibitor. We also studied the sensitivity of gastric cancer cells to BET bromodomain inhibitors. Several studies have been performed to investigate whether Brd4 inhibition causes cell death in solid tumors, including breast cancer, prostate cancer, lung cancer, colon cancer, and hepatocellular cancer, whereas few studies have focused on gastric cancer [18–20]. In this study, we suggest that Brd4 inhibitors can induce cell death in gastric cancer cells. In addition, aristoyagonine is effective on gastric cancer cells that are resistant to I-BET-762, which is under clinical trial.

2. Materials and methods

2.1. Molecular cloning & protein expression, and purification

Brd4 cDNA was provided by Dr. Stefan Knapp from the University of Oxford. N-terminal GST-tagged and C-terminal His-tagged BD1 (GST-BD1-His_6) was expressed in *E. coli* and purified. BD1 spans 47–170 amino acids. The pGEX 6P-1 vector was digested with EcoRI and XhoI restriction enzymes. BD1 PCR was performed with the BD1_Forward primer (5′- ATC TAG GAA TTC CCC CCA GAG ACC TCC AAC CC -3′) and BD1_Rev primer (5′-ATC TAG CTC GAG TTA GTG GTG GTG GTG GTG GTG TTC GAG TGC GGC CGC AAG CTC GGT TTC TTC TGT GGG TA-3′). BL21 Star (DE3) was transformed and induced by 0.1 mM IPTG overnight at 18 °C. The cells were lysed with lysozyme (1 mg/mL) and sonicated in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and adjusted pH to 8.0 by NaOH) and centrifuged at 8000 rpm for 30 min. The supernatant was incubated with Ni-NTA beads (Qiagen) for 2 h at 4 °C and proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and adjusted pH to 8.0 by NaOH). Purified His-tag proteins were further purified by size exclusion chromatography on a superdex 16/600 Hiload column (GE Healthcare) using buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl).

2.2. Alpha-screen enzyme assay

The alpha-screen assay was performed in accordance with the manufacturer's protocol (PerkinElmer, USA), by using a buffer (50 mM HEPES, 100 mM NaCl, 0.1% BSA, pH 7.4 supplemented with 0.05% CHAPS) and OptiPlate™−384 plate (PerkinElmer, USA). Briefly, 2.5 μL of compound solution and 5 μL of peptide solution [SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRK-biotin] were added to 5 μL of glutathione-S-transferase (GST) and His-tagged BD1 or BD2 in OptiPlate™−384 plate. Streptavidin-coated donor beads and anti-GST alpha-screen acceptor beads were added under low-light condition. Plate was incubated at 25 °C for 60 min using a Thermomixer C (Eppendorf, USA), and read using a Fusion-Alpha™ Multilabel Reader (PerkinElmer, USA). The alpha-screen results were confirmed by using alpha-screen TruHit kits (PerkinElmer, USA).

2.3. ELISA assay

Streptavidin coated 384-well plates (Thermo Fisher Scientific, USA) were rinsed by phosphate buffered saline/Tween-20 (PBST). Biotin-tagged tetra acetylated or non-acetylated peptides were added and incubated in a cold chamber overnight. The plate was washed by PBST and blocking was performed by using a 1% bovine serum albumin solution. 10 μL of compound solution, and 40 μL of glutathione-S-transferase (GST) and His tagged BD1 were added to each well. After 1 h, 25 μL of primary GST-antibody (0.01 ng/ml, Abcam, USA), and 25 μL of 2nd HRP conjugated antibody (Thermo Fisher Scientific, USA) were added. Between stages, the plate was washed in PBST. Finally, the absorbance of the wells was measured by using an EnVision Multilabel Plate Reader (PerkinElmer, USA).

2.4. Western blot

For immunoblotting, cells were washed in PBS, lysed in 1 × sample buffer (50 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 3% β-mercaptoethanol), and boiled for 10 min. Lysates were subjected to SDS-PAGE followed by blotting with the indicated antibodies and detection by Western blotting substrate ECL reagent (Thermo Scientific). Images were produced using a SensiQ-2000 and Image software. The following antibodies were obtained from Cell Signaling Technology: c-Myc (Catalog No. 5605). Tubulin antibody (Catalog No. T6199) was purchased from Sigma-Aldrich. HRP-conjugated anti-mouse (Catalog No. NCI1430KR), and HRP-conjugated anti-rabbit (Catalog No. NCI1460KR) antibodies were obtained from Thermo Scientific.

2.5. c-Myc knockdown

The pLKO.1 vector was digested with AgeI and EcoRI. pLKO.1 The primers for c-Myc shRNA 2 were: CCGG GAT GAG GAA GAA ATC GAT G CTCGAG C ATC GAT TTC TTC CTC ATC TTTTT and AATTAAAAA GAT GAG GAA GAA ATC GAT G CTCGAG C ATC GAT TTC TTC CTC ATC. The primers for pLKO.1 c-Myc shRNA 4 were: CCGG CCT GAG ACA GAT CAG CAA CAA CTCGAG TTG TTG CTG ATC TGT CTC AGG TTTTT and AATTAAAAA CCT GAG ACA GAT CAG CAA CAA CTCGAG TTG TTG CTG ATC TGT CTC AGG. pLKO.1 c-Myc shRNA plasmids were co-transfected with packaging plasmid and envelope plasmid into 293T cells to produce lentiviral particle.

2.6. Cell cytotoxic assay

For the viability experiments, cells were seeded in 96-well plates at 30% confluency and exposed to chemicals the next day. After 72 h, WST-1 reagent was added, and absorbance at 450 nm was measured by using a Spectramax spectrophotometer (Molecular Devices, US) in accordance with the manufacturer's instructions. The IC_{50} values were calculated by using GraphPad Prism version 5 for Windows. The curves were fitted using a nonlinear regression model with a log (inhibitor) versus response formula.

2.7. In vivo xenograft

Female athymic BALB/c (nu/nu) mice (6 weeks old) were obtained from Charles River of Japan. Animals were maintained under clean room conditions in sterile filter top cages and housed on high efficiency particulate air-filtered ventilated racks. Animals received sterile rodent chow and water ad libitum. All of the procedures were conducted in accordance with guidelines approved by the Laboratory Animal Care and Use Committee of Korea Research Institute of Chemical Technology. Ty82 cells (5×10^6 in 100 μL) were implanted subcutaneously (s.c.) into the right flank region of each mouse and allowed to grow to the designated size. Once tumors reached an average volume of 200 mm^3, mice were randomized and dosed via oral gavage daily with the indicated doses of compounds for 14 days. Mice were observed daily throughout the

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