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AZD5153, a novel BRD4 inhibitor, suppresses human thyroid carcinoma cell growth *in vitro* and *in vivo*

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ABSTRACT

The development of novel anti-papillary thyroid carcinoma agents is urgent. AZD5153 is a novel and specific Bromodomain-containing protein 4 (BRD4) inhibitor. Here, we show that AZD5153 dose-dependently inhibited survival, proliferation and cell cycle progression in TPC-1 cells and primary human thyroid carcinoma cells. Yet, it was non-cytotoxic to the primary thyroid epithelial cells. AZD5153 induced caspase-3/-9 and apoptosis activation in TPC-1 cells and primary cancer cells. Its cytotoxicity in TPC-1 cells was significantly attenuated with co-treatment of the caspase inhibitors. BRD4 expression was elevated in TPC-1 and primary human thyroid carcinoma cells, but was low in the thyroid epithelial cells. BRD4-regulated proteins, including c-Myc, Bcl-2 and cyclin D1, were significantly downregulated following AZD5153 treatment in TPC-1 and primary cancer cells. *In vivo*, oral administration of AZD5153 at well-tolerated doses significantly inhibited TPC-1 xenograft growth in severe combined immunodeficient (SCID) mice. BRD4-dependent proteins, Myc, Bcl-2 and cyclin D1, were also downregulated in AZD5153-treated tumor tissues. Collectively, the results suggest that targeting BRD4 by AZD5153 inhibits human thyroid carcinoma cell growth *in vitro* and *in vivo*.

1. Introduction

Thyroid cancer is one health threat globally [1,2]. Its incidence has been rising in the past decades [1,2]. The fast majority of thyroid cancer is papillary thyroid carcinoma [1,2]. Studies have implied that radiation and/or genetic susceptibility are important for the initiation and progression of human papillary thyroid carcinoma [1,2]. The prognosis for the advanced and/or metastatic thyroid carcinoma is poor, with the current treatment options extremely limited. Molecularly-targeted therapy is vital for the efficient treatment of papillary thyroid carcinoma [3,4].

Bromodomain and extraterminal (BET) family has at least four members, including Bromodomain-containing protein (BRD) 2, BRD3, BRD4 and the testis-specific isoform BRDT [5,6]. BRD4 is one abundant and major BET family protein [7–9]. BRD4 binds to acetylated-histones, which functions as a key epigenetic regulator [7–10]. BRD4 regulates chromatin structure in daughter cells [7–9]. BRD4 can also recruit P-TEFb (the positive transcription elongation factor b), and phosphorylating the RNA polymerase II, which are both required for transcription elongation [9]. Several BRD4-regulated/-dependent proteins have been indentified, including Bcl-2, Myc, cyclin D1 and several pro-cancerous genes [7–12]. BRD4 is upregulated in human cancers, which is associated with cancer progression [11,12].

Recent studies have characterized AZD5153 as a novel, potent and bivalent BRD4 inhibitor [13–15]. AZD5153's unique bivalent binding to BRD4 sets it apart from other well-known BRD4 inhibitors. Its activity against human papillary thyroid carcinoma cells is tested in this study.

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2. Materials and methods

2.1. Chemicals and reagents

AZD5153 was obtained from Sigma (Beijing, China). The other BRD4 inhibitors JQ1 and CPI203 were obtained from Selleck (Shanghai, China). The caspase inhibitors, Z-DEVD-fmk, Z-LEHD-fmk and Z-VAD-fmk, were purchased from Calbiochem (La Jolla, CA). The antibodies utilized in the current study were purchased from Cell Signaling Tech (Beverly, MA). All tissue culture plates were purchased from Corning Co. (Shanghai, China).

2.2. TPC-1 cell culture

The human thyroid cancer cell line TPC-1 was provide by the Cell Bank of Chinese Academy of Science (Shanghai, China). TPC-1 cells were culture in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) with necessary anti-biotic. The reagents for cell culture were provided by Gibco Co. (Suzhou, China).

2.3. Primary human cells

Two primary PTC human patients (56/49-year old, male), with written-informed consent, provided the tumor tissues at the time of thyroidectomy. Tumor tissues and surrounding normal thyroid epithelial tissues were separated carefully under surgical microscopes. The tissues were minced, followed by digestion with collagenase I, and were filtered by a 75-μm nylon cell strainer. The primary human cells were cultured in the complete DMEM/F12 medium for primary human cells (Biyuntian, Wuxi, China). The fibroblasts and blood vessels were removed carefully. In this study, two different primary PTC cells ("C1/C2") and two different thyroid follicular epithelial cells ("E1/E2") were established. Validation of human thyroid cancer and epithelial cell markers was described previously [16]. Primary cells at passage of 3–8 were utilized. The protocols were in accordance with the Declaration of Helsinki and the Institutional Ethics Review Board.

2.4. MTT assay

Cells were plated onto 96-well tissue-culture plates at 5000 cells per well. After the applied AZD5153 treatment, cell survival was tested by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT, Sigma) dye assay. MTT's absorbance was measured by a microplate reader (Bio-Rad, Basel, Switzerland) at 550 nm.

2.5. Clonogenicity assay

THP-1 cells with the indicated AZD5153 treatment were placed on 10-cm tissue-culture dishes at 20,000 cells/dish. AZD5153-containing medium was renewed every two days for five rounds (10 days). Afterwards, the number of remaining THP-1 colonies was counted manually.

2.6. Hoechst-33342 nuclei staining of apoptotic cells

Cells were plated onto 24-well tissue-culture plates at 20,000 cells per well. After the indicated AZD5153 treatment, cells were further stained with Hoechst-33342 (Sigma). Non-apoptotic nuclei displayed the faint delicate chromatin staining. But the nuclei showing intensified Hoechst-33342 condensation/brightness or fragmentation were labeled as apoptotic nuclei. For each treatment, 200 cells of 5 random views (1: 100) were included to calculate the apoptotic nuclei percentage.

2.7. BrdU ELISA assay

Cells were initially plated onto the 96-well tissue culture plates at 5000 cells per well. Cells were treated with AZD5153 in the presence of BrdU (10 μM). Afterwards, cellular BrdU incorporation was then determined via an ELISA kit (Cell Signaling Tech, Shanghai, China). BrdU ELISA optical density (OD) at 405 nm was recorded.

2.8. Cell cycle distribution analysis

Following the treatment, cells were fixed in ice-cold ethanol, which were then stained with 10 μg/mL propidium iodide (PI, Invitrogen) and 100 μg/mL RNase (Invitrogen). DNA content was analyzed with a flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cell cycle distribution (G0-G1, S and G2-M) percentages were recorded.

2.9. Caspase activity assay

After treatment, 30 μg of cytosolic protein extracts were mixed with the caspase assay buffer and the caspase (−3/-8/-9) substrate (10 μg/mL) with 7-amino-4-trifluoromethylcoumarin (AFC, Calbiochem, Darmstadt, Germany). The release of the fluorogenic AFC was measured using Infinite 200 PRO reader (Tecan Group Ltd., Männedorf, Switzerland) at 400 nm excitation and 505 nm emission.

2.10. Western blotting assay

The cultured cells or tissues were incubated with the tissue lysis buffer (Biyuntian, Wuxi, China). The quantified lysates (30 μg proteins per treatment of each lane) were separated by 10–12% SDS-PAGE gels, which were then transferred to the PVDF membrane (Biyuntian). After blocking, the applied primary antibody and the corresponding secondary antibody were added to the blot. ECL reagents were utilized to visualize the targeted protein band using X-Ray films. ImageJ software (NIH) was utilized to quantify the intensity of each band, and its value normalized to the corresponding loading control.

2.11. Xenograft assay

The female severe combined immunodeficient (SCID) mice at 6–7 weeks old and 17.5–18.2 g weight were obtained from the Experimental Animal Center of Soochow University (Suzhou, China). TPC-1 cells (6 million cells per mouse, in 200 μL, Matrigel/Serum free medium, 1:1 ratio) were subcutaneously (*s.c.*) injected to the right flanks. Within 16–18 days, the TPC-1 xenografts were established (the volume around 100 mm^3). The SICD mice were thereafter randomly assigned into three groups, with 10 mice of each group ($n = 10$). The tumor volumes and the mice body weights were recorded every five days for a total of 35 days (7 rounds). All studies were performed in accordance with the standards of ethical treatment approved by the Institutional Animal Care and Use Committee (IACUC) of all authors' institutions.

2.12. Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistics were analyzed by one-way ANOVA followed by the Scheffe' and Tukey Test using SPSS software (19.0, Chicago, CA). Significance was chosen as $\boldsymbol{P} < 0.05$. To determine significance between only 2

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