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I-7ab inhibited the growth of TNBC cells via targeting HDAC3 and promoting the acetylation of p53

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ABSTRACT

Triple negative breast cancer (TNBC) is a heterogenous disease with high aggressive and poor outcome. The lack of biomarkers and targeted therapies makes it a challenge for the treatment of TNBC. Histone deacetylase inhibitors (HDACis) are emerging as novel anti-tumor agents in many types of human cancers. In this study, we found that I-7ab, a novel HDACi, inhibited the cell viability of TNBC cells and induced the cell apoptosis. Mechanistically, I-7ab specifically decreased the expression of HDAC3 and promoted the acetylation of p53 at both Lys373 and Lys382 amino acids. The up-regulated acetylation of p53 promoted the transcriptional activity of p53 and induced the expression of p21, which consequently caused cell cycle arrest at G1 phase. Administration of I-7ab inhibited the colony formation of TNBC cells. Collectively, these results indicated I-7ab as a promising anti-cancer agent in the treatment of TNBC.

1. Introduction

The triple negative breast cancer (TNBC) accounts for about 10–15% of all breast cancers, which is characterized by lacking of the expression of estrogen receptor (ER), the progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Compared with other subtypes of breast cancer, TNBC is a heterogeneous disease presenting early relapse, aggressive behavior and poor prognosis [1–3]. Due to the lack of effective targets, standard chemotherapy is the mainly treatment protocol of TNBC patients. The pathological complete response rate of TNBC is less than 30% and most patients suffered drug-resistant metastatic disease [4]. Therefore, it is quite necessary to explore novel therapeutic targets and approaches to improve the outcome of TNBC patients.

The initiation and development of cancer is tightly associated with the aberrant gene expression. Epigenetic modification to chromatin is a critical regulatory mechanism of gene expression by regulating the chromatin structure and recruitment of transcription factors [5,6]. Dysfunction of epigenetic modification has been demonstrated to be involved in human diseases, especially cancers. Among all the different types of modifications, acetylation and deacetylation are critical epigenetic processes that control the gene expression [7–9]. It has been well documented that histone acetylation catalyzed by the histone acetyltransferases (HATs) triggers the gene transcription, and histone deacetylation mediated by the histone deacetylases (HDACs) results in transcriptional repression [10–12]. Four classes containing 18 HDACs have been identified until now according to their sequence homology to the yeast original enzymes [3]. Notably, compared with class II and III, class I HDACs, comprising HDAC 1–3 and HDAC8, play critical roles for the uncontrolled tumor cell proliferation [13]. Overexpression of class I HDACs have been observed in a various of human cancers [14]. And down-regulation of HDAC1 inhibited the cancer cell growth. As the close association between HDACs and cancer development, HDACs are emerging as promising targets for anti-cancer drugs.

A large number of HDAC inhibitors have been synthesized chemically or isolated from the natural materials, which display target specificity, pharmacokinetic characteristics and anti-cancer activity both *in vitro* and *in vivo* study [15–19]. Based on the clinical structure, HDACi are classified into five groups including hydroximates (vorinostat, givinostat, abexinostat, panobinostat, belinostat and trichostatin A), depsipeptide (romidespin), benzamides (entinostat), alphatic acids, and ketones [3,20]. Among these HDACis, vorinostat and belinostat has been approved by the U.S. Food and Drug Administration (FDA) for the clinical treatment of cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma, respectively [21–23]. Studies investigating the anti-cancer efficiency of other forms of HDACis are undergoing.

The N-hydroxyacrylamide derivative, (*N*-hydroxy-3-(2-phenyl-4-((2-(piperidin-1-yl)ethyl)amino)-2H-pyrazolo[3,4-c]quinolin-8-yl)

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acrylamide, also named I-7ab, was identified as a novel class I HDAC inhibitor [24]. I-7ab down-regulated the cyclin B1-CDK1 complex and arrested cell cycle progression in colorectal cancer cells [24]. Additionally, I-7ab was found to increased Bax/Bcl-2 ratio and suppressed the nuclear translocation of NF-κB, which finally induced cell apoptosis [24]. However, the anti-tumor effect of I-7ab in other types of cancers has not been illustrated. In this study, we explored the influence of I-7ab on the growth of TNBC cells and characterized the underlying molecular mechanisms of I-7ab in regulating the growth of TNBC cells.

2. Materials and methods

2.1. Regents

The I-7ab was generated with the protocol as previously described [24] and diluted with the dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to make a 100 mg/ml stock solution before diluted in a complete medium to the working concentration.

2.2. Cell culture

The TNBC cell lines MDA-MB-231 and BT-20 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell viability assay

TNBC cells were plated in the 96-well plate with the density of 1000 cells per well. I-7ab was added into the medium with the indicated concentration for 12, 24, 36,48, 60, 72, 84 and 96 h. The cytotoxic effect of I-7ab on TNBC cells was determined using the Cell Counting Kit-8 (CCK-8, CK054, Donjindo Laboratories, Japan) according to the manufacturer's instructions. Briefly, 10 μl of CCK-8 reagent was added into the cell culture medium and incubated at 37 °C for 2 h. After this, the absorbance of each well at 450 nm was detected with the microplate reader.

2.4. Cell apoptosis

Both MDA-MB-231 and BT-20 cells were treated with 100 μg/ml I-7ab for 48 h. Cells were harvested and the cell apoptosis rate was determined with the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, cells were resuspended with 500 μl of binding buffer and incubated with 2.5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) at room temperature for 15 min in the dark. The cell apoptosis rate was measured with the FC500 Flow Cytometer (Beckman Coulter, USA).

2.5. Activity of caspase-3

TNBC cells were treated with 100 μg/ml I-7ab for 48 h and then cells were harvested to detect the activity of caspase-3 with the Caspase 3 Assay Kit (Colorimetric, ab39401, Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, 3×10^6 cells were re-suspended with 50 μl of pre-cold cell lysis buffer and incubated on ice for 10 min. Cells were centrifuged at 100,000 *g* for 1 min at 4 °C and then the supernatant was collected. The protein concentration of each samples were measured and 50 μg protein was used for the following steps. 50 μl of 2 × reaction buffer and 5 μl of 4 mM DEVD-*p*-NA were added into the samples and incubated at 37 °C for 1 h. The absorbance at 405 nm was measured by the microplate reader.

2.6. Cell cycle analysis

TNBC cells were cultured in 6 cm dishes and incubated with the I-7ab with the indicated dose. When cells grown to approximately 75% confluent, cells were collected and fixed with 75% ethanol at 4 °C overnight. Cells were washed twice with pre-cold PBS and then stained with the propidium iodide (Sigma-Aldrich, Bornem, Belgium) in the darkness for 30 min at room temperature. The percentages of cells distributed in G0/G1, S, and G2/M phases were detected by the FACScan flow cytometer (BD Biosciences, Erembodegem, Belgium). The cell cycle profiles were analyzed with the ModFit LT version 3.1 (Verity Software House Inc.).

2.7. Western blot

Cells were incubated with the indicated concentration of I-7ab. Cells were collected and lysed with the NP-40 lysis buffer (Beyotime Biotechnology, Shanghai, China) on ice for 10 min. 30 μg of total cell lysates were loaded and separately by the 15% SDS-PAGE. The proteins were then transferred onto the nitrocellulose membrane (Millipore Billerica, MA, USA). The membrane was firstly blocked with 5% non-fat milk for 1 h at room temperature (RT) and then incubated with the indicated primary antibody for 2 h at RT. And then the membrane was incubated with HRP-labeled anti-mouse antibody (1:5000; Proteintech Group, Chicago, IL, USA) for 1 h at RT. The western blot bands were detected with the Pierce TM ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibodies against HDAC1 (1:2000; #34589S, Cell Signaling Technology, Danvers, MA, USA), HDAC2 (1:2000; #5113S, Cell Signaling Technology, Danvers, MA, USA), HDAC8 (1:2000; ab187139, Abcam, Cambridge, UK), HDAC3 (1:2000; #3949S, Cell Signaling Technology, Danvers, MA, USA), p53 (1:500, #9282, Cell Signaling Technology, Danvers, MA, USA), p21 (1:1000, #2947, Cell Signaling Technology, Danvers, MA, USA), Acetyl-p53 (Lys382)(1:3000; #2525S, Cell Signaling Technology, Danvers, MA, USA) and Acetyl-p53 (Lys373)(1:3000; orb311582, biorbit, Wuhan, Hubei, China) and anti-GAPDH mAb (1:5000, 3H12, MBL, Japan) were commercially purchased.

2.8. Quantitative real-time PCR

Total RNA was extracted with the TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RNA concentration was detected by the NanoDrop2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Reverse transcription was performed with the TranScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (ABSCI, AB452, USA). Real-time quantitative PCR reaction was performed with the TaqMan Multiplex Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the Roche LightCycler® 96 (LC96) real-time PCR system. The p21 primers were: 5′-TGTCCGTCAGAACCCATGC (forward) and 5′-AAAGTCGAAGTTCCATCGCTC-3′ (Reverse); The PUMA primers were: 5′-ATGGCGGACGACCTCAAC (forward) and 5′-AGTCCCATGAAGAGATTGTACATGAC (Reverse) ; The Bax primers were 5′-ATGTTTTCTGACGGCAACTTC (forward) and 5′-AGTCCAATGTCCAGCCCAT (Reverse); the GAPDH primers were 5′-TGACAACTTTGGTATCGTGGAAGG-3′ (forward) and 5′-GCAGGGATGATGTTCTGGAGAG-3′ (Reverse). Relative expression of p21 was normalized to that of GAPDH and the fold change was calculated with the $2-^{\Delta\Delta Ct}$ method.

2.9. Statistical analysis

All the data were presented as mean ± standard deviation (SD) and analyzed by the Student's *t*-test or One-way ANOVA with the SPSS 19.0 (IBM SPSS Inc., NY, USA). $P < 0.05$ was considered as statistical significance.