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Research Paper

Inhibition of HDACs-EphA2 Signaling Axis with WW437 Demonstrates Promising Preclinical Antitumor Activity in Breast Cancer

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ABSTRACT

Histone deacetylase inhibitors (HDACi) are small molecules targeting epigenetic enzymes approved for hematologic neoplasms, which have also demonstrated clinical activities in solid tumors. In our present study, we screened our internal compound library and discovered a novel HDACi, WW437, with potent anti-breast cancer ability *in vitro* and *in vivo*. WW437 significantly inhibited phosphorylated EphA2 and EphA2 expression. Further study demonstrated WW437 blocked HDACs-EphA2 signaling axis in breast cancer. In parallel, we found that EphA2 expression positively correlates with breast cancer progression; and combined use of WW437 and an EphA2 inhibitor (ALW-II-41-27) exerted more remarkable effect on breast cancer growth than either drug alone. Our findings suggested inhibition of HDACs-EphA2 signaling axis with WW437 alone or in combination with other agents may be a promising therapeutic strategy for advanced breast cancer.

1. Introduction

Histone deacetylase inhibitors (HDACis) are a series of compounds capable of inducing epigenetic changes *via* targeting epigenetic enzymes [1]. To date, four classes of HDACi have been discovered or developed, including short-chain fatty acids (butyrate, valproic acid), hydroxamic acids (trichostatin A, vorinostat, and pracinostat), tetrapeptides (romidepsin) and benzamidines. Among them, vorinostat and romidepsin are approved for the treatment of cutaneous T-cell lymphoma (CTCL), and belinostat has been approved for the treatment of peripheral T-cell lymphoma (PTCL) [2–4]. Histone deacetylase inhibitors have also demonstrated clinical activities either alone or in combination with other agents in solid tumors [5].

Eph receptor tyrosine kinase signaling regulates cancer initiation and metastatic progression through multiple mechanisms [6]. Among the distinct EphA receptors, EphA2 is an important modulator of tumor growth, angiogenesis, and metastasis [7,8]. The role of EphA2 differs in distinct tumor types. Several researches suggest EphA2 plays an oncogenic-suppressive role in cancer and deletion of EphA2 receptor tyrosine kinase leads to increased susceptibility to carcinogenesis in mouse skin [9]. However, in lung cancer, genetic and pharmacologic inhibition of EphA2 results in increased tumor cell death *in vitro* and decreased tumor burden *in vivo* [10]. EphA2 is proved to promote tumor cell migration/invasion and can be considered as a poor prognostic marker in colorectal cancer [11]. In parallel, EphA2 amplification has been found in >80% of breast cancer clinical samples [12,13]. Previous studies reported that targeting EphA2 in ERBB2-driven murine mammary tumor models resulted in inhibited tumor formation and metastatic progression [12]. Targeting EphA2 using shRNA or inhibitor intervention impairs cell cycle progression and growth in basal-like/triple-negative breast cancer [8].

Breast cancer is a serious health problem and the second leading cause of cancer-related death among women. Epigenetic changes in cancer are common and have been involved in breast cancer occurrence and development [14,15]. Several HDACis are being determined as single agents or combined with conventional therapies in clinical trials of metastatic breast cancer [16,17]. In these preclinical and clinical settings, it is necessary to develop novel HDAC inhibitors as well as investigate their exact mechanisms.

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Here, we identified a novel HDACi, WW437, which demonstrates potent anti-breast tumor activity *in vitro* and in preclinical animal model. Mechanistically, we found WW437 significantly inhibits HDACs-EphA2 signal axis. Our results suggest that HDACs-EphA2 signaling axis may represent a novel target in breast cancer.

2. Materials and Methods

2.1. Cell Lines, Cell Culture, and Reagents

The breast cancer cell line MDA-MB-231 (MDA231), BT549 and 4 T1 were purchased from ATCC (Manassas, VA, USA). MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. BT549 and 4 T1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All breast cancer cells were maintained at 37 °C under a humidified 5% CO_2 incubator. Mycoplasma contamination was monitored periodically.

Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Matrigel was purchased from BD Bioscience (Pasadena, CA, USA). Antibodies against acetyl-histone H3, acetyl-histone H4, HDAC1, HDAC2, HDAC3, HDAC5, HDAC6, E-cadherin, Zeb1, Vimentin, c-Myc, p21, cleaved PARP, Sp1, EphA2, Phospho-Tyrosine (p-Tyr-1000), Acetylated-Lysine and Flag were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibody against HDAC4 was purchased from Abcam (Hong Kong, China). Antibody against actin and dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Sigma-Aldrich, Inc., Shanghai, China). The detailed information of the antibodies we used in our study was shown in supplementary 1. WW437 were synthesized as described in the Supplementary Information (Supplementary Fig. 1). The synthetic route of SAHA was described previously [18]. The stock solutions of compound were prepared in dimethyl sulfoxide (DMSO) at a concentration of 50 mM and stored at −80 °C. Breast cancer tissue array were obtained from Alenabio (Alenabio, Xian, Shanxi, China).

2.2. HDAC Inhibitor Activity Assay

HDAC inhibitor activity assay was executed using the HDAC inhibitor drug screening kit (BioVision, Inc.) as described previously [19]. Briefly, HDACi candidates were incubated with HDAC enzymes (HeLa nuclear extract or MDA-MB-231 cell lysates) and HDAC fluorometric substrates at 37 °C for 1 h. The lysine developer was used to stop the reaction and the fluorescence units were obtained at Ex/Em 355/460 nm.

2.3. Cell Viability Assay

Breast cancer cells were seeded in 96-well plates. After 24 h, the cells were treated with different concentrations of WW437, and the cell viability was measured by MTS assay as described previously [20].

2.4. Western Blotting

Western blot analysis was performed as previously described [21]. Cell lysates were prepared in RIPA lysis buffer containing protease and phosphatase inhibitors.

2.5. Immunofluorescent Staining

Immunofluorescent staining was conducted as previously described [20].

2.6. Colony Formation Assay

Colony formation assay was conducted as previously reported [19]. Breast cancer cells were seeded in a 6-well plate and treated with or without WW437. Culture medium was refreshed every other day. All the cells were cultured for 10 days. Then the clones were stained with 0.1% crystal violet and counted manually.

2.7. Assessment of Apoptosis

Apoptosis was assessed using the Apoptosis Detection Kit (BD Biosciences) according to the manufacture's guidelines.

2.8. Wound Healing Assay

Wound-healing migration assay was performed as described previously [22]. When tumor cells grew to full confluence, the “wounds” were created by a sterile 100 μL pipette tip. Next, fresh medium was added containing different concentrations of WW437. After 12 h incubation, cells were fixed and photographed. The migrated cells were manually quantified.

2.9. Invasion Assay

Invasion assay was performed as described previously [19]. In brief, a total of 5×10^4 cells (for MDA-MB-231 cells) or 1×10^5 cell (for 4T1 cells) in 100 μL of FBS-free medium were added in the upper chamber, and 500 μL of complete medium was added at the bottom. Indicated concentrations of WW437 were added to both chambers. After 12 h, invaded cells were stained with 0.1% crystal violet and counted manually.

2.10. Fluorescent-Gelatin Degradation Assay

This assay was performed as reported previously [23]. Briefly, Cells were seeded on coverslips (precoated with FITC-gelatin) with different concentrations of WW437, incubated for 12 h, and followed by immunofluorescence. Gelatin degradation was quantified using Image-Pro Plus 6.0 software.

2.11. Three-Dimensional on-Top Assay

Three-dimensional on-top assay was performed as previously described [21].

2.12. Animal Model

All animal care and experimental studies were performed according to the guidelines and approval of the Animal Investigation Committee of the Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine. Female BALB/c mice were bred and maintained at the animal center in Shanghai General Hospital (21 °C, 55% humidity, on a 12-h light–dark cycle).

4 T1 cells (1×10^5) resuspended in 0.1 mL PBS were injected subcutaneously into the 4th abdominal mammary fat pad of BALB/c mice. On day 7, the mice were randomly divided into four groups ($n = 7$ per group) and received i.p. injection of WW437 (10 mg/kg/day and 30 mg/kg/day) and SAHA (30 mg/kg/day) as compared with mice injected with DMSO (control group). Tumor size was measured every one week, and tumor volume was calculated according to the formula: $V = L \times W^2 \times 0.52$, where L and W refers to length and width, respectively. After 35 day treatment, all the mice were killed. Lung metastases were manually counted using a dissecting microscope by three individuals who do not have personal biases with the experiment. The lungs were fixed and prepared for H&E staining. The primary tumors were weight and then prepared for western blotting assay.

Another independent animal experiment ($n = 5$ per group) was performed to determine the potential toxicity of WW437 on mice.