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RETRACTED ARTICLE: Belinostat suppresses cell proliferation by inactivating Wnt/β-catenin pathway and promotes apoptosis through regulating PKC pathway in breast cancer

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ABSTRACT

Belinostat is a histone deacetylase inhibitor drug capable of regulating cell growth in diverse cancers. Nonetheless, little information clarified the role of Belinostat in breast cancer. Hence, the functions of Belinostat in breast cancer cells survival was disclosed in this study. Belinostat at 50 and 100 μM were applied to manage MCF-7 cells, cell viability, Ki67 positive cells, cell cycle and apoptosis were monitored via MTT, immunohistochemistry and flow cytometry. Furthermore, the apoptosis-related factors, Wnt/β-catenin pathway and PKC pathway were tested through western blot and qRT-PCR. Lastly, in vivo effect of Belinostat was determined by a murine model. The results showed that Belinostat dampened cell viability, decreased the proportion of Ki67 positive cells and arrested cells at G0/G1 phase. The decreases of Wnt/β-catenin, CCND2 and Myc were observed in MCF-7 cells after Belinostat stimulation. Additionally, Belinostat induced cell apoptosis, meanwhile dampened Bcl-2 and raised Bax and Cleaved caspase 3 in a dose and time-dependent manner. Additionally, Belinostat activated PKC pathway by upgrading PKCα and PS3 expressions. Furthermore, Belinostat restrained tumour weight and volume in vivo. In summary, this study depicted that Belinostat prohibited proliferation and evoked apoptosis via mediating Wnt/β-catenin and PKC pathways in MCF-7 cells.

Introduction

Breast cancer is the prevalent malignant tumour that develops from breast epithelial tissues [1]. Approximately 99% cases of breast cancer occur in women, and the morbidity rate is second only to uterine cancer [2]. It has become a predominant problem that threatens women’s physical and mental health in the current society [3]. The cause of breast cancer is complicated that is often linked to inheritance and also affected by various environmental factors [4]. Recently, surgery along with chemotherapy or radiation therapy is often applied for the therapy of breast cancer [5], however, some adverse effects remain inevitable. Therefore, to look for an effective therapy for remedying breast cancer is urgently needed. Recent studies have corroborated that several chemotherapy agents have been applied to reduce mortality of breast cancer [6–8]. These medications worked by destroying cancer cells fast grow or replicates, and also by causing DNA damage upon replication [9]. Belinostat is a neoteric histone deacetylase (HDAC) inhibitor that has been broadly exploited to treat peripheral T-cell lymphoma (PTCL) [10]. As a new anti-tumour drug, Belinostat was also evaluated for the treatment of miscellaneous solid cancers, such as hepatocellular carcinoma [11], pancreatic cancer [12], and renal cancer [13]. Emerging evidence confirmed that Belinostat showed significant regulatory impacts on cell survival and metastasis [14]. As Kong et al. found that Belinostat emerged the anti-tumour cytotoxicity induced cells apoptosis by repression of MAPK pathway in lung squamous cell carcinoma [15]. In addition, Wang et al. testified that Belinostat could induce pancreatic cancer cells apoptosis and prohibit growth by activation of TAK1-AMPK signal pathway [16]. Whereas, the roles of Belinostat in breast cancer remains unclear.

Herein, we attempted to unveil the influence of Belinostat in cell growth of breast cancer via analyzing proliferation, apoptosis and corresponding signal pathways. Our research found that Belinostat dampened cell proliferation via down-regulation of Wnt/β-catenin pathway and expedited apoptosis through up-regulation of the PKC pathway in breast cancer. In vivo experiment further revealed the inhibition effect of Belinostat on tumour growth of breast cancer. All these data may indicate that Belinostat exhibits the anti-tumour effect and contributes to breast cancer remedy.

Materials and methods

Murine studies

The male BALB/c nude mice (6-week-old) were achieved from the HFK Biosciences (Beijing, China) maintained under specific pathogen-free conditions. For tumour propagation,......
1 x 10^5 indicated tumour cells were subcutaneously injected with 50 μM of Belinostat into BALB/c nude mice on the back, and then the weight and volume of breast cancer tumour were measured 5 weeks post-injection. Animal health and management were conformed to the guidance of the Institutional Animal Care and Use Committee (IACUC) of Binzhou Medical University Hospital. The studies involving animals were endorsed by the Ethics Committee of Binzhou Medical University Hospital.

**Cell culture**

MCF-7 cells were procured from the American Type Culture Collection (ATCC, Manassas, VA), as well as cultivated in DMEM (Life Technologies, Carlsbad, CA) with 10% foetal bovine serum (FBS, Life Technologies) at 37°C placing in a wettish incubator with 5% CO2. Different concentrations of Belinostat (0, 50 and 100 μM, Topotarget, København, Denmark) were utilized for the management of MCF-7 cells and 50 μM of Belinostat was applied to treat MCF-7 for different times (0, 24 and 48 h) in this study.

**Cell viability**

The viability of MCF-7 cells was measured via conducting MTT colorimetric assay referring to the foregone description [17]. After management with different concentrations of Belinostat, 5 x 10^3 MCF-7 cells were cultivated in 96-well plate, in the meantime fostered for 1–4 d at 37°C. Afterward, 20 μl of MTT (Sigma-Aldrich, St. Louis, MO) was replenished in cell culture and further cultivated for another 4 h at 37°C and 5% CO2. The 150 μl DMSO (Sigma-Aldrich, St. Louis, MO) was subsequently supplemented to each culture well and oscillated for 10 min. After dissolving the formazan crystals, the absorbance was monitored at 490 nm via conducting a Microplate Reader (Bio-Rad, Hercules, CA).

**Immunohistochemical analysis of Ki67 positive cells**

After administration with Belinostat (0, 50 and 100 μM) for 24h, MCF-7 cells were laundered with PBS and immobilized in frappe methanol-acetone (1:1) for 10 min. Subsequently, the above-mentioned samples were laundered with PBS again and were sealed with 3% BSA for 1 h in a humidified CO2. After this, the cells were co-trained with anti-Ki67 antibody (ab16667, Abcam, Cambridge, UK) for 20 h at 4°C. Next, these cells were laundered with PBS, meanwhile cultivated with an anti-rabbit antibody (ab205718, Abcam, Cambridge, UK) for extra 4 h at 37°C. The 3,3’-diaminobenzidine tetrahydrochloride (DAP) was then utilized for dying above cells for 10 min. Finally, the percentage of the positively stained cells was calculated using a microscope (Nikon, Tokyo, Japan).

**Cell cycle assay**

Cell cycle was determined through conducting flow cytometry trial as the previous study described [18]. After management with 100 μM of Belinostat, these above-treated cells were collected and laundered two times with pre-cooling PBS, as well as immobilized with 70% ethanol at 4°C for all night. Then, 100 μg/ml of RNase A was supplemented and dipped for 30 min in a water bath at 37°C. Next, 400 μl propidium iodide (PI, Thermo Scientific, Waltham, MA) was added and incubated with cells for 30 min under dodging light circumstance. The ratios of above cells at the disparate cell cycle phases were counted and analyzed via a FACSscan flow cytometer (Becton Dickinson, San Jose, CA).

**Cell apoptosis assay**

In brief, 1 x 10^5 cells were fostered in 6 well-plates, and then laundered twice with PBS. Afterward, these cells were re-suspended in 100 μl of binding buffer. Subsequently, above-mentioned cells were double-dyed with 10 μl FITC-Annexin V (20 μg/ml) and 5 μl PI (50 μg/ml, all from Biosea, Beijing, China) under dodging light circumstance for 30 min. The early or late apoptotic cells were quantified through conducting flow cytometer (Beckman Dickinson, San Jose, CA).

**Quantitative real time RT-PCR (qRT-PCR)**

For abstracting total RNA from MCF-7 cells managed by Belinostat, TRIZol reagent (Invitrogen) was utilized in this process. The PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) was applied for compounding the cDNAs. For mRNA detection, qRT-PCR was conducted by employing SYBR Premix Ex Taq II (Takara, Tokyo, Japan). GAPDH was exploited for normalization of the correlative mRNA expression, and data from this experiment were computed via employing the 2^-ΔΔCT method [19].

**Western blot assay**

After treatment with diverse concentrations of Belinostat, these correlative cells were laundered twice with pre-cooling PBS, in the meantime lysed in RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors (Roche Diagnostics, Basel, Switzerland). Employing the BCA™ Protein Assay Kit (Pierce, Appleton, WI), the protein concentrations were evaluated. The above samples were resolved via conducting SDS-PAGE, as well as shifted to nitrocellulose membranes (Whatman, Dassel, Germany). Primary antibodies, including β-catenin (ab16051), Cyclin D2 (CCND2, ab79482), Myc (ab32072), Bcl-2 (ab47489), Bax (ab81083), Caspase 3 (ab32150), Cleaved caspase 3 (ab32042), Phosphorylated-Protein kinase C delta (P)-PKCδ (ab76181), Protein kinase C delta (PKCδ) (ab182126), P53 (ab32389), and GAPDH (ab181602, Abcam, Cambridge, UK) (the dilution all 1:1000) were prepared, and were co-cultivated with the membrane after 5% BSA sealing at 4°C for all night long. Subsequently, the membrane was laundered with PBS and fostered with the corresponding secondary antibody (ab97051, the dilution all 1:2000, Abcam, Cambridge, UK) for 1 h at room temperature. The protein bands were imaged utilizing the WEST-ZOL plus Western Blot Detection System (iNtRON Biotechnology, Seoul, Korea).
**Statistical analysis**

The results in this study are presented as the mean ± SD. SPSS 19.0 statistical software (SPSS Inc., Chicago, IL) was conducted for computing statistical consequences. The p values were ciphered out via conducting ANOVA or Student's t-test. p-value < .05 was ascertained to be a statistically significant consequence.

**Results**

**Belinostat prohibited breast cancer cell proliferation**

Foregone research has testified that Belinostat exerts the anti-tumour effects on miscellaneous cancers [20], nonetheless, functions of Belinostat in breast cancer cells have not been understood. To clarify the anti-proliferative activity of Belinostat in breast cancer cells, the different doses of Belinostat (0, 50 and 100 μM) were utilized to treat MCF-7 cells and cell viability, cell proliferation marker Ki67 and cell cycle were evaluated through MTT, immunolocalization and flow cytometry. As results are shown in Figure 1(A), Belinostat significantly reduced cell viability at 50 and 100 μM on cultured 3 d (p < .05) and 4 d (p < .01). The percentage of Ki67 was also decreased by Belinostat at 50 μM (p < .05) and 100 μM (p < .01, Figure 1(B)). Furthermore, after management with 100 μM of Belinostat for 24 h, the proportion of MCF-7 cells was increased in the G0/G1 phase (Figure 1(C)). These above-involved data displayed the anti-proliferative effect of Belinostat on breast cancer cells.

**Belinostat impeded Wnt/β-catenin signal pathway**

It is well known that Wnt/β-catenin pathway emerges a vital role in adjusting cell proliferation [21]. Therefore, we supposed whether Belinostat affects cell proliferation by regulation of Wnt/β-catenin signal pathway. The protein levels of nuclear β-catenin and β-catenin were analyzed at diverse doses of Belinostat (0, 50 and 100 μM) and disparate treatment times (0, 24 and 48 h). As displayed in Figure 2(A,B), the levels of nuclear β-catenin and β-catenin were both remarkably down-regulated by Belinostat in a dose-dependent manner. Moreover, after treatment with 50 μM Belinostat, the levels of nuclear β-catenin and β-catenin were also decreased by Belinostat in a time-dependent manner. Next, we tested the target genes of β-catenin mRNA and protein levels of CCND2 and Myc through conducting qRT-PCR and western blot. We discovered that Belinostat restrained mRNA and protein levels of CCND2 and Myc at 50 μM (p < .05 or p < .01) and 100 μM (p < .001, Figure 2(C,D)). These data confirmed our conjectures that Belinostat could prohibit cell proliferation possibility through blocking Wnt/β-catenin signal pathway.

**Belinostat accelerated breast cancer cell apoptosis**

We next explored the functions of Belinostat in cell apoptosis of MCF-7 cells. After management with different doses of Belinostat for 24 h, flow cytometry assay was conducted to calculate the quality of early and late apoptotic cells. Results showed that Belinostat significantly increased cell apoptosis in a dose-dependent manner (p < .05). Further, dose-response and time-response studies of apoptosis-related factors were examined by western blot. As displayed in Figure 3(B,C), the expression levels of Bcl-2 and Caspase 3 were remarkably decreased however Bax and Cleaved caspase expressions were increased by Belinostat at a dose-dependent and time-dependent manner. The consequences uncovered that Belinostat expedited cell apoptosis in MCF-7 cells.

**Belinostat activated PKC signal pathway**

A recent study has reported that PKC signal pathway is linked to cell apoptosis process [22], however, whether Belinostat promotes cell apoptosis via regulation of PKC signal pathway remain unclear. qRT-PCR was conducted for the evaluation of the mRNA expressions of PKCδ and P53 in Belinostat treated cells with 50 and 100 μM. PKCδ and P53 expression levels were both enhanced by Belinostat at dose-dependent manner (p < .05, Figure 4(A)). Western blot experiment disclosed that Belinostat obviously promoted P-PKCδ, PKC and P53 expressions in a dose-dependent and time-dependent manner (Figure 4(B,C)). These data indicated that Belinostat promoted cell apoptosis may via regulation of PKC signal pathway in MCF-7 cells.
Belinostat repressed tumour formation in vivo

To further explore the anti-tumour activity of Belinostat in breast cancer, in vivo experiment was utilized to measure tumour formation. After treatment with 50 µM of Belinostat, tumour weight and volume were detected. As results showed in Figure 5(A,B), Belinostat treatment significantly inhibited tumour weight and decreased tumour volume ($p < .05$). These observations demonstrated that Belinostat could inhibit tumour formation in vivo, further indicating the anti-tumour effect of Belinostat on breast cancer.
Discussion

Herein, we disclosed that Belinostat prohibited cell proliferation and induced apoptosis through the regulation of Wnt/β-catenin and PKC signal pathways in dose-dependent and time-dependent manners. Furthermore, in vivo experiment further verified the anti-tumour activity of Belinostat by suppressing tumour weight and volume.

Recent studies have uncovered that Belinostat exerted the anti-proliferation function in a kind of cancers. One study found that Belinostat potently prohibited the growth of prostate cancer cell lines and induced cells at G2/M phase [23]. Buckley et al. imparted in bladder cancer that Belinostat inhibited cell growth and increased cells in the G0/G1 phase [24]. Furthermore, abundant studies clarified that Ki67 was a good marker for evaluation of cell proliferation that was related to the occurrence and development of breast cancer [25,26]. Therefore, we analyzed cell viability, Ki67 positive cells and cell cycle to probe the anti-proliferation impacts of Belinostat on breast cancer. Our study indicated that Belinostat exerted the anti-proliferation effect on breast cancer through inhibiting cell viability, decreasing Ki67 cells and arresting cells at the G0/G1 phase.

Wnt/β-catenin pathway has been corroborated to exhibit a vital role in cell proliferation of different cancers [27,28]. A recent study revealed that the inhibition of Wnt/β-catenin pathway and its downstream targets are closely linked to the development of tumours [29]. In breast cancer, Shao et al. found that triptolide restrained breast cancer cells proliferation via hindering Wnt/β-catenin signalling pathway. Moreover, several studies reported that downstream components of c-Myc and Cyclin D1 were activated in breast tumours [30,31]. Accordingly, to make clear the importance of Wnt/β-catenin pathway in Belinostat-suppressed cell proliferation, relative expression of β-catenin, CCND2 and Myc were tested. The findings in our study were partially similar to these previous studies that Belinostat blocked Wnt/β-catenin pathway and also inhibited CCND2 and Myc expression, indicating that Belinostat suppressed cell proliferation might be by repression of Wnt/β-catenin pathway in breast cancer.

Belinostat has been widely confirmed to have an effective anti-tumour ability in the treatment of different cancers by regulation of cell apoptosis [16,17]. For example, Wang et al. found the anti-tumour effect of Belinostat through inducing apoptosis and inhibition of cancer growth on pancreatic cancer [16]. Makoto et al. reported that Belinostat and ritonavir synergistically induced apoptosis, meanwhile restrained renal cancer cells survival [32]. Based on these studies, we supposed that Belinostat could suppress breast cancer cell growth via regulation of cell apoptosis. Just as we thought, Belinostat dramatically induced cell apoptosis in MCF-7 cells at dose-dependent and time-dependent manner.

It is proverbially reported that PKC is a serine/threonine kinase, which emerges a momentous role in cell proliferation, differentiation and apoptosis [33]. Activation of PKC is also a key link in the cell signal transduction pathway, which regulates cellular signals, participates in genes expressions, and affects cell cycles [34]. All these functions of anti-cell information maybe provide a broaden prospect for anti-tumour therapy. As Deka et al. displayed that Alkyl Cinnamates promoted breast cancer cells apoptosis through induction of PKC translocation [35]. Similar with the study, we found that Belinostat obviously activated PKC and P53 expression that might be indicated that Belinostat promoted apoptosis through activation of the PKC pathway in breast cancer.

Several pre-clinical tumour models have been established to further investigated the anti-tumour effect of Belinostat in various cancers [36]. As Jensen et al. demonstrated in ovary cancer xenrafts in mice, Belinostat decreased ovary tumour volumes [37]. Dmitriy et al. study showed inhibition of tumour growth by Belinostat in pancreatic cancer in vivo [12]. Nevertheless, the role of Belinostat in breast cancer has not been evaluated. Herein, we found that Belinostat inhibited tumour formation in vivo, further indicated the anti-cancer effect of Belinostat on breast cancer.

Taken together, our findings revealed that Belinostat suppressed cell proliferation via hindering Wnt/β-catenin pathway and promoted apoptosis through up-regulation of the PKC pathway in breast cancer. It is indicated that Belinostat may be a novel anti-cancer medicine for the treatment of breast cancer. Further study is needed to explore the underlying mechanism.

Disclosure statement

Authors declare that there is no conflict of interests.
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