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Valproic acid, a histone deacetylase inhibitor, induces apoptosis in breast cancer stem cells

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ABSTRACT

Cancer stem–like cells (CSCs) are a cell subpopulation that can reinitiate tumors, resist chemotherapy, give rise to metastases and lead to disease relapse because of an acquired resistance to apoptosis. Especially, epigenetic alterations play a crucial role in the regulation of stemness and also have been implicated in the development of drug resistance. Hence, in the present study, we examined the cytotoxic and apoptotic activity of valproic acid (VPA) as an inhibitor of histone deacetylases (HDACs) against breast CSCs (BCSCs). Increased expression of stemness markers were determined by western blotting in mammospheres (MCF-7s, a cancer stem cell-enriched population) propagated from parental MCF-7 cells. Anti-growth activity of VPA was determined via ATP viability assay. The sphere formation assay (SFA) was performed to assess the inhibitory effect of VPA on the self-renewal capacity of MCF-7s cells. Acetylation of histon H3 was detected with ELISA assay. Cell death mode was performed by Hoechst dye 33342 and propidium iodide-based flouresent stainings (for pyknosis and membrane integrity), by M30 and M65 ELISA assays (for apoptosis and primary or secondary necrosis) as well as cytofluorimetric analysis (caspase 3/7 activity and annexin-V-FITC staining for early and late stage apoptosis). VPA exhibited anti-growth effect against both MCF-7 and MCF-7s cells in a dose (0.6–20 mM) and time (24, 48, 72 h) dependent manner. As expected, MCF-7s cells were found more resistant to VPA than MCF-7 cells. It was observed that VPA prevented mammosphere formation at relatively lower doses (2.5 and 5 mM) while the acetylation of histon H3 was increased. At the same doses, VPA increased the M30 levels, annexin-V-FITC positivity and caspase 3/7 activation, implying the induction of apoptosis. The secondary necrosis (late stage of apoptosis) was also evidenced by nuclear pyknosis with propidium iodide staining positivity. Taken together, inhibition of HDACs is cytotoxic to BCSCs by apoptosis. Our results suggested that targeting the epigenetic regulation of histones may be a novel approach and hold significant promise for successful treatment of breast cancer.

1. Introduction

Breast cancer is the most commonly diagnosed malignancy and a major cause of cancer-related mortality among women worldwide [1]. Advances in local treatment (surgery, radiotherapy) and adjuvant therapy or combined treatment strategies have increased the survival rate in early breast cancer, however almost a fifth of patients will develop local or distant recurrence within 5 years of diagnosis [2]. Cancer recurrence and subsequent death from metastasis may occur because of a subpopulation of cancer cells known as cancer stem cells (CSCs), also known as cancer initiating cells or cancer stem-like cells.

CSCs are able to self-renew and replicate into the heterogeneous population in a manner similar to normal tissue stem cells which are also critical for tumor initiation and growth [3]. Furthermore, the expression of tissue-specific cell surface markers, ability of anchorage-independent growth, activation of stemness-related pathways in addition to anti-apoptotic pathways are other hallmarks defined for CSCs [4,5]. Owing to their relative resistance to radiotherapy and chemotherapy, they are believed to be responsible for metastasis, relapse of the disease and represent an important therapeutic target [6].

Epigenetic reprogramming plays a crucial role in the regulation of stemness and tumorigenicity that especially occurs through the DNA methylation and/or histone modifications, as well-defined mechanisms [7]. It is known that alterations in post-translational histone

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modifications and the loss of specific histone acetylation/methylation markers are related with the breast cancer [8]. Modulation of the histone acetylation program is mediated by a dynamic/reversible equilibrium between histone acetyltransferase (HAT) and histonedeacetylase (HDAC) enzyme families that interferes with the important cellular events [9]. Several studies reported the elevated expression of HDACs and global loss of histone acetylation in many cancer types by correlation with poor prognosis [10–14]. Therefore, HDAC enzymes have been the target of potential drugs to avoid epigenetic repression and trigger the transcriptional changes such as the re-activation of tumor suppressor genes [15]. During the last decade, a range of HDAC inhibitors have been demonstrated in the induction of growth arrest, differentiation, and/or apoptosis *in vitro* as well as the inhibition of tumor growth and metastasis *in vivo* [16–19]. Furthermore, it has been shown that they are also able to target CSCs derived from different tumors [20–22]. Several HDAC inhibitors, either alone or in combination with chemotherapeutic drugs, are currently in different stages of clinical trials for both hematological and solid tumors [23].

Valproic acid (VPA, 2-propylpentanoic acid), a short-chain fatty acid, has been widely used in the treatment of epilepsy and other neuropsychiatric diseases since the last two decades and, more recently, defined as a potent HDAC inhibitor with a strong anti-tumor activity [24]. It has been reported in preclinical studies that VPA modulates the biology of various cancer types through the induction of differentiation, cell cycle arrest and apoptosis as well as the inhibition of metastasis and angiogenesis [24–26]. On the other hand, it seems that therapeutic efficacy of VPA varies in BCSCs depending on the dose and cell type used [21,27]. However, it suggests a more effective approach as chemosentisizer when combined with standard therapy and is currently being investigated in breast cancer clinical trials [28,29]. By taking into consideration the differential effect of VPA on BCSCs, we observed in the present study that VPA prevents mammosphere formation and triggers caspase-dependent apoptosis in accordance with the increased histone H3 acetylation. This is the first report regarding the effect of VPA on BCSCs cell death and warranting further *in vivo* studies.

2. Materials and methods

2.1. Chemicals

VPA, a clinically available HDAC inhibitor, was purchased from Sigma (Catalog no: P4543, St. Louis, MO, USA) and dissolved in deionized water at a concentration of 200 mM as a stock solution. The stock solution of VPA was frozen in aliquots at −20 °C and further dilutions were made in culture medium. Hoechst dye 33342 and Propidium Iodide (PI) were obtained commercially (Sigma Aldrich, USA). The pan-caspase inhibitor Z-Val-Ala-dl-Asp(OMe)-fluoromethylketone (zVAD-fmk) was from Enzo Life Sciences (USA) and Necrostatin-1 (Nec-1) was obtained from Santa Cruz Biotechnology (USA).

2.2. Culture of MCF-7 cell line

The MCF-7 breast cancer cells were maintained as monolayer culture in Roswell Park Memorial Institute 1640 (Lonza, Verviers, Belgium) medium supplemented with 5% fetal bovine serum (Gibco, USA), 100 U/ml penicillin +100 µg/ml streptomycin (Gibco, Grand Island, NY, USA) and 2 mM L-glutamine (Gibco, Grand Island, NY, USA), at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Mammosphere (stem cell-enriched population) culture from MCF-7 cell line

For mammosphere culture, single cell suspension of MCF-7 cells were seeded at 2.5 × 10^5 cells/ml in T-25 Ultralow attachment cell culture flasks (Corning Inc., Corning, NY) in a serum-free mammary epithelial basal medium (MEBM; Lonza, Switzerland) containing 1 × B27 supplement w/o vitamin A (50 × , Gibco, USA), 2 µg/ml Heparin (0.2%, Stem Cell Technologies, Canada), 0.05% Hydrocortisone (Stem Cell Technologies, Canada), 0.5% Primocin (Invivogen, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown for approximately 3–4 days to form mammosphere structures. At the end of 3–4 days, mammospeheres were collected by centrifugation (1.200 rpm, 10 min) and dissociated enzymatically (10 min in Tryple Select; Gibco, USA) and mechanically, using a pasteur pipette. The cells obtained from dissociation were analyzed microscopically for single-cellularity. If cells were not dissociated, mechanical dissociation was repeated. Single cells were then re-plated for subsequent passages.

2.4. Detection of stemness markers

For detecting expression of stem cell markers, cell lysates were prepared from MCF-7 and mammosphere cultures using RIPA lysis buffer (Santa Cruz Biotechnology Inc., USA), containing protease inhibitors. Equal amounts of protein (20 µg protein/lane) from each lysate was loaded onto a 12% SDS polyacrylamide gel and then separated by electrophoresis which was followed by transfer to nitrocellulose membrane. Western blotting was performed using rabbit anti-Oct-4 antibody, rabbit anti-Sox2 monoclonal antibody and rabbit anti-β-actin monoclonal antibody at 1:1000 dilution (Cell Signaling, USA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies (1:2000 dilution; Cell Signaling, USA) were used to detect primary antibodies and Phototope®-HRP Western Blot Detection System (Cell Signaling, USA) was used for detection of secondary antibody according to the manufacturer's instructions. Bound antibodies were visualized on Fusion FX-7 imaging device (Vilber Lourmat, France).

2.5. The ATP viability assay

The luminogenic ATP assay determines the level of cellular ATP as an indirect measure of the number of viable cells. Briefly, MCF-7 and MCF-7s were seeded at a density of 3 × 10^3 cells per well of 96-well ultralow surface cell culture plates in 100 µl medium. The untreated cells received only the medium. Cells were grown for approximately 3–4 days to form mammosphere structures. The cells were treated with different concentrations of the VPA (0.6–20 mM) prior to the incubation at 37 °C for 24 h, 48 h and 72 h in a humidified atmosphere containing 5% CO_2. Furthermore, VPA toxicity was also examined after cells were pre-treated with 40 µM zVAD-fmk (zVAD) and 25 µM Necrostatin-1 (Nec-1) for 24 h. At the end of treatment, to extract intracellular ATP from the cells, 50 µl 5 × ATP-releasing reagent (a detergent-based reagent) was added to all the wells in the 96-well plate and the cells were then incubated at room temperature for 20–30 min. 50 µl of suspension was transferred into white opaque 96-well plate and 50 µl luciferin–luciferase mixture (FLAAM, Sigma Aldrich, USA) was added. Luminescent signal was measured at luminometer (Bio-Tek, USA) and the result was expressed in RLU (relative light units). Cell viability of treated cells was calculated in reference to the untreated control cells using the formula as viability (%) = 100 × (Sample Abs)/(Control Abs). All the experiment was repeated twice in triplicate.

2.6. Acetylated histone H3 level

Acetylated histone H3 activity was determined by using PathScan Acetylated Histone H3 Sandwich ELISA kit (Cell Signaling Technology) that detects endogenous levels of acetylated lysines on histone H3. After the cells are treated with VPA, the assay was performed according to the manufacturer's instructions. Briefly, adding the reagent to well resulted in cell lysis. After incubation with cell lysates, histone H3 is captured by the coated antibody. Following washing, an acetylated-lysine mAb is added to detect the acetylated lysines on the histone H3 protein. Anti-mouse IgG is then used to recognize the bound detection