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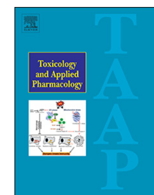

The vanillin derivative 6-bromine-5-hydroxy-4-methoxybenzaldehyde induces aberrant mitotic progression and enhances radio-sensitivity accompanying suppression the expression of PLK1 in esophageal squamous cell carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer in China. Since chemotherapy is the standard clinical intervention for advanced ESCC, the development of highly effective and minimal/non-toxic drugs is essential to improve the clinical outcome and prognosis of the patients. A novel derivative of vanillin, 6-bromine-5-hydroxy-4-methoxybenzaldehyde (BVAN08), has been recently reported to activate different cell death pathways in cancer cells. In this study, we demonstrate that BVAN08 exhibits a potent anti-proliferation effect on ESCC cells (TE-1 and ECA-109) by inhibiting the expression of PLK1, an important mitotic kinase. Consistent with this, BVAN08 induces mitotic arrest and chromosomal misalignment in ESCC cells. The disruption of microtubule nucleation around centrosomes is also observed in BVAN08 treated ESCC cells. Furthermore, BVAN08 enhances radio-sensitivity of ESCC cells by prolonging DNA damage repair. These findings underscore the potential value of BVAN08 in cancer therapeutics and demonstrate the underlying mechanism by which BVAN08 induces mitotic catastrophe and enhances radio-sensitivity in ESCC cells.

1. Introduction

Esophageal cancer (EC) is one of the most common cancers worldwide with high mortality rates. In 2017 alone, EC accounted for nearly 1,688,780 new cancer cases and 600,920 deaths in the United States (Siegel et al., 2017) and for 78% of the esophageal cancer-related mortalities in China (Chen et al., 2016). There are two main types of EC: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, of which ESCC is more common in the Chinese population (Chen et al., 2016). At present, the treatments against ESCC include surgical resection, radiotherapy, chemotherapy and targeted therapy. Although these treatments greatly improve the survival rate of patients, the overall 5-year survival rate still remains under 25% (Pennathur et al., 2013). The early stage of EC can usually be treated with surgical resection whereas chemotherapy is the main recourse to improve clinical outcomes for the patients who have already lost the opportunity of surgery (Pennathur et al., 2013; Samson and Lockhart, 2017). Currently, the DNA-damaging agents (DDAs) and microtubule-targeting agents (MTAs), such as cisplatin and taxol-related compounds, are commonly used in ESCC chemotherapy. However, traditional chemotherapy often fails, or is interrupted prior to completion, due to varying long- or short-term side effects such as gastrointestinal reactions, bone marrow suppression and neurological toxicities (Ruppert et al., 2010; Kim et al., 2013; Janowitz et al., 2016). Therefore,

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identifying novel chemotherapy agents, especially targeting specific molecules, with decreased adverse effects is still an essential strategy for ESCC treatment.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a secondary metabolite and the main component of vanilla, a flavoring agent used worldwide. It has been reported that vanillin displays both antioxidant and anti-tumor activities (Walton et al., 2003; Bezerra et al., 2016). BVAN08 (6-bromine-5-hydroxy-4-methoxybenzaldehyde), a derivative of vanillin, exhibits anti-proliferative activity and induces apoptosis in hepatoma and leukemia cells (Yan et al., 2007; Zhang et al., 2011; Pan et al., 2013). Yan et al. demonstrated that BVAN08 induced ROS generation and cell cycle arrest in HepG2 cells (Yan et al., 2007; Pan et al., 2013). Their study also revealed that BVAN08 could activate DNA damage response by promoting cleavage of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Yan et al., 2007; Pan et al., 2013). Zhang et al. used proteomic profiling to analyze the protein signature of the BVAN08-induced mitotic catastrophe and spindle disruption. Several differentially expressed proteins were identified in BVAN08 treated HepG2 cells, including regulators of DNA damage response, cell cycle progression, macromolecule complex subunit organization and cell death (Huang et al., 2005; Zhang et al., 2011).

In the present study, we used ECA-109 and TE-1 cells as in-vitro models to explore the mechanistic basis of the inhibitory effect of BVAN08 on ESCC cells. We found that BVAN08 significantly inhibited the proliferative and clonal ability of ESCC cells. BVAN08 induced mitotic arrest and caused various mitotic aberrations in ESCC cells, such as misaligned chromosomes and multipolar spindle structure. Furthermore, BVAN08 inhibited the expression of an essential mitotic kinase PLK1 which was consistent with the BVAN08 mediated inhibition of microtubule growth around centrosomes. BVAN08 treatment also increased radio-sensitivity and prolonged DNA damage repair in ESCC cells.

2. Materials and methods

2.1. Chemicals

The vanillin derivative 6-bromine-5-hydroxy-4-methoxybenzaldehyde, designated BVAN08, was generously provided by Dr. Lin Wang (Beijing Institute of Radiation Medicine). For the experiments, BVAN08 was reconstituted with DMSO to a stock concentration of 40 mM. Chemical structures of vanillin and BVAN08 are shown in Fig. 1A and B.

2.2. Cell culture and treatment

The human ESCC cell lines ECA-109 (wild type p53)and TE-1 (homozygous p53 gene mutation) were kindly provided by Dr. Jundong Zhou of the Nanjing Medical University Affiliated Suzhou Hospital (Suzhou, China). The cells were maintained in DMEM medium containing 10% FBS (HyClone, Hudson, NH, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. The cells were treated with various concentrations of BVAN08 for varying durations. For the radio-sensitivity study, cells were exposed to various doses of X-ray irradiation from the X-ray tube (RadSource Technologies, Suwanee, GA, USA) at a rate of 1.15 Gy/min.

2.3. Plasmids

The PLK1 coding sequence was amplified by PCR from an expressed sequence tag (EST) clone (GenBank™ accession No. BE 900300, obtained from Research Genetics) and cloned into the pCMV-HA vectors to generate pCMV-HA-PLK1.

2.4. Cell viability assay

ECA-109 and TE-1 cells were seeded in 96-well plates at a density of 3×10^3 cells per well and after 24 h, were treated with 5, 10, 20, 40 μM BVAN08 for a further 24 h or 48 h. The negative control groups were incubated with an equal volume of DMSO. The Cell Counting Kit-8 (CCK-8) assay was carried out according to the manufacturer's instructions. A volume of 10 μl CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated for 2 h at 37 °C. The absorbance of each well was measured using a microplate reader at 450 nm excitation wavelength.

2.5. Colony-forming assay

ECA-109 and TE-1 cells were seeded into 6-well plates at densities of 100 to 5000 cells per well in triplicates depending on the BVAN08 concentrations or radiation doses. After 24 h, cells were treated with 0, 5, 10, 20, 40 μM BVAN08 for 24 h or 20 μM BVAN08 for 12 h followed by 0, 2, 4, 6 and 8 Gy radiation, replaced with fresh medium and cultured for another 7 days for colony formation. The colonies were then fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of colonies containing > 50 cells was counted.

2.6. Mitotic index analysis and immunofluorescent staining

The cells were seeded and cultured on coverslips in 6-well plates at a suitable density. The adherent cells were treated with different concentrations of BVAN08 for 24 h and then washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were permeabilized with 0.5% Triton-X-100/PBS for 30 min and then blocked with 5% bovine serum albumin (BSA)/PBS at room temperature for an additional 30 min. Immunostaining was performed by incubating slides with antibodies against α-tubulin, γ-tubulin (Sigma, St Louis, MO, USA), phosphorylated H3 (pSer 10), Aurora B (Cell Signaling Technology, Beverly, MA, USA), PLK1 or γ-H2AX (Abcam, Cambridge, MA, USA) for 4 h at room temperature. All primary antibodies except anti-PLK1 were used at a 1000-fold dilution while the anti-PLK1 antibody was used at a 100-fold dilution. After incubation, the slides were washed three times in PBS and incubated with Alexa-488 conjugated anti-rabbit or Alexa-568 conjugated anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. The slides were then counterstained with DAPI (Vector Laboratories, Burlingame, CA, USA) to visualize the DNA. Images were obtained using a LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

2.7. Microtubule regrowth assay

The cells were cultured on coverslips and treated with ice-cold medium containing 1 μg/ml nocodazole (Sigma, St Louis, MO, USA) for 1 h. Preheated fresh medium containing 60 μM BVAN08 was added after washing with cold PBS to allow the microtubules to regrow. After the indicated times of incubation (0, 2, 4 min), the cells were fixed with ice-cold methanol for 30 min and subjected to immunostaining as described above. The re-grown microtubules were labeled with anti-α-tubulin and γ-tubulin antibody.

2.8. Western blotting analysis

The cells were cultured in 6 cm dishes and treated with different concentrations (0, 10, 20, 40 μM) of BVAN08 for 24 h. The treated cells were then harvested and lysed with RIPA Lysis Buffer (strong) (Beyotime Institute of Biotechnology, Haimen, China) with Complete™ Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA) and the total protein was extracted. Protein concentrations were assayed using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen,

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