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Proteomic profiling revealed the functional networks associated with mitotic catastrophe of HepG2 hepatoma cells induced by 6-bromine-5-hydroxy-4-methoxybenzaldehyde

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ABSTRACT

Mitotic catastrophe, a form of cell death resulting from abnormal mitosis, is a cytotoxic death pathway as well as an appealing mechanistic strategy for the development of anti-cancer drugs. In this study, 6-bromine-5-hydroxy-4-methoxybenzaldehyde was demonstrated to induce DNA double-strand break, multipolar spindles, sustain mitotic arrest and generate multinucleated cells, all of which indicate mitotic catastrophe, in human hepatoma HepG2 cells. We used proteomic profiling to identify the differentially expressed proteins underlying mitotic catastrophe. A total of 137 differentially expressed proteins (76 upregulated and 61 downregulated proteins) were identified. Some of the changed proteins have previously been associated with mitotic catastrophe, such as DNA-PKcs, FoxM1, RCC1, cyclin E, PLK1-pT210, 14-3-3σ and HSP70. Multiple isoforms of 14-3-3, heat-shock proteins and tubulin were upregulated. Analysis of functional significance revealed that the 14-3-3-mediated signaling network was the most significantly enriched for the differentially expressed proteins. The modulated proteins were found to be involved in macromolecule complex assembly, cell death, cell cycle, chromatin remodeling and DNA repair, tubulin and cytoskeletal organization. These findings revealed the overall molecular events and functional signaling networks associated with spindle disruption and mitotic catastrophe.

Introduction

Mitotic catastrophe is a type of cell death resulting from abnormal mitotic events and is characterized by the formation of large, nonviable cells with multiple micronuclei and de-condensed chromatin (Karsenti and Vernos, 2001; Ha et al., 2009; Singh et al., 2010). In mammalian cells, mitotic catastrophe has been shown to be a consequence of premature mitosis (Heald et al., 1993; Fotedar et al., 1995; Niida et al., 2005) or a failure to undergo complete mitosis (Chan et al., 1999; Nitta et al., 2004; Shang et al., 2010). However, mitotic catastrophe has also been defined as abnormal mitosis leading to cell death via necrosis or apoptosis rather than an alternative form of cell death (Chu et al., 2004; Nitta et al., 2004). Mitotic catastrophe has been found to be closely related to the occurrence of multiple centrosomes and spindle disruption (Nitta et al., 2004; Eriksson et al., 2007; Ha et al., 2009; Shang et al., 2010; Wu et al., 2010). Spindle formation is a key functional event driving proper chromosomal segregation during mitosis and employs a variety of kinesins, dyneins and microtubule polymers to generate bipolar spindle assembly and chromosomal motility (Karsenti and Vernos, 2001; Wadsworth and Khodjakov, 2004; Niethammer et al., 2007). The formation of bipolar spindles is tightly associated with the duplicated centrosomes, and spindle structure is accurately maintained (Bieling et al., 2010; Song and Rape, 2010). Spindle disruption can generate cells with multiple micronuclei or bi-nucleated giant cells and eventually results in mitotic catastrophe (VanderPorten et al., 2009; Shang et al., 2010). Presently, mitotic catastrophe is considered to be a new strategy to overcome drug or radiation resistance in cancer therapy (Sekhar et al., 2007; Singh et al., 2010). A number of cancer therapeutic agents have already been shown to induce spindle disruption and mitotic catastrophe, including DNA damaging agents, such as ionizing radiation (Nitta et al., 2004; Huang et al., 2005) and the DNA topoisomerase II inhibitors, etoposide (VP-16)(Rello-Varona et al., 2006) and doxorubicin (Eom et al., 2005; Park et al., 2005), DNA binding compound (Cahuzac et al., 2010), histone acetyltransferase-depleting agents (Ha et al., 2009), spindle or microtubule disruption agents (Cenciarelli et al., 2008; Dowling et al., 2005; Ho et al., 2008; Vitale et al., 2007), and the DNA polymerase inhibitor, aphidicolin (Nitta et al., 2004). Mitotic catastrophe has been shown to share

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several biochemical hallmarks of apoptosis, such as mitochondrial membrane permeabilization and caspase activation (Castedo et al., 2004), and apoptotic cell death was assumed to be the final outcome of mitotic catastrophe (Chu et al., 2004; Meng et al., 2007). The induction of mitotic catastrophe has been demonstrated by the inactivation of some proteins associated with cell-cycle checkpoints and DNA damage responses, such as BRCA1, CHK2(Castedo et al., 2004; Vakifahmetoglu et al., 2008), 14-3-3δ (Vakifahmetoglu et al., 2008), BRCA2-interacting protein (BCCIP) (Meng et al., 2007), Plk1 (Petronczki et al., 2008), the transcription factor, forkhead box M1 (FoxM1) (Wonsey and Follettie, 2005), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Shang et al., 2010). However, it is still largely unknown that precisely how mitotic catastrophe is regulated and what signaling networks are involved.

In the present study, we assessed the induction of DNA damage and spindle disruption by a vanillin derivative, 6-bromine-5-hydroxy-4-methoxybenzaldehyde (6-bromoisovanillin, BVAN08), which generates DNA double-stranded breaks and mitotic arrest and suppresses DNA-PKcs. We use proteomics, gene ontology and network analyses to identify and characterize the proteins and functional signaling networks or pathways related to mitotic catastrophe. A set of altered proteins have been identified, including, for example, 14-3-3, NPM1, DSTN, RCC1, SMARCE1, PXMP3, GMPS, FoxM1, RAD23B, DNA-PKcs and FEN1, etc., that function in the DNA damage response, microtubule organization and spindle assembly, macromolecule complex subunit organization and cell death. Multiple functional networks were found to be perturbed upon spindle disruption and mitotic catastrophe.

Materials and methods

Cell culture. Human hepatocellular carcinoma HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37 °C in 5% CO_2. After treatment with BVAN08 for the indicated times, cells were harvested for further experiments. For the proteomic analysis, normal growing HepG2 cells in about 70% confluence status were treated with 60 μM BVAN08 for 12 h. The treatment time and dosage of BVAN08 for other experiments were separately described in the corresponding sections of Materials and methods or Results.

Chemicals and antibodies. The vanillin derivative, 6-bromine-5-hydroxy-4-methoxybenzaldehyde (6-bromoisovanillin, BVAN08), was provided by Dr. L. Wang from the laboratory of Materia Medical Chemical Laboratory at the Beijing Institute of Radiation Medicine. The following antibodies were purchased commercially: anti-DNA-PKcs (sc-9051, H163, Santa Cruz, CA), anti-phospho-H2AX-S139 (05-636, Upstate Biotechnology, Lake Placid, NY, USA), anti-HSP70 (sc-24, Santa Cruz), cyclin E Ab-1 (RB-012-PO, NeoMarkers, Fremont, CA), anti-phospho-histone H3 (Ser-10) (6G3) (#9706, Cell Signaling Technology, Danvers, MA), anti-γ-tubulin (ab11317, Abcam, Cambridge, UK), anti-α-tubulin (ZM0438, Zhongshan, Beijing, China), anti-Plk1 (37-7000, Invitrogen, Carlsbad, CA), anti-RCC1 (13225-1-AP, Proteintech, Chicago), anti-14-3-3σ (#9636, Cell Signaling Technology), anti-phospho-Plk1 (pT210) (ab39068, Abcam), anti-phospho-Chk2 (Thr68) (#2661, Cell Signaling Technology), anti-Chk2 (#2662,Cell Signaling Technology), anti-Chk1 (#2345,Cell Signaling Technology), anti-cyclin E (NeoMarkers, Fremont, CA), anti-BrdU (Ab-2) (ZBU30, Calbiochem), HRP-conjugated anti-mouse IgG (ZB2305, Zhongshan), HRP-conjugated anti-rabbit IgG (ZB2301, Zhongshan), TRITC-conjugated anti-rabbit IgG (ZF-0316, Zhongshan), TRITC-conjugated anti-mouse IgG (ZF0313, Zhongshan), FITC-conjugated anti-mouse IgG (ZF0312, Zhongshan) and FITC-conjugated goat anti-rabbit IgG (ZF0311, Zhongshan).

Clonogenic survival assay. The normal growing HepG2 cells in 80% confluence status were trypsinized, counted. An appropriate number of cells (3×10^2 to 1×10^4) were plated into 60 mm diameter petri dishes in triplicate, and incubated in the culture medium containing different concentration (0–80 μM) of BVAN08 for 24 h, then cultured in the fresh growing medium without BVAN08. After total of 10 days culture, cells were fixed with methanol, stained with Giemsa solution, and colonies consisting of more than 50 cells were counted. The colony-forming rates were corrected with the cells numbers plated. Resulting survival plots were fitted. All experiments were repeated 3 times.

Cell cycle analysis by flow cytometry. When HepG2 cells were growing in 70% confluence status, the cells were treated with 60 μM BVAN08 for a given time. The treated or untreated HepG2 cells were harvested and fixed with 75% ethanol. The cells were resuspended in PBS containing 0.1% saponin and 1 μg/ml RNase A (Sigma, St. Louis, MO, USA), incubated for 20 min at 37 °C, and stained with 25 μg/ml propidium iodide (PI) (Sigma). The cell cycle distribution was evaluated by flow cytometry, and more than 10,000 cells per sample were enumerated.

Determining M phase cells using H3. The cells were treated with BVAN08, rinsed with PBS, and fixed in 70% ethanol. After washing with PBS, the cells were incubated in 50 μl of 0.5% Triton X-100 at room temperature for 15 min. The cells were then incubated in 50 μl of Triton X-100 containing a 1:100 dilution of an anti-phospho-histone H3 (Ser10) (6 G3) mouse mAb for 1 h. Cells were washed twice with PBS and incubated in 50 μl of a 1:100 dilution of a FITC-conjugated goat anti-mouse antibody for 1 h. The cells were then resuspended in PBS containing 0.1% saponin and 1 μg/ml RNase A, incubated for 20 min at 37 °C, stained with 25 μg/ml PI and analyzed by flow cytometry.

Immunofluorescence confocal microscopy. Cells were grown on poly-D-lysine-coated culture slides (BD Pharmingen, USA), washed in PBS, fixed in PBS containing 4 or 0.5% paraformaldehyde (PFA) for 15 min, and permeabilized in Triton buffer (0.1% Triton X-100 in PBS). The fixed cells were blocked in blocking solution (2% bovine serum albumin, 0.1% Tween, PBS) for 30 min at 37 °C in a humidified chamber. Immunostaining was performed using anti-α-tubulin and anti-γ-tubulin antibodies for 2 h at room temperature in a humidified chamber, and the resulting solution was washed three times in blocking buffer. The cells were then incubated with the following secondary antibodies: goat anti-mouse tetramethylrhodamine isothiocyanate (TRITC)-conjugated IgG (Zhongshan, ZF0313) and goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated IgG (Zhongshan, ZF0311). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 33258 (H-33258, Sigma) in a mounting solution. Immunofluorescence confocal microscopy was performed on an LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

Immunoblotting assay. The cells were harvested and washed twice in an ice-cold PBS after being treated with BVAN08, and the total protein was extracted using the M-PER Mammalian Protein Extraction Reagent (Pierce 78501). Equal amounts of protein (50 μg) were loaded and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes for western blot analysis.

Comet assay to assess DNA double-stranded breaks. After being treated with BVAN08, cells were collected and mixed with low melting point agarose at 37 °C. This mixture was overlaid onto 0.5% normal melting point agarose on a slide, covered with a coverslip and returned to 4 °C until solid. The coverslip was gently removed, and NMP agarose was added onto the slide. The slide was covered again and placed at 4 °C