Title: 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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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.

Keywords: mitotic catastrophe, spindle assembly, microtubule, cell cycle arrest, DNA damage, therapeutic target, vanillin derivative
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 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-bromoisoovanillin, 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% CO₂. 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 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 × 10² to 1 × 10⁴) 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 minutes 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) (6G3) 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 minutes 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 until the mixture was solid. The slide was placed in neutral lysis buffer and subjected to electrophoresis. Thereafter, the slides were gently washed with neutralization buffer, stained with ethidium bromide (2 μg/ml) and visualized and analyzed under a fluorescence microscope. **A CaspLab-1.2.2 software (University of Wroclaw, Poland) was used to analyze the comets results.** DNA damage was characterized as tail moment combining comet tail length and the proportion of DNA migrating into the tail.
**Pulsed-field gel electrophoresis (PFGE).** PFGE was also employed to detect DNA double-stranded breaks induced by BVAN08. Briefly, the cells were collected by centrifugation, resuspended in DMEM and mixed with an equal volume of 1.2% low melting point agarose prepared with DMEM at 37°C. The mixture was pipetted into the plug mold (80 ml/well, 4 x 10^5 cells) and allowed to solidify at 4°C for 10 min. The plugs were incubated in DMEM growth medium containing 60 μM BVAN08 for 4–24 h, incubated in lysis buffer (0.5 M EDTA, pH 8.0, 2 mg/ml proteinase K, 0.5% SDS, 10 μM Tris buffer, pH 8.0) at 50°C for 48 h, and rinsed three times with TE solution. Half of each DNA plug was inserted into the wells of a 0.9% agarose gel containing 0.5 mg/ml ethidium bromide. The gels were subjected to PFGE (field angle 120°) and run in circulating 0.5X TBE buffer at 14°C and 1.5 V/cm with a switching time of 15 min for 72 h. After electrophoresis, the gel section was observed and photographed under ultraviolet. The DNA released from the well represented the double-strand break DNA and was quantified using the electrophoresis gel imaging system.

**Two-dimensional gel electrophoresis (2-DE).** Cells were harvested after 60 μM BVAN08 treatment for 12 in DMEM supplemented with 10% fetal bovine serum. The nuclear protein was extracted with KEYGEN nuclear-cytosol extraction kit (KGP-1-50, China). Chromatographic analysis was performed, and concentration of proteins was determined by the modified Bradford method.

For 2-DE, first-dimensional electrophoresis was performed on an IPGphor IEF system (Amersham Biosciences). Approximately 600 μg of protein was mixed with rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% Pharmalyte 3–10) and applied to 18 cm Immobiline DryStrips with a linear pH 3–10 gradient by in-gel rehydration. The focusing
program was set as follows: 30 V for 12 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, gradient to 8,000 V for 30 min, finally 8,000 V for 4 h. After IEF, the strip was immediately equilibrated for 15 min in reducing equilibration buffer that contained 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% v/v glycerol, 2% w/v SDS, a trace of bromophenol blue, and 1.0% w/v DTT and then for 15 min in alkylation eequilibration buffer that contained 2.5% w/v iodoacetamide instead of 1.0% w/v DTT. The reduced and alkylated strip was then transferred onto a 13% self-cast SDS-PAGE gel (200 × 200 × 1 mm). The second-dimensional gel electrophoresis was performed on a Protein II (BioRad) vertical electrophoresis unit for 30 min at a constant current of 15 mA/gel and then for 30 mA/gel until the bromophenol blue band reached the bottom of the gels at 15 °C.

**Gel staining and image analysis.** The gel was immersed in 250 ml of fixation solution (50% methanol, 10% acetic acid) with gentle agitation at least twice for 30 min each. Following image acquisition, the gel was stained with Coomassie brilliant blue (CBB) G-250 for at least 12 h and then destained in distilled water. This image was scanned using an Image Scanner apparatus (Amersham Biosciences) and stored as TIF files. Subsequent analysis of spot detection, matching, quantitation, and normalization were performed by the software package of Image Master 2D Platinum (Version 5.0, Amersham Biosciences). Protein spots were detected using an automated procedure from the software combined with manual editing to remove artefacts. The intensity of each protein spot was normalized to the total intensity of the entire gel image.

**In-gel trypsin digestion.** After the image analysis, the protein spots of interest were cut out of the gel and minced into pieces. The gel pieces were destained with 50% acetonitrile.
(ACN) in 25 mM ammonium bicarbonate until transparent and dried in a vacuum centrifuge. Protein spots were incubated with 60-80 μL of trypsin (0.01 mg/ml, 25 mM ammonium bicarbonate) for 16-18 h at 37°C. The gel spots were immersed in 50 ml of 0.5% trifluoroacetic acid (TFA) and 50% ACN for 1 h at 37°C followed by another 50 ml of 0.5% TFA and 50% ACN for 1 h at 37°C. The supernatants were combined and vacuum-dried.

**MS analysis and database search.** The peptides were resuspended in 2 μl of 0.1% TFA and immediately spotted onto a MALDI target plate with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. The sample was analyzed using the Applied Biosystems 4800 Proteomics Analyzer, and the resulting data were applied to the IPI database (Human_3.51) search using GPS Explorer v.3.6 software (Applied Biosystems, Framingham, MA, USA). The search parameters were as follows: 10 ppm mass tolerance; trypsin digestion with two missed tryptic cleavage sites; MH+ and monoisotopic; and carbamidomethyl as a fixed modification for cysteines and oxidation as a variable modification for methionines. The criteria for protein identification were as follows: the protein score was significant \( p < 0.05 \) and higher than 60 score. Multiple proteins within a single spot were classified in the same group if they matched at least four peptides.

**Bioinformatics analysis.** Gene ontology analysis of the differentially expressed proteins was done with online Slim Mapper tools on the website, Gene Ontology (http://www.geneontology.org/). Proteins for which no biological process could be assigned were omitted from the chart. Significance refers to the log \( p \)-value), which was determined by right-tailed Fisher’s exact test using the Ingenuity program (Ingenuity® Systems, www.ingenuity.com). The threshold is at \( 0.205 = -\log (p = 0.05) \).
Results

DNA double-stranded breaks and mitotic arrest of hepatocellular carcinoma HepG2 cells induced by BVAN08

The cytotoxicity of BVAN08 (6-bromoisoavanillin) in human hepatocellular carcinoma HepG2 cells was examined by colony-forming assays, and the results after treatment with BVAN08 for 24 h are shown in Figure 1A. Cell viability was reduced in a concentration-dependent manner, and the IC$_{50}$ was 23.1 μM for the 24 h treatment. The DNA double-stranded breaks (DSBs) were detected by pulsed-field gel electrophoresis (Figures 1B & C) and neutral single cell gel electrophoresis (comet assay) (Figures 1D & E). A significant amount of DNA DSBs was induced in BVAN08-treated HepG2 cells as early as 4 h at concentrations of 40–60 μM. The increased level of γH2AX, a molecular marker of DSBs (Redon et al., 2009; Tanaka et al., 2009), further indicated the induction of DSBs upon treatment with BVAN08 (Figure 1F).

The induction of G2/M arrest upon treatment with 60 μM BVAN08 was shown by flow cytometric analysis (Figure 2A & C). To identify whether BVAN08 blocked HepG2 cells at the G2/M or the mitotic phase, mitotic cells were measured by flow cytometric analysis of cells labeled with an anti-phospho-histone H3 antibody, which is a known marker for mitotic cells (Juan et al., 1998). As shown in Figures 2B & D, the amount of phospho-histone H3-positive cells was dramatically increased in BVAN08-treated cells, suggesting that mitotic arrest was induced. Reduction of p-H3 positive cells percentage after 48 h could be attributed to death of some long term arrested mitotic cells. In addition, an increased
induction of multiploidy was demonstrated in HepG2 cells 24 h after the 60 μM BVAN08 treatment (Figure 2A).

*Spindle disruption and mitotic catastrophe in HepG2 cells treated with BVAN08*

By staining with antibodies against α-tubulin and/or γ-tubulin, we were able to visualize the spindles. BVAN08 treatment resulted in a dose-dependent increase in proportions of mitotic catastrophe cells (Figures 3A & C). Moreover, a significantly increased proportion of cells with multicentrosomes or aberrant spindle patterns (asymmetric dipolar spindles and/or tripolar and tetrapolar spindles) were detected in BVAN08-treated cells (Figures 3A, B and D). In addition, a pattern of nuclear aberrations were also observed, including micronuclei and multinucleated giant cells (Figure 3E).

*2-DE analysis and identification of differentially expressed proteins in BVAN08-treated HepG2 cells*

To investigate the early molecular events and signaling that result in spindle disruption and mitotic catastrophe, 2-DE quantitative image analysis and mass spectrometry (MS) sequencing were performed to identify the protein profile associating with mitotic catastrophe in HepG2 cells. After 60 μM BVAN08 treatment for 12 h, about 35 % of cells arrested at mitotic stage and were at the early stage of mitotic catastrophe (Figure 2 and Figure 3). Therefore, this dosage and treatment time of BVAN08 were adopted for following proteomic profiling analysis. Three replicate analytic gels of control and BVAN08-treated cells were compared by image analysis. A total of 93 differentially expressed protein spots, including 45 spots representing upregulated proteins and 48 spots representing downregulated proteins, were identified and subjected to trypsin digestion and MS analysis.
Figure 4 shows some representative differentially expressed protein spots from the 2D gels. The 76 upregulated proteins and 61 downregulated proteins are summarized in Supplementary Tables 1 and 2, respectively. A unique protein was identified in most of the spots, but in some, two or more proteins were identified.

**A number of proteins, which have been already known as targets associating with mitotic catastrophe, were detected by western blot analysis (Figure 5).** The results showed decreased expression of DNA-PKcs, the regulator of chromosome condensation 1 (RCC1), cyclin E and FoxM1, and increased expression of phosphorylated PLK1-pT210, 14-3-3σ and HSP70. Although the expression of 14-3-3σ initially increased at 12 h, but declined 24 h after the 60 μM BVAN08 treatment. The dose-dependent alteration in expression of 14-3-3σ, HSP70, and RCC1, three proteins identified in 2-DE gels, is also shown in Figure 5.

**Protein database search, classification and bioinformatics analysis**

To better understand the putative signaling pathways and functional networks related to spindle disruption and mitotic catastrophe, the biological processes for the identified proteins from BVAN08-treated HepG2 cells were identified and classified according to the IPI database (Human_3.51) and gene ontology analysis (http://www.geneontology.org) (Figure 6A). The most abundant protein group (22.6%) in response to the BVAN08 treatment was the protein metabolic/post-translational modification group, followed by the cell communication/signaling group (17.5%) and the organelle organization group (13.9%). It is interesting that a considerable number of identified proteins are involved in the DNA damage response (chromatin organization, cell cycle, DNA repair) and in microtubule organization. The pathway significance was analyzed through the use of Ingenuity Pathway Analysis.
(Ingenuity® Systems, www.ingenuity.com), and the 14-3-3-mediated signaling pathway showed a highly significant enrichment. Enrichments were also observed for proteins involved in the following signaling pathways: PI3K/Akt, actin cytoskeleton, IGF-1 and ERK/MAPK (Figure 6B). The functional network analysis further demonstrated the abundance of identified proteins in 14-3-3-mediated signaling (Figure 7), macromolecule complex assembly (Supplementary Figure 1), cell death (Supplementary Figure 2), and in figures not shown, cell cycle, cytoskeleton assembly and tubulin assembly.

Discussion

BVAN08, a derivative of the natural flavoring agent vanillin, has previously been demonstrated to have antiproliferative effects on various cancer cell lines (Yan et al., 2006; Yan et al., 2007). Here, we further observed that BVAN08 induced mitotic arrest, multipolar spindles, and multinucleated cells in HepG2 cells. More than 60% of the mitotic cells were aberrant spindles cells after a 24-h treatment with 40 μM BVAN08. The formation of multinuclear cells and multipolar spindles has been considered to be the dominant feature of cells undergoing mitotic catastrophe (Huang et al., 2005; Wonsey and Follettie, 2005; Dodson et al., 2007). DNA damaging agents, including ionizing radiation (Nitta et al., 2004; Huang et al., 2005) as well as other chemical DNA damaging agents (Vakifahmetoglu et al., 2008; Cahuzac et al., 2010) and spindle-disrupting agents (Vitale et al., 2007; Cenciarelli et al., 2008; Ho et al., 2008) are two major kinds of agents that induce mitotic catastrophe. Similar to our previous report (Yan et al., 2007), BVAN08 treatment leads to DNA double-stranded breaks (Figure 1) and decreased the expression of DNA-PKcs (Figure 5).
in HepG2 cells. While inactivation of DNA-PKcs was confirmed to promote the spindle disruption and mitotic catastrophe in response to DNA damage (Shang et al., 2010). Therefore, both DNA damage induction and DNA-PKcs depression are two major triggers of mitotic catastrophe in HepG2 cells by BVAN08.

We used comparative proteomic profiling to identify the differentially expressed proteins related to spindle disruption and mitotic catastrophe in response to DNA damage induced by BVAN08. A total of 137 proteins, including 76 upregulated proteins and 61 downregulated proteins, were identified (Supplementary Tables 1 and 2). The analyses of the biological processes for these identified proteins indicated that: the most abundant protein group (22.6%) is the protein metabolic/post-translational modification group, followed by the cell communication/signaling group (17.5%) and the organelle organization group (13.9%). It is interesting that a considerable number of identified proteins are involved in the DNA damage response (chromatin organization, cell cycle, DNA repair) and in microtubule organization.

The altered expression of DNA-PKcs, FOXM1, RCC1, cyclin E, PLK1-pT210, 14-3-3σ and HSP70 have been confirmed by western blot analysis, among which the altered expression of RCC1, 14-3-3σ and HSP70 was observed in the proteomic-MS study. All these proteins have already been reported to associate with the induction of mitotic catastrophe, and further indicating that the cellular model is reliable for the mechanistic study of mitotic catastrophe.

FoxM1 is a transcription factor that, through binding with and phosphorylation by Plk1, mediates Plk1-dependent regulation of cell cycle progression. The genes regulated by FoxM1
are essential for proper chromosomal segregation and mitosis, and which include Plk1, cyclin B1, Aurora B (Fu et al., 2008), Cdc25B, survivin, centromere protein A (CENPA), CENPB (Wang et al., 2005; Wonsey and Follettie, 2005), Nek2, KIF20A (Wonsey and Follettie, 2005). Loss of FoxM1 expression generates mitotic spindle defects, delays cells in mitosis, and induces mitotic catastrophe (Wonsey and Follettie, 2005; Fu et al., 2008).

RCC1 is a Ran guanine nucleotide exchange factor for the Ran GTPase and has a pivotal role in mitosis as well as nucleocytoplasmic transport and nuclear envelope assembly. The amino-terminal serine or proline residue of RCC1 was found to be uniquely methylated on its alpha amino group, and the defect resulted in less efficient binding to chromatin during mitosis, which caused spindle pole defects (Chen et al., 2007). Inhibition of binding of RCC1 to the mitotic chromosome by actinomycin D led to a reduction in Ran GTP levels and resulted in severe spindle defects and mitotic catastrophe (Ho et al., 2008).

Cyclin E, an activator of cyclin-dependent kinase (Cdk) 2, accumulates in the G1/S phase of the cell cycle, where it stimulates functions associated with entry into and progression through S phase (Ekholm and Reed, 2000). It was recently shown that overexpression of full-length cyclin E (EL) prolonged cell cycle arrest in prometaphase, whereas overexpression of low molecular weight cyclin E (LMW-E), a tumorigenic cyclin E isoform that lacks a portion of the EL amino-terminus containing a nuclear localization sequence, reduced the length of mitosis; accelerated mitotic exit; generated binucleated or multinucleated cells with amplified centrosomes; promoted chromosome missegregation during metaphase; generated anaphase bridges during anaphase and resulted in failed cytokinesis and polyploidy (Bagheri-Yarmand et al., 2010a). Other studies found that targeting the cyclin E-Cdk2
complex markedly inhibited growth through the induction of multipolar anaphases that then triggered anaphase catastrophe (Galimberti *et al.*, 2010), and deregulation of cyclin E impaired mitotic progression through premature activation of Cdc25C (Bagheri-Yarmand *et al.*, 2010b). Thus, reduced RCC1, cyclin E and FOXM1 levels should be closely related to the spindle disruption and mitotic catastrophe induced by BVAN08.

In addition, our proteomic analysis data include another two changed proteins, the upregulated RAN and downregulated NPM1, which may also function in spindle or centrosome organization. It was reported that NPM1 inactivation led to unrestricted centrosome duplication and genomic instability (Grisendi *et al.*, 2005). In Kaposi’s sarcoma herpesvirus (KSHV)-infected cells, NPM1 was phosphorylated by cyclin-dependent kinase (CDK) 6 after its activation by KSHV D-type cyclin, resulting in the accumulation of cells with supernumerary centrosomes and abnormal chromosomal segregation (Cuomo *et al.*, 2008).

With regard to DNA damage and mitotic arrest induced by BVAN08, there were some proteins found to be involved in the functional networks of the DNA damage response, including chromatin remodeling, DNA repair (FEN1, RAD32B, HSPA1B), and cell cycle and death signaling (Figure 9). Some identified proteins function in multiple networks. One example of such a protein is EIF5A (eukaryotic translation initiation factor 5A). EIF5A is a nucleocytoplasmic shuttle protein, and overexpression of EIF5A led to p53-dependent apoptosis or sensitized cells to induction of apoptosis by chemotherapeutic agents (Li *et al.*, 2004). Moreover, apoptosis induced by EIF5A1 was mediated by the mitochondrial apoptotic pathway (Sun *et al.*, 2010). EIF5A clearly plays
a role in both macromolecule complex assembly (Supplementary Figure 1) and cell death (Supplementary Figure 2).

Heat shock proteins (HSPs) are a group of proteins encoded by a set of so-called stress genes that play an essential role as molecular chaperones; HSPs assist in the proper folding of nascent and stress-accumulated misfolded proteins and prevent their aggregation. As shown in Supplementary Table 1, a number of HSP70 and HSP90 isoforms, including HSPA2, HSPA1B, HSPA8, HSP90AA1, HSP90AB1, HSP90AB3P and HSP90B1, were upregulated upon treatment with BVAN08. The western blot analysis also showed increased expression of HSP70 (Figure 5). The induction of HSPs reflects the stress response of HepG2 cells to BVAN08 toxicity. Importantly, a number of reports have demonstrated the direct involvement of HSPs in mitotic events. Under the stress of heat shock, HSP70 was rapidly recruited to mitotic centrosomes, and normal progression through mitosis was observed immediately after release of HSP70 from centrosomes (Hut et al., 2005). HSP70 and HSP90 have been shown to associate with the components of the centrosome complex, including CRM-1, γ-tubulin, eEF-1A and HOPS (Pieroni et al., 2008), which are intimately involved in centrosome regulation and mitotic spindle formation (Pieroni et al., 2008; Taylor et al., 2008). HSP90 inhibition sensitizes cells to centrosome abnormalities and anaphase cell death by the mitotic disruptor, arsenite. Therefore, HSPs function to protect cells against mitotic stress (Glowala et al., 2002; Taylor et al., 2008).

Protein modification is an important molecular event regulating protein activity. After BVAN08 treatment, a number of post-translational modification proteins were affected, such as the above-mentioned HSP isoforms, PPIA, CCT6A, and CCT1; these proteins have roles in
protein folding. Other proteins affected were those involved in proteolysis and peptidyl-lysine modification/hypusine (EIF5A2, EIF5A) and other modifying proteins, including PSMC2, UFD1L, KRT1, SH3D19 and PHB.

Protein complex assembly is the structural basis for subcellular organization and includes microtubules, spindles, and actin filaments. A considerable number of the identified proteins (SF3A1, EIF4B, HSP90AA1, HMGA1, SMARCE1, SEPT11, CCT1, EIF5A, RCC1, NPM1, CAPZA1, CAPZA2, SEPT7, PHB, CBX3) were found to be involved in the network of macromolecule complex assembly/disassembly (Figure 8), such as chromatin organization (HMGA1, SMARCE1, CBX3, PHB) and microtubule organization (RCC1, NPM1, RAN). In addition, multiple isoforms of tubulins, including TUBA1A, TUBA1B, TUBA1C, TUBA3C, TUBA3E, TUBA4A and TUBA8, were found to be increased in our proteomic analysis.

The pathway significance analysis of our proteomic data demonstrated that the 14-3-3-mediated signaling category was highly enriched for the differentially expressed proteins (Figure 6b & Figure 7). Multiple 14-3-3 isoforms, including YWHAG (14-3-3γ), YWHAH (14-3-3η), YWHAQ (14-3-3θ), and YWHAZ (14-3-3ζ/δ) were upregulated. As shown in Figure 7 and Supplementary Table 1, a group of HSP and tubulin (TUB) isoforms as well as CCT8, PPIA, CLIC4, ACTN4, and FKBP4 proteins were upregulated in the 14-3-3 network. In this network, there were also a number of proteins (EEF1B2, PDCD6IP, TCP1, PKM2, PDIA3, PKG1, and CCT6A) that were decreased upon treatment with BVAN08. The 14-3-3 proteins represent a highly conserved protein family comprising seven mammalian isoforms (β, γ, η, τ, σ, ε, and ζ) that are involved in a
wide variety of biological processes, such as cell death, cell cycle response to DNA damage (Wilker et al., 2007; Hosing et al., 2008; Mohammad and Yaffe, 2009; Telles et al., 2009; Kasahara et al., 2010; Zuo et al., 2010), and mitogenic signal transduction (Du et al., 2005; Tzivion et al., 2006; Zuo et al., 2010). 14-3-3 also plays an important role in controlling mitotic progression through association with Cdc25C (Margolis et al., 2006; Hosing et al., 2008) and binds to a variety of translation/initiation factors, including eukaryotic initiation factor 4B, during mitosis (Wilker et al., 2007). 14-3-3σ knockout cells were unable to maintain cell cycle arrest and died from mitotic catastrophe upon entry into mitosis (Chan et al., 1999). Lack of 14-3-3σ resulted in reduced mitosis-specific expression of the internal ribosomal entry site-dependent form of the cyclin-dependent kinase, Cdk11, leading to loss of polo-like kinase 1 (PLK-1) at the midbody and impaired cytokinesis (Wilker et al., 2007). 14-3-3γ forms a complex with Chk1 phosphorylated at Ser296 and mediates the interaction between Chk1 and Cdc25A. This ternary complex formation has been shown to be essential in Cdc25A phosphorylation and degradation to block premature mitotic entry after DNA damage (Kasahara et al., 2010). Finally, 14-3-3 isoforms σ (Moreira et al., 2008), ε and γ (Pietromonaco et al., 1996) were found to localize in centrosomes, and 14-3-3σ also localized to the midbody during cytokinesis (Moreira et al., 2008). More interestingly, the association of 14-3-3σ with tubulin was dependent on microtubule (MT) assembly, because MT breakdown led to increased abundance of the nuclear form of the 14-3-3σ protein (Moreira et al., 2008). The upregulation of multiple 14-3-3 isoforms by BVAN08 strongly suggested that 14-3-3-mediated signaling plays an important role in the early stage of cellular processes of spindle disruption and mitotic catastrophe.
In conclusions, in this study, we used proteomic profiling to identify differentially expressed proteins associated with the induction of spindle disruption and mitotic catastrophe in human hepatoma HepG2 cells induced by BVAN08, a vanillin derivative. The functional pathway analysis demonstrated that the 14-3-3-mediated signaling network was the most highly enriched for the differentially expressed proteins. The modulated proteins were involved in pathways associated with macromolecule complex assembly, cell death, cell cycle, chromatin remodeling and DNA repair, tubulin and cytoskeletal organization. These findings elucidated the overall molecular events and signaling pathways associated with spindle disruption and mitotic catastrophe.

Conflict of interest statement

No potential conflict of interests was disclosed.

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Figures legends

Fig. 1. Induction of DNA double-stranded breaks in HepG2 cells by BVAN08. (A) Clonogenic survival of HepG2 cells treated with different concentration of BVAN08 for 24 h. Data represent the mean with standard deviation from four independent experiments. (B) A representative gel pattern of DNA double-stranded breaks (DSBs, DNA entered into gel) induced by 60 μM BVAN08 treatment for 0-24 h in HepG2 cells, detected by pulsed-field gel electrophoresis (PFGE). (C) Quantitative measurement of BVAN08-induced DNA DSBs detected by PFGE. Data represent the mean with standard deviation from four independent experiments. #p < 0.01 as compared with the untreated control. (D) A representative comet image of DNA DSBs in HepG2 cells treated with 40 μM BVAN08 for different times, detected by neutral single-cell gel electrophoresis (SCGE). (E) DNA DSBs expressed as the comet tail moment of SCGE assays. Data represent the mean with standard deviation from three independent experiments. *p < 0.05, #p < 0.01 as compared with control cells. (F) Western blot analysis of γ-H2AX levels in HepG2 cells treated with 60 μM BVAN08 for the indicated times.

Fig. 2. Accumulation of mitotic cells induced by BVAN08. (A) Representative flow cytometric histograms indicate the induction of G2/M arrest in HepG2 cells after treatment with 60 μM BVAN08. (B) Representative flow cytometric histograms of cells stained with a phospho-histone H3 (Ser10) antibody to determine mitotic cells. HepG2 cells were treated with 60 μM BVAN08 for the indicated times and collected and fixed for flow cytometric analysis. (C) Changes in G0/G1, S and G2/M phases in cells as detected by flow cytometry at
different timepoints after treatment with 60 μM BVAN08. Data represent the mean of three independent experiments. (D) Quantitative measurement of mitotic cells by histone H3 staining and flow cytometric analysis. Data represent the mean with standard deviation from three independent experiments *p < 0.05, #p < 0.01 as compared with the untreated cells.

**Fig. 3.** Aberrant multipolar spindles and multinucleated cells induced by BVAN08. (A) Representative images demonstrating the aberrant spindle patterns in BVAN08-treated HepG2 cells. After 24 h of treatment with 60 μM BVAN08, the cells were fixed, immunostained with an antibody against α-tubulin, and analyzed using confocal immunofluorescence. Nuclei were visualized with DAPI staining. Accumulation of mitotic cells and a considerable proportion of cells with aberrant spindles can be seen. (B) BVAN08-induced accumulation of mitotic cells determined by immunostaining with an anti-α-tubulin antibody and assessed by confocal immunofluorescence. Cells were treated with 0-60 μM BVAN08 for 24 h. Data represent the mean with standard deviation of three independent experiments. (C) A representative image showing the aberrant tetrapolar spindle induced by BVAN08. The cells were fixed, co-stained with antibodies against α-tubulin and γ-tubulin (staining centrosomes), and analyzed using confocal immunofluorescence. (D) Quantitative analysis of the mitotic cells with aberrant spindles or multicentrosomes. The data represent the mean with standard deviation from three independent experiments. (E) A representative image showing the multinucleated cells induced by BVAN08.
Fig. 4. Representative images of the differentially expressed protein dots from the two dimensional electrophoresis gels derived from control cells and cells treated with 60 μM BVAN08 for 12 and 24 h. Red square indicates the upregulated protein dots and green ring indicates the downregulated protein dots.

Fig. 5. Western blot analyses demonstrate the BVAN08-mediated decrease in expression of DNA-PKcs, cyclin E, RCC-1 and FoxM1 and BVAN08-mediated increase in expression of phospho-PLK1/T210, 14-3-3σ and HSP70. HepG2 cells were treated with 60 μM BVAN08 for 0–24 or 48 h or with different concentrations of BVAN08 for 12 h. β-actin expression was used as a control.

Fig. 6. Functional classification and significance analysis of BVAN08-responsive proteins. (A) Gene ontology analysis of the differentially expressed proteins was performed with online Slim Mapper tools on the website, Gene Ontology (http://www.geneontology.org/). Proteins for which no biological process could be assigned were omitted from the chart. Categories with more than four assigned proteins are shown. (B) Significance refers to the log (p-value), which was determined by right-tailed Fisher’s exact test using the Ingenuity program. The threshold is at 0.205 = -log (p = 0.05).

Fig. 7. Enrichment of BVAN08-responsive proteins in the 14-3-3-mediated signaling network. Proteins in red and green nodes were upregulated and downregulated by BVAN08, respectively. Network modeling was done using the Ingenuity Pathway program.
Supplementary Data

**Supplementary table 1.** The complete list of upregulated proteins in HepG2 cells induced by BVAN08

**Supplementary table 2.** The complete list of downregulated proteins in HepG2 cells induced by BVAN08

**Supplementary Fig. 1.** The macromolecule complex assembly network generated with BVAN08-responsive proteins.

**Supplementary Fig. 2.** The cell death network generated with BVAN08-responsive proteins.
References


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Figure 1

A. Graph showing survival (%) against concentration (μM). 

B. Gel image showing 40 μM BVAN08 treatment with time points 0, 4, 8, 12, 24 (h) and arrow indicating DSB.

C. Bar graph showing percentage of DSB DNA (in lane) against time of treatment (h).

D. Images showing tail moment at 0h, 4h, 8h, 24h.

E. Bar graph showing tail moment against time of treatment (h) with significance levels indicated.

F. Western blot showing γ-H2AX and β-actin expression over time (h).
Figure 2

A

Control  60 µM, 2h  60 µM, 4h  60 µM, 8h  60 µM, 12h  60 µM, 24h  60 µM, 48h

B

Control  60 µM, 2h  60 µM, 4h  60 µM, 8h  60 µM, 12h  60 µM, 24h  60 µM, 48h

C

The time of treatment (h)

Ratio of cell cycle distribution

G0-G1  G2-M  S

D

p-H3 positive cells (%)

p-H3 positive

The time of treatment (h)
Figure 3

A) α-tubulin, DAPI, Merge

B) α-tubulin, γ-tubulin, DAPI, Merge

C) Percentage of Mitotic cells

D) Multipolar spindle cells (%)

E) α-tubulin, Hoche33258, Merge