Vanillin derivative 6-bromine-5-hydroxy-4-methoxybenzaldehyde-elicited apoptosis and G2/M arrest of Jurkat cells proceeds concurrently with DNA-PKcs cleavage and Akt inactivation

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Abstract. Vanillin, a naturally occurring food component, has been reported to have anti-mutagenic and anti-metastatic potentials, and to inhibit DNA-PKcs activity. However, vanillin itself exhibits very weak antiproliferative activity. We explored the effects of bromovanin (6-bromine-5-hydroxy-4-methoxybenzaldehyde), a novel vanillin derivative, on survival and cell-cycle progression of human Jurkat leukemia cells. Treatment with >10 μM bromovanin significantly elicited apoptosis and G2/M arrest in Jurkat cells in a dose- and time-dependent manner. Bromovanin-induced DNA double-strand breaks (DSB) were demonstrated by means of comet assay as well as detection of phosphorylated H2AX, a sensitive indicator of DNA DSBs. Immuno-hybridiztion analysis revealed that the cleavage of procaspase-3 and DNA-PKcs occurred concurrently with bromovanin-induced apoptosis. Furthermore, phosphorylated Akt protein (Ser473), which is catalyzed by DNA-PKcs, as well as phosphorylated GSK3ß (a substrate of activated Akt), markedly decreased in bromovanin-treated Jurkat cells, suggesting that bromovanin leads to inactivation of Akt pathway via cleaving DNA-PKcs. These multiple effects, associated with the regimen of cancer therapeutic strategies, make bromovanin very appealing for future development as a novel anticancer drug.

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavor compound in food and personal products. It displays antioxidant and antimicrobial properties (1-4), and has consistently proved to be an antimutagen and an anti-carcinogen against a variety of chemical and physical agents (5-9). For example, it was shown to decrease the number of small intestinal tumors induced by several carcinogens (8), and reduce the number of preneoplastic glutathione S-transferase π isoenzyme-positive foci induced by 2-amino-3-methylimidazo[4,5]quinoline in a hepatocarcinoma model (9). More recently, vanillin was shown to inhibit the invasion and migration of cancer cells *in vitro*, and to inhibit the metastasis of mouse breast cancer cells *in vivo* (10). It was also reported that vanillin inhibited the activity of DNA-dependent protein kinase (DNA-PK) and enhanced the sensitivity of cancer cells to cisplatin (11) and trichostatin A (a hydroxamic acid-based HDAC inhibitor) (12). DNA-PKcs (DNA-PK catalytic subunit) has recently been proposed to be an ideal target for designing and developing novel anticancer drugs (13-17). Inhibition of DNA-PK is not only a valid approach to increase the tumor cell killing of other anticancer agents, e.g. cisplatin, topo II poisons and ionizing radiation (14-16), but also leads to down-regulation of proto-oncogene c-myc protein (16) as well as some genes associated with survival, proliferation and invasion, e.g. death-associated protein kinase 3 (DAPK3), the nuclear factor of activated T cells (NFAT) family members NFATc1 and NFATc3 (17). Depressing DNA-PKcs also confers the up-regulation of N-myc downstream regulated gene 1 (NDRG1), CDKN1B (p27/Kip1), CDKN1A (p21/Cip1) and some interferon signaling pathway components (17).

The inhibiting effect of vanillin on DNA-PKcs offers a potential for developing novel anticancer drugs from vanillin derivatives. As vanillin itself exhibits little cytotoxicity, we have recently explored and compared the antiproliferative effect of vanillin and its derivatives, and found that the derivative bromovanin (6-bromine-5-hydroxy-4-methoxybenzaldehyde) displayed a powerful antiproliferative effect as well as an inhibitory effect on DNA-PKcs kinase activity. Based on these observations, we investigated the ability of bromovanin to induce DNA damage, apoptosis and cell-cycle changes in human Jurkat leukemia cells, as well as its related mechanisms.

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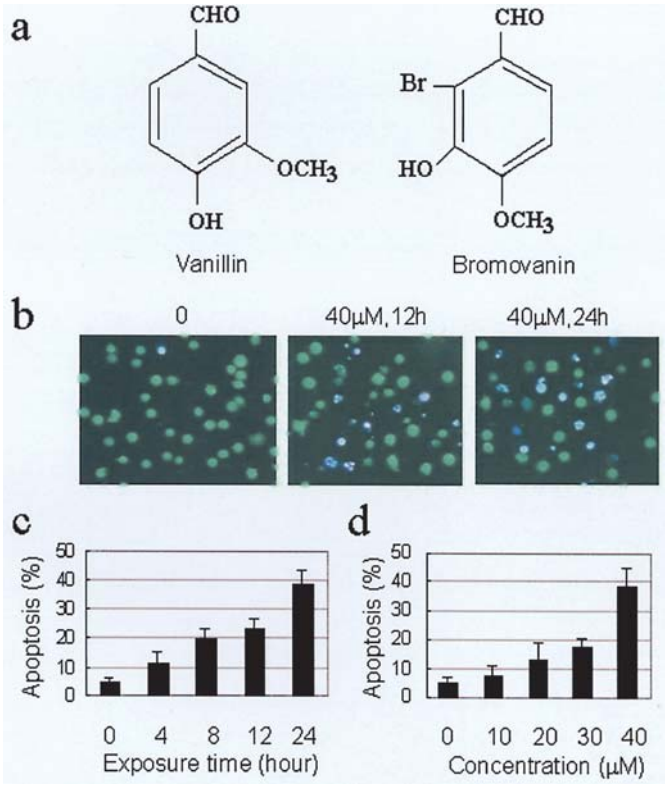

Figure 1. Detection of bromovanin-induced apoptosis by fluorescent dye staining. (a) Chemical structures of vanillin and bromovanin; (b) morphological characteristics of apoptosis displayed under fluorescence microscopy. In this assay, cells were stained simultaneously with Hoechst 33255, fluorescein diacetate (FDA) and propidium iodide (PI). Only viable cells can metabolize FDA to fluorescein that elicits green fluorescent light under fluorescence microscope. Apoptotic or dead cells were stained weakly or not stained by FDA, but stained by Hoechst 33255 to elicit a bright blue fluorescent light (condensed or wreckage nuclei); (c) the percentage of apoptosis after different lengths of 40 μm bromovanin treatment; (d) dose-response effect of bromovanin-induced apoptosis after 24-h treatment.

Materials and methods

Chemicals. Vanillin (4-hydroxy-3-methoxybenzaldehyde) was purchased from Sigma Chemical Co. (St. Louis, MO). Vanillin derivative, 6-bromine-5-hydroxy-4-methoxybenzaldehyde, designated bromovanin, was provided by Dr Lin Wang (Beijing Institute of Radiation Medicine). Chemical structures are shown in Fig. 1.

Cell culture. Human Jurkat leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37˚C and 5% CO_2 in a humidified incubation chamber.

Cell-cycle analysis. After treatment with bromovanin for certain lengths of time, the cells were harvested and fixed with 75% ethanol. Cells were resuspended in PBS plus 0.1% saponine and 1 μg/ml RNase A (Sigma), incubated for 20 min at 37˚C, and stained with 25 μg/ml propidium iodide (PI) (Sigma). Cell-cycle distribution was then evaluated by flow cytometry (>10,000 cells per sample).

Apoptosis detection. For detecting apoptosis, the treated cells were harvested, and stained simultaneously with multiple fluorescent dyes, Hoechst 33258, fluorescein diacetate (FDA) and PI for 15 min (18). In this assay, FDA (20 μg/ml), a vital dye, was used to stain the cytoplasm of the viable cells; Hoechst 33258 (10 μg/ml) was used to stain the nuclei of viable and apoptotic cells. Normal viable cells were stained an even weak blue under fluorescent microscopy, whilst apoptotic cells were stained bright blue due to chromatin condensation; PI (10 μg/ml) was used to stain necrotic cells or late-stage apoptotic cells. Apoptotic cells were also determined through measuring the sub-G1 population by flow cytometry.

Comet assay. After treatment with bromovanin for certain lengths of time, cells were collected and mixed with low melting point (LMP) agarose at 37˚C. This mixture was placed on the top of the previous layer of 0.5% normal melting point (NMP) agarose on the slide, then covered with a coverslip and returned to 4˚C until solid. The coverslip was gently removed and some NMP agarose was added to the slide. The slide was then covered again with a coverslip and placed at 4˚C until the mixture was solid. The slide was placed in chilled alkaline lysis buffer (for DNA single-strand break detection) or neutral lysis buffer (for DNA double-strand break detection), and subjected to electrophoresis. Thereafter, the slides were gently washed with neutralization buffer, then stained with ethidium bromide, and visualized and analyzed under a fluorescence microscope. DNA damage was expressed as tail moment combining comet tail length and the proportion of DNA migrating into the tail.

DNA fragmentation detection. Jurkat cells were treated with different concentrations of bromovanin for 24 h. The cells were harvested and lysed and the genomic DNA was isolated using the Genomic DNA purification kit (Promega). The DNA fragments were subjected to 2.0% agarose gel electrophoresis, stained with ethidium bromide and observed under UV light.

Immuno-hybridization analysis. The cells were harvested and washed twice in ice-cold phosphate-buffered saline. Cell pellets were treated with the lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1% Noridet P40, 0.5% sodium deoxycholate, 150 mmol/l NaCl, 1 piece of protease inhibitor cocktail tablet in 50 ml solution; Roche Co., USA). The total protein (20-40 μg) was resolved on SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane for Western blotting. Immuno-hybridization was performed with anti-DNA-PKcs antibody (#H-163; Santa Cruz Biotechnology), or anti-γH2AX antibody (#868p407c; NeoMarkers)/anti-actin antibody (#SC1616; Santa Cruz Biotechnology)/anti-Akt antibody (#9272; Cell Signaling Technology)/anti-phosphorylated Akt (Ser437) antibody (#4051; Cell Signaling Technology)/anti-caspase-3 antibody (#E-8; Santa Cruz Biotechnology)/anti-GSK3ß antibody (#9332; Cell Signaling Technology)/anti-phosphorylated GSK3ß antibody (#9336; Cell Signaling Technology). The secondary antibody, IgG-HRP, was purchased from Zhongshan Co. (Beijing, China). Protein expression levels were detected using luminal analysis reagents (Santa Cruz Biotechnology) according to the manufacturer's instructions.

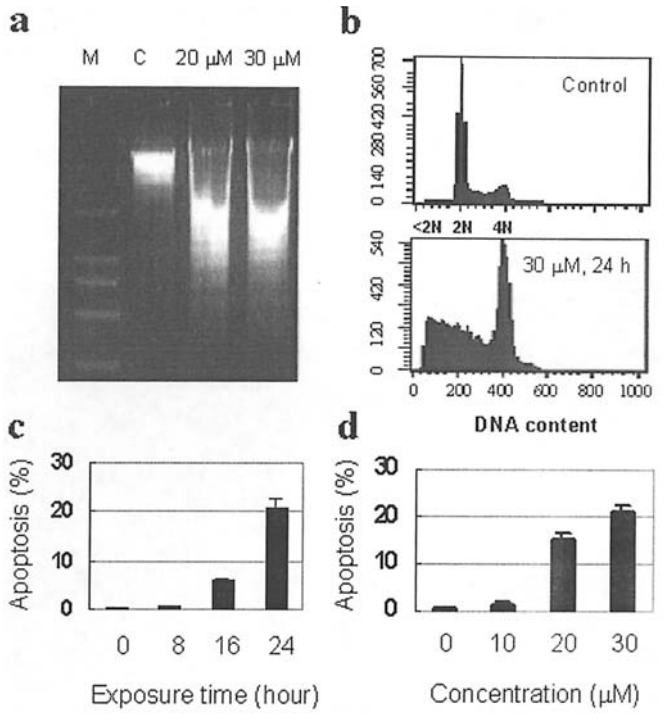

Figure 2. Bromovanin-induced apoptosis as demonstrated by genomic DNA fragmentation assay (a) and flow cytometry (b-d). (a) Genomic DNA was extracted from cells treated with 0-30 μm bromovanin for 24 h and analyzed by electrophoresis on a 2% agarose gel; (b) flow cytometry was performed to assess the apoptotic cells (sub-G1 population or '<2N') induced by 30 μm bromovanin treatment; (c) the percentage of apoptotic cells (sub-G1) after different times of 30 μm bromovanin treatment; (d) the percentage of apoptotic cells (sub-G1) detected at 24-h treatment with different concentrations of bromovanin.

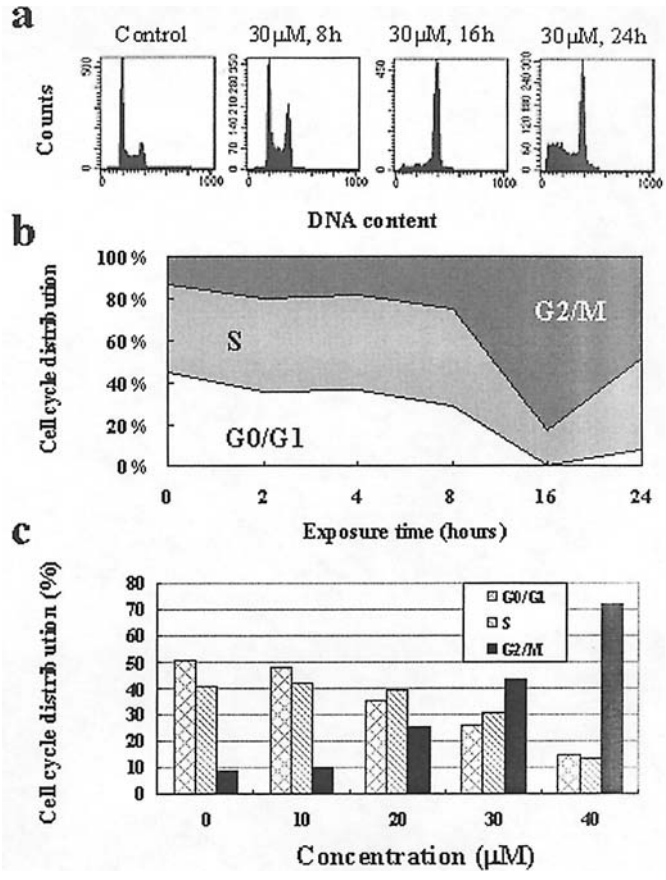

Figure 3. Cell-cycle analyses of Jurkat cells treated with bromovanin by flow cytometry. (a) Histograms of flow cytometry; (b) the distribution changes of G0/G1, S and G2/M phase cells at different exposure times with 30 μM of bromovanin; (c) percentage of cells in G2/M phase detected at 24-h treatment with different concentrations of bromovanin.

Results

Bromovanin-elicited apoptosis. The chemical structure of bromovanin is shown in Fig. 1a. Bromovanin-induced apoptosis of Jurkat cells was first detected by means of fluorescent dye staining. In this assay, the nuclei of apoptotic cells was stained bright blue with Hoechst 33258 due to chromatin condensation. Apoptotic cells, characterized as shrunk or wrecked nuclei, were observed in bromovanin-treated cells (Fig. 1b). The time-course of apoptosis induced by 40 μm bromovanin was shown in Fig. 1c. The induction of apoptosis at 24-h treatment of bromovanin was shown in a dose-dependent manner (Fig. 1d).

Bromovanin-elicited apoptotic cell death of Jurkat cells was also demonstrated by endonucleolytic cleavage of genomic DNA (Fig. 2a), a biochemical indicator of apoptosis, and increased sub-G1 population of flow cytometry assay (Fig. 2b and c).

G2/M arrest of Jurkat cells by bromovanin. Flow cytometry was performed on bromovanin-treated Jurkat cells for cell-cycle analysis. As shown in Fig. 3, bromovanin resulted in a substantial accumulation of G2/M population, whereas the G1 population was dramatically decreased. The amount of G2/M phase cells reached the highest level at 16-h treatment of 30 μM bromovanin (Fig. 3a and b), and then decreased possibly due to apoptotic cell death, which was expressed by the increased sub-G1 population at the 24 h-time point (Fig. 3a). The accumulation of G2/M occurred in a dose-dependent manner (Fig. 3c). In addition, there emerged a great number of aneuploid cells after 24-h treatment, e.g. the ratio of aneuploid reached 26.6% and 60% in 30 μM- and 40 μM-treated cells respectively.

Induction of DNA damage by bromovanin. It is clear that DNA damage is an effective signal to elicit apoptosis and cell-cycle arrest. We investigated the ability of bromovanin to induce DNA damage by single-cell gel electrophoresis (comet assay). We first detected DNA single-strand breaks (SSBs) by alkaline comet assay, and found that treatment with over 10 μM of bromovanin-induced SSBs in a dose-dependent manner (data not shown). DNA double-strand breaks (DSBs) were then measured by neutral comet assay. As shown in Fig. 4a, the amount of DNA DSBs in 40 μM bromovanin-treated cells increased with the exposure time. The yield of bromovanin-induced DNA DSBs after 12-h treatment increased with the drug concentrations (Fig. 4b and c). γH2AX (phosphorylated histone H2AX) has been proved to be a sensitive indicator of DNA DSBs. We then detected the alteration of γH2AX by immuno-hybridization assay. The result indicated that the γH2AX level dramatically increased after 12- to 24-h treatment of bromovanin (Fig. 4d), further suggesting that bromovanin results in DNA DSBs in Jurkat cells.

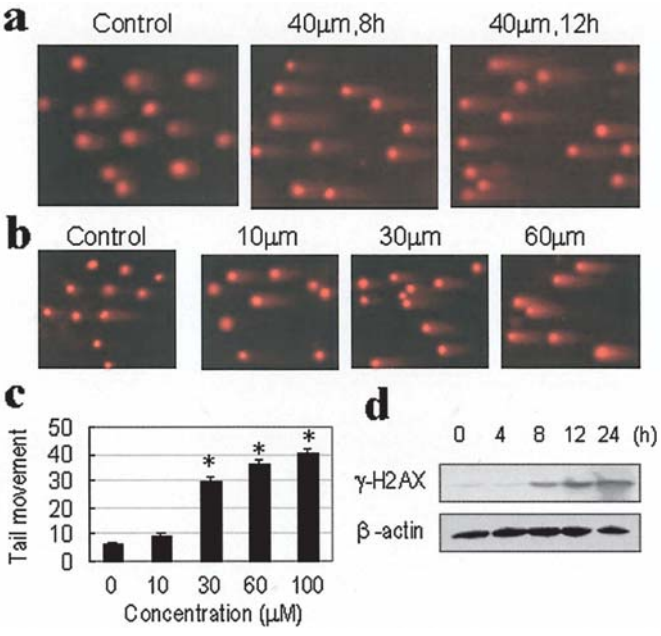

Figure 4. Detection of bromovanin-induced DNA damage. (a) Comet images of DNA double-strand breaks at 8- and 12-h treatment with 40 μm bromovanin; (b) comet images of DNA double-strand breaks after 12-h treatment with different concentrations of bromovanin. (c) Dose-dependent responses of bromovanin-induced DNA double-strand breaks expressed as comet Olive tail movement after 12-h treatment. Mean ± SE, $^{*}P<0.01$ vs untreated cells; (d) immuno-hybridization assay of phosphorylated H2AX (γH2AX).

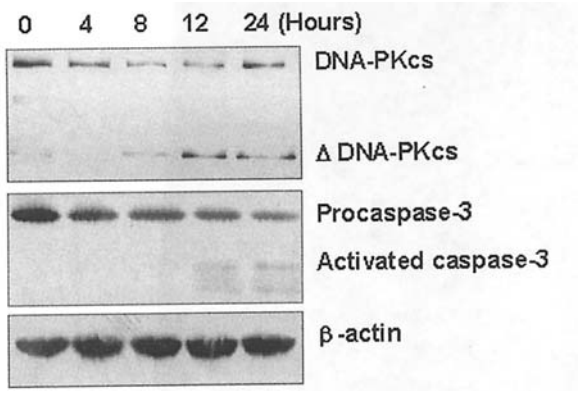

Figure 5. The cleavage of DNA-PKcs and procaspase-3 detected by immuno-hybridization in bromovanin-treated cells. After treatment with 40 μm bromovanin for the indicated amounts of time, the cells were harvested and the cell lysates were prepared and subjected to Western blot analysis.

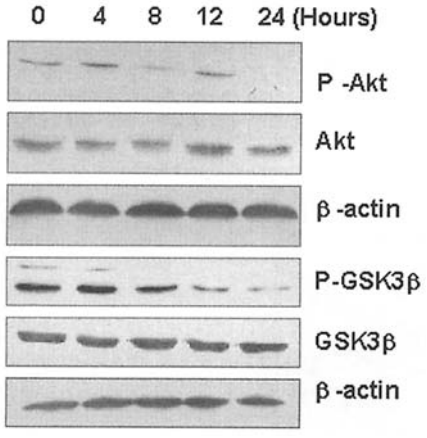

Figure 6. Immuno-hybridization analyses of phosphorylated Akt protein and phophorylated GSK3ß protein. After treatment with 40 μm bromovanin for the indicated amounts of time, the cells were harvested and the cell lysates were prepared and subjected to immuno-hybridization analyses with antibodies recognizing phosphorylated (Ser473) or non-phosphorylated Akt, or antibodies recognizing phosphorylated or non-phosphorylated GSK3ß.

Cleavage of DNA-PKcs and Akt inactivation in bromovanin-treated cells. Bromovanin-induced DSBs might be caused by the enhancement of DNA damage and/or the inhibition of DNA damage repair. There are two distinct but complementary mechanisms for DNA DSB repair, i.e. non-homologous end joining (NHEJ) and homologous recombination (HR), both of which are complicated cascades involving various repair proteins. DNA-PKcs was identified to play an important role in both repair pathways (19). It was reported that vanillin was a potential inhibitor of DNA-PKcs, and the IC_{50} value was ~1.5 mM (11). We observed the alteration of DNA-PKcs protein in bromovanin-treated cells by immuno-hybridization analysis. As shown in Fig. 5, the protein level of full-length 465-kDa DNA-PKcs was obviously decreased in 40 μm bromovanin-treated cells. Meanwhile, there appeared to be a gradually increased fragment of cleaved DNA-PKcs.

It has been reported that DNA-PKcs was cleaved into two fragments of 305 kDa and 160 kDa at its apoptotic cleavage site by the ICE homologue caspase-3, in the cells exposed to agents triggering apoptosis (20,21). So that, we further investigated the potential alteration of caspase-3. Upon treatment with 40 μM bromovanin, the amount of procaspase-3 was reduced, and concurrently 17 kDa of cleaved caspase-3 was yielded (Fig. 5), suggesting that caspase-3 was activated. These results are consistent with the apoptosis observation.

Akt was reported to be a phosphorylated substrate of DNA-PKcs at the Ser473 site. We then measured the level of phosphorylated Akt with the antibody recognizing phosphorylated Akt (Ser473). As shown in Fig. 6, the amount of phosphorylated Akt was visibly reduced in bromovanin-treated cells, whereas total Akt remained unchanged. Furthermore, we detected the phosphorylation level of GSK3ß that is the phosphorylated substrate of the activated Akt protein. The result indicated that phosphorylated GSK3ß also markedly decreased in bromovanin-treated cells, while the level of total GSK3ß remained unchanged. This result is consistent with the alteration of Akt protein.

Discussion

In this report, we have revealed the induction of apoptosis and G2/M arrest of Jurkat leukemia cells by bromovanin (6-bromine-5-hydroxy-4-methoxybenzaldehyde), a novel vanillin derivative. DNA damage was observed in bromovanin-treated cells by means of comet assay. The increased level of phosphorylated histone H2AX, which was proved to be a sensitive indicator of DNA double-strand breaks (22,23), further suggests that bromovanin can result in DNA damage. DNA double-strand break (DSB) is a major threat to cell survival and genomic integrity, and it is also a major lesion signal for eliciting apoptosis and cell-cycle arrest. DNA DSBs

can arise from exposing to exogenous DNA damaging agents, such as ionizing radiation, as well as through endogenous cellular metabolic events, such as recombination, replication stalling or reactive oxygen species (ROS) produced during normal cellular metabolisms. There are two biochemical pathways to repair DNA DSBs in eukaryotic cells, the homologous recombination (HR) and non-homologous end joining (NHEJ) (19). The NHEJ pathway of DNA DSBs is initiated by the DNA-PK complex, which consists of a DNA-PK catalytic subunit (DNA-PKcs), and the Ku DNA-binding subunits, Ku70 and Ku80 (19). A recent study suggested that vanillin could inhibit non-homologous DNA end joining, and switch the pathway of double-strand break repair from NHEJ to HR (11). Furthermore, vanillin promoted toxicant-induced homologous recombination in the proliferative cells of *Drosophila* (24). It is likely that the blockage of NHEJ by vanillin is due to the inactivation of DNA-PKcs (11,12). In this study, we have observed the cleavage of DNA-PKcs in bromovanin-treated cells, suggesting that inactivation of DNA-PKcs by vanillin derivatives might be the consequence of DNA-PKcs degradation. Bromovanin-induced cleavage of DNA-PKcs not only results in the degradation of this protein, but also the yielding of non-functional competitive fragments. For example, the cleaved fragment containing a leucine zipper-like sequence (aa 1503-1538) can interact with the Ku subunits of DNA-PK, and competitively inhibits the activity of DNA-PKcs. We have also measured the levels of Ku70 (another subunit of DNA-PK complex), and found that it remained unaltered in bromovanin-treated cells (data not shown).

In addition to DNA repair reaction, the onset of apoptosis and the operation of cell-cycle checkpoints are another two important cellular responses to DNA damage. On the basis of cell morphology changes, endonucleolytic cleavage of genomic DNA, procaspase-3 expression and cleavage as well as flow cytometry assay, it is clear that bromovanin can effectively elicit apoptosis and G2/M arrest of Jurkat cells. The induction of apoptosis obviously coincided with the cleavage of DNA-PKcs, which was well documented in the process of other DNA damage agent-induced apoptosis (25) or in Fas-mediated apoptosis (20,26).

When examining the apoptotic cells by flow cytometry, we unexpectedly observed a dramatic accumulation of G2/M population in bromovanin-treated cells. This G2/M arrest might be triggered by bromovanin-induced DNA damage. It was reported that DNA-PKcs inhibitor increased the induction of G2/M blockage by the topo II poison agent, etoposide (15). More recently, Holgersson *et al* (27) compared the cell-cycle progression in response to DNA double-strand break-inducing agents between two human glioma cell lines, DNA-PKcs defective M059J and DNA-PKcs proficient M059K. They found that M059J cells, but not M059K cells, displayed substantial G2/M accumulation after 4 Gy low LET γ-ray irradiation. In contrast, high LET ^{14}N ion exposure or bleomycin treatment resulted in G2/M accumulation for both cell lines. However, G2/M blockade remained for a longer time with M059J cells than with M059K cells. They proposed that this distinct cell-cycle block and release was dependent on the complexity of the induced DNA damage as well as the status of DNA-PKcs activity (27). Therefore, we suggest that the G2/M arrest induced by bromovanin might also be associated with the inactivation of DNA-PKcs. In addition, it is worth noting that H2AX was recently reported to be phosphorylated at the M phase in HeLa cells, even without DNA damage (28). We do not exclude the possibility that the increased γH2AX level in bromovanin-treated cells was associated with the accumulation of G2/M phase cells.

Akt protein was reported to be constitutively activated in various human cancers, including leukemia cells (29-31). The major biological consequences of Akt activation are anti-apoptotic and pro-proliferative in cancer cells (32-34), and Akt also plays a role in tumor-induced angiogenesis (35). Our study also showed a high constitutive level of phosphorylated Akt in untreated Jurkat leukemia cells. Considering recent reports that DNA-PKcs phosphorylates Akt protein at the Ser473 site (36,37), we employed an antibody recognizing phosphorylated Akt (Ser473) to detect the level of phosphorylated Akt in Jurkat cells after bromovanin treatment. The result indicated that phosphorylated Akt protein was reduced in bromovanin-treated cells, which in turn led to the decreased phosphorylation level of GSK3ß, which is a substrate of activated Akt (38). It is likely that bromovanin can lead to inactivation of Akt pathway via the cleavage of DNA-PKcs.

In conclusion, we herein demonstrated for the first time that bromovanin (6-bromine-5-hydroxy-4-methoxybenzaldehyde), a novel vanillin derivative, induces apoptosis and G2/M arrest in human Jurkat leukemia cells, which proceeds concurrently with the induction of DNA DSBs, cleavage of DNA-PKcs and procaspase-3, and the inactivation of Akt pathway. Overall, these multiple effects of bromovanin make it very appealing for future development as a novel anticancer drug.

Acknowledgements

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