

Inhibitory Effects of BVAN08 on the Growth of Experimental Tumor Cell Lines Both *in vivo* and *in vitro*

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Abstract

In order to study the mechanism of a novel vanillin derivative BVAN08, provide evidence and experimental data for developing it as a new potential anticancer drug. Hepatic cancer HepG2 cells and normal LO2 cells were used to investigate cytotoxicity of BVAN08. The MTT and colony-forming ability assays showed that BVAN08 significantly sensitized HepG2 cells to radiation rather than LO2 cells. Moreover, BVAN08 inhibited the growth of HepG2 cells in nude mice and exerted no effects on body weight and the number of the peripheral white blood cells. The immunohistochemistry results indicated that the DNA-PKcs expression the BVAN08 group tumor was lower than that of control group. BVAN08 obviously inhibits proliferation of HepG2 cells *in vitro* and *in vivo* supporting it as a promising anticancer drug candidate.

Keywords: Vanillin derivative; Apoptosis; DNA damage; DNA-PKcs; Antiproliferation

Introduction

As a natural phenolic compound, vanillin has been reported to have antioxidant and anti-mutagenic activities, and has also been proved to exert weakly inhibitory roles on the activities of DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [1]. To obtain more information regarding the structure-activity relationship of vanillin derivatives, a series of vanillin derivatives were identified. A vanillin derivative BVAN08 (6-bromine-5-hydroxy-4-methoxybenzaldehyde) selected as a promising candidate compound. BVAN08 displayed obvious inhibitory activity on anti-proliferation, inhibited DNA-PKcs kinase activity and inactivated Akt in human Jurkat leukemia cells [2]. BVAN08 exhibited a potent antiproliferative effect on a broad spectrum of cancer cell lines, but it's antiapoptotic roles demonstrated a large variation on different cancer cell lines. BVAN08-induced cleavage and inactivation of DNA-PKcs was found to occur concurrently with a rapid destruction of c-Myc oncoprotein [3]. Moreover, the induction of DNA damage and spindle disruption by BVAN08, which generates DNA double-stranded breaks and mitotic arrest [4].

DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a key component involves in the non-homologous end joining (NHEJ) pathway of DNA double-strand breaks and has important role in protecting the stabilization of chromosome telomere [5,6]. Many researches indicated that protein levels of DNA-PKcs were highly expressed in many kinds of tumors. So DNA-PKcs was a potential therapeutic target to cure cancer [7]. It was also reported that inhibition of DNA-PKcs was a valid approach to increase the tumor cell killing of some anticancer agents such as cisplatin, Top II poisons and ionizing radiation [7-11].

Previously revealed that BVAN08, exhibited a potent antiproliferative effect on a broad spectrum of cancer cell lines, Herein we further confirmed the BVAN08's significantly antiproliferative effect on HepG2 cells but lower cytotoxic activity on LO2 cells. Meanwhile, our results also demonstrated that BVAN08 resulted in DNA-PKcs cleavage and DNA double-strand breaks. We further found that BVAN08 have the efficiency to induced apoptotic death. By means of molecular image

system, we have found that BVAN08 obviously inhibited the growth of tumor *in vivo*.

Materials and Methods

Chemicals and cell culture

Vanillin derivative BVAN08 (6-bromine-5-hydroxy-4-methoxybenzaldehyde) was provided by Dr. Lin Wang (Beijing Institute of Radiation Medicine). Chemical structures were shown previously [2]. Human liver cancer HepG2 cells and LO2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in a humidified chamber at 37°C in 5% CO₂.

Antibodies

All antibodies were purchased commercially: anti-DNA-PKcs (H-163, Santa Cruz, CA), anti-β-actin (I-19-R, Santa Cruz, CA), anti-Rabbit IgG(H+L)/HRP (ZB-2301, Zhongshan, Beijing, China), anti-Mouse IgG(H+L)/HRP (ZB-2305, Zhongshan, Beijing, China), anti-tubulin (ZM0438, Zhongshan, Beijing, China), anti-γ-tubulin (ab11317, ABCAM), anti-phospho-Histon H2AX(ser139) (mouse monoclonal IgG), Upstar (05-636, Rb IgG(H+L)/FITC-conjugated anti-rabbit IgG (ZF-0311, Zhongshan, Beijing, China, Mo IgG (H+L)/TRITC-conjugated anti-mouse IgG (ZF-0313, Zhongshan, Beijing, China).

MTT Assay

Cells (10⁴ cells/well) were seeded in 200 μl of medium and cultured

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at 37°C for 24 h, then the medium was replaced with fresh media containing testing agents. After further 24 h incubation, 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4 h. The medium was discarded and 150 µl of DMSO was added to each well and incubated for 20 min. The OD at 570 nm was measured and the cell growth inhibitory ratio was calculated *via* following formula and described with cell growth inhibitory curve. The half cell growth inhibitory concentration (IC50) was calculated with the curve. Cell Growth inhibitory ratio=[1-(average absorbance of experimental group/average absorbance of contrast group)] × 100%. The experiments were repeated thrice.

Clonogenic survival

The cells were trypsinized, counted, and diluted to certain concentrations. An appropriate number of cells (3×10^3 to 1×10^4) were plated into 60 mm diameter petri dishes in triplicate, and incubated in the culture medium containing different concentrations of BVAN08 for 24 h, then continuously incubated in the growth medium without BVAN08. After total of 2 weeks culture, cells were fixed with methanol, stained with Giemsa solution, and colonies consisting of more than 50 cells were counted. Resulting survival plots were fitted. All experiments were repeated at 3 times and the mean values for each group were compared using Student's t-test at $P < 0.05$.

Cell-cycle analysis

HepG2 cells and LO2 cells were treated with different concentration BVAN08 for certain time, the cells were harvested and fixed with 75% ethanol. Cell 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

Cells were harvested after BVAN08 treatment for certain time. KGA-212 kit was used to detect apoptosis. Washed in PBS and re-suspended in 500 µl binding buffer at a concentration of 1×10^6 cells/ml. And then 5 µl of annexin-V-FITC and 5 µl PI was added. The tubes were incubated for 20 min at room temperature in the dark. Flow cytometric analysis was performed immediately after supravital staining. The amounts of early apoptosis and late apoptosis/necrosis were determined, respectively, as the percentage of Annexin V+/PI- or Annexin V+/PI+.

Generation of stable cell lines which expressed GFP protein

HepG2 cells were transfected with pEGFPN1 expression plasmid for stable expression of GFP protein. Transfection was performed using Lipofectamin2000 according to manufacturer's instructions. Forty-eight hours later, cells were split in 1:10 in selection medium (complete medium with 0.5 mg/ml G418). After 3-weeks' selection, G418-resistant colonies were expanded in 24-well plates and maintained in medium supplement with 0.1 mg/ml G418. The stable cell lines expressing GFP protein were screened by fluorescence microscope.

Animal and experimental design

Female BALB/c nude mice, 4–6 weeks old and weighing 18–20 g, were supplied by the laboratory Animal Center of the Academy of Military Medical Sciences. They were kept in autoclaved cage with polyester fiber filters to avoid contacting with the pathogens in 12 h light–dark cycle at 22–25°C and were fed with standard laboratory food and water *ad libitum*. Transplantable tumors (induced by HepG2-GFP cells, subcutaneous inoculation of a mixture of 2×10^7 actively growing HepG2-GFP cells in 200 µl PBS to the dorsal side of each nude

mouse) were chopped into fragments (about 1.5 mm³), each of which was transplanted into the right axillary fossa of 24 nude mice. Palpable tumors of volume 100–200 mm³ were developed after 15 days and the mice were randomly divided into 4 groups (with 6 mice per group): negative control group; BVAN08 2, 5, and 10 mg/kg groups. The negative control group received 0.9% normal saline containing 1% DMSO. All of these drugs were injected intraperitoneally every day up to 15 days. Tumor size was measured once every 2 days in two perpendiculars and tumor volume (TV) was calculated using the formula: $(ab^2)/2$, where a and b refers to the longer and shorter dimensions, respectively. The body weight of the animals was measured three times a week at the same time as the tumor dimension measurement and the mortality was monitored daily. The tumor growth inhibition rate was calculated using the formula: inhibition rate (%)=(mean negative control group–mean tumor weight of treatment group)/mean tumor weight of negative control group.

Fluorescence imaging

The tumor size was monitored by Berthold LB983 NightOwl System (NC100, Germany) in expression of EGFP. The excitation source is filtered using an HQ 470 band pass filter (Chroma Technology Corp, Rockingham, VT) and uniformly illuminates the field of view of the mouse. The emission spectrum is filtered using an HQ 525 band pass filter (Chroma Technology) to enhance the GFP fluorescence relative to the autofluorescence signal from endogenous tissue. The fluorescent images of the tumor were taken after tumor transplanted for 7 days, and at first day treatment immediately was taken image, and on days 4, 9, 13 days were totally taken image 3 times, after 15 days treatment we dislodge tumor and take image of fluorescence. The mice were sedated (50 mg/kg pentobarbital by i.p. injection), positioned in the light-tight chamber, and imaged with an exposure time of 100 ms. And the data were analyzed by the WinLight32 software supplied with the instrument.

Immunohistochemistry analysis of DNA-PKcs

The tumors of nude mice from one cell transplantation groups (n=6) and control groups (n=6) were used for immunohistochemistry after treatment period. To perform immunohistochemistry, the tumors were immersed in 10% neutral formalin, and postfixed at room temperature for 48 h. The tumors were then embedded in paraffin. Paraffin sections (2 µm) were obtained with an ultramicrotome (Leica, Germany) and attached to glass slides. The sections (a small portion of sections were dedicated to hematoxylin and eosin (HE) staining) were then incubated overnight at 4°C with DNA-PKcs (polyclonal antibody; Santa Cruz Biotechnology (H-163); 1:500) primary antibodies. After reaction with the primary antibodies, the specimens were washed 3 times with PBS for 5 min and incubated with the corresponding secondary antibodies (horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG) for 20 min in a humid box in the dark at room temperature. Coloration was made with 3, 3'-diaminobenzidine (DAB) solution and the positive product was labeled brown. Slides were rinsed completely and nuclei were counterstained with hematoxylin. Subsequently, specimens were dehydrated and mounted with neutral resin and cover slips for histologic analysis. The cells whose cytoplasmic granules appear brown granules were regarded as positive cells, then, we selected five fields of vision that was magnified 400 times to count the number of positive cells in 100 cells: <5% as negative (-), 6%–25% as positive (+), 26%–50% as moderately positive (++) , and >50% as highly positive(+++). A cytoplasmic staining was considered nonspecific.

Lencocyte counts

At the end of treatment, nude mice were collected blood by vena

then count peripheral blood leucocyte by complete blood count instrument.

Results

Antiproliferative effect of BVAN08 on HepG2 cell lines

Cell viability was assessed by MTT assay. HepG2 cells were treated with BVAN08 for 4 h, 8 h, 12 h, 24 h, and 48 h. The results indicated that 40 μ M BVAN08 inhibited the proliferation of HepG2 cells and 80 μ M BVAN08 could obviously inhibit the proliferation of HepG2 cells in 24 h ($p < 0.01$). Moreover, 40 μ M BVAN08 also obviously inhibited cell proliferation at 48 h

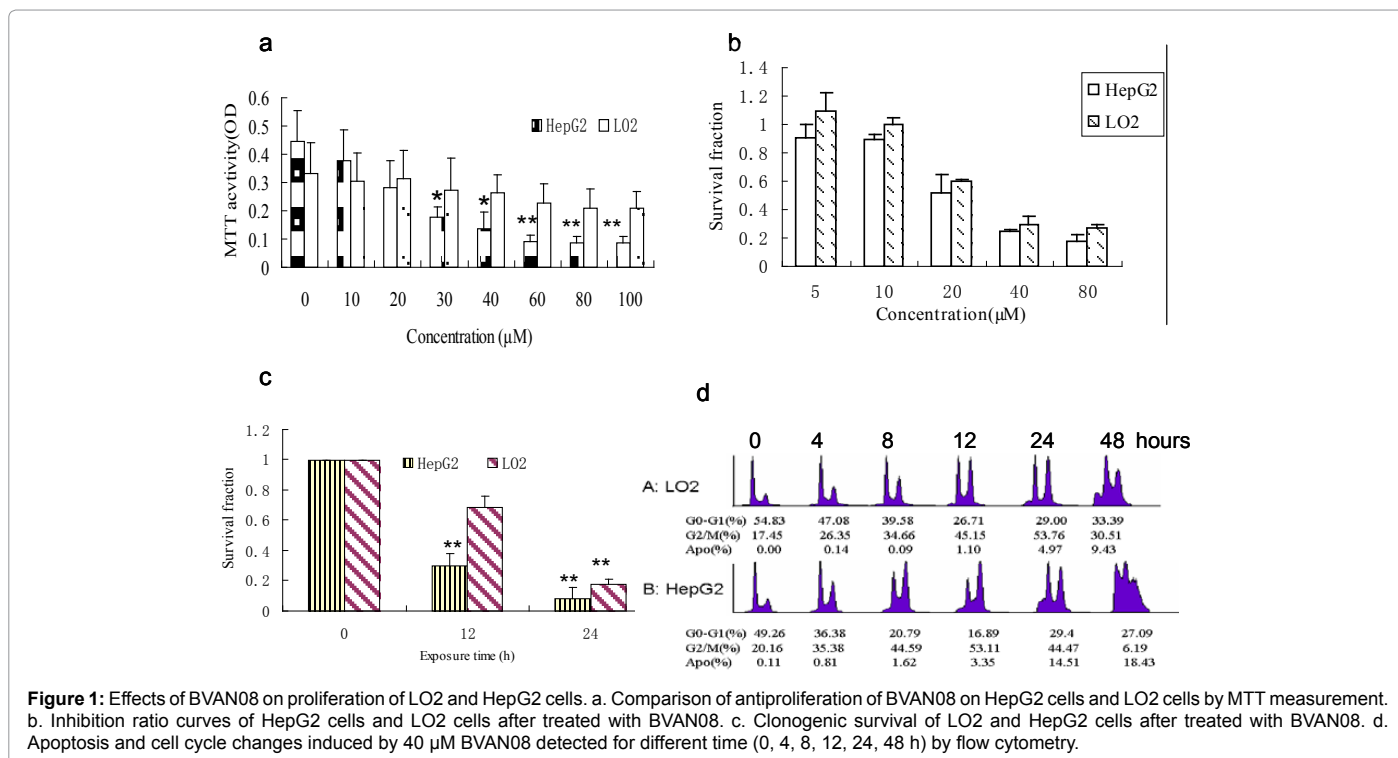
indicated that BVAN08 possessed potent anticancer activity ($p < 0.01$), and the inhibition ratio was in a time-dependent manner of cell proliferation (Table 1). As normal liver cell line the effects of BVAN08 on LO2 cells were weak, even 80 μ M BVAN08 exerted no influence at 48 h which indicated the antiproliferative activity on LO2 cell lines was very minor (Table 2). In addition, we also compared the antiproliferation under various concentrations of BVAN08 at 48 h of HepG2 cells and LO2 cells. As showed in Figure 1a, antiproliferative effect of BVAN08 characterized by a dose-dependent manner both on HepG2 and LO2 cells. The inhibition ratio of HepG2 cells was up to 80% (Figure 1b). The IC50 of BVAN08 was 32.6 μ M, however on LO2 cells was 86.5 μ M (data not shown).

Concentration (μ M)	Culture Time (h)				
	4	8	12	24	48
0	0.443 \pm 0.007				
1	0.492 \pm 0.085	0.493 \pm 0.070	0.473 \pm 0.070	0.505 \pm 0.064	0.479 \pm 0.044
5	0.462 \pm 0.055	0.444 \pm 0.061	0.421 \pm 0.043	0.451 \pm 0.050	0.408 \pm 0.064
10	0.452 \pm 0.072	0.398 \pm 0.062	0.403 \pm 0.064	0.409 \pm 0.031	0.362 \pm 0.013
20	0.406 \pm 0.058	0.445 \pm 0.046	0.510 \pm 0.049	0.520 \pm 0.033	0.495 \pm 0.185
40	0.500 \pm 0.064	0.461 \pm 0.015	0.404 \pm 0.086	0.300 \pm 0.058*	0.231 \pm 0.055**
80	0.479 \pm 0.067	0.428 \pm 0.057	0.372 \pm 0.071	0.180 \pm 0.032**	0.119 \pm 0.015**

Table 1: The effect of vanillin derivative BVAN08 on proliferation of HepG2 cells (OD).

Concentration (μ M)	Culture Time (h)				
	4	8	12	24	48
0	0.419 \pm 0.005				
1	0.448 \pm 0.078	0.418 \pm 0.056	0.426 \pm 0.052	0.446 \pm 0.039	0.411 \pm 0.050
5	0.420 \pm 0.061	0.421 \pm 0.065	0.427 \pm 0.065	0.443 \pm 0.071	0.429 \pm 0.064
10	0.420 \pm 0.056	0.451 \pm 0.059	0.467 \pm 0.044	0.467 \pm 0.421	0.381 \pm 0.040
20	0.412 \pm 0.073	0.456 \pm 0.061	0.459 \pm 0.012	0.429 \pm 0.059	0.464 \pm 0.068
40	0.429 \pm 0.056	0.409 \pm 0.021	0.402 \pm 0.070	0.365 \pm 0.059	0.330 \pm 0.023
80	0.421 \pm 0.052	0.397 \pm 0.063	0.407 \pm 0.061	0.322 \pm 0.045	0.305 \pm 0.006*

Table 2: The effect of vanillin derivative BVAN08 on proliferation of LO2 cells (OD).



The antiproliferative activity of BVAN08 was detected by clone forming analysis experiment. The LO2 and HepG2 cells were pretreated by 40 μ M BVAN08 for 12 and 24 h, after 15 days we analyzed the clone formation. Our results indicated that 40 μ M BVAN08 could inhibit HepG2 cells' proliferation of at 12 h but have no effect on the LO2 cells. Even though the survival rate of LO2 cells was also degraded, it still much higher than that of HepG2 cells (Figure 1c). We indicated that BVAN08 have antiproliferative effect on HepG2 cell lines, but small cytotoxicity on LO2 cells.

Flow cytometry was performed on BVAN08-treated HepG2 cells and LO2 cells for cell-cycle analysis. The results revealed that BVAN08 induced obviously G2/M arrest and in a time-dependent manner. After 48 h the G2/M phase cells of HepG2 cells were decreased. In addition, there emerged a great number of aneuploid cells in HepG2 cells after 40 μ M BVAN08 treated (Figure 1d).

BVAN08-induced apoptosis in HepG2 cells

The apoptosis of HepG2 cells induced by BVAN08 was firstly detected by means of fluorescent dye staining. In this assay, the nuclei of apoptotic cells was stained bright blue with Hoechst 33342 due to chromatin condensation, and the necrosis cells was stained bright red with PI (Figure 2a). The time-dependent induction of necrosis at 60 μ M BVAN08 treatment was observed, but the apoptosis was obviously higher as compared to control. Results of apoptosis and necrosis after 60 μ M BVAN08 treatment for different times were shown in Figure 2b.

BVAN08-elicited apoptotic cell death of HepG2 cells was also

demonstrated by flow cytometry. The early apoptotic cells were marked by Annexin V-FITC (Figure 2c). The results indicated that BVAN08 induced apoptosis in HepG2 cells; the percentage of apoptosis was obviously increased after 72 h treatment (Figure 2d). Our results indicated that BVAN08 inhibited the growth of HepG2 cells, at least in part, by induction of apoptosis.

Fluorescence imaging and *in vivo* tumor growth

HepG2 cells stabilized expressed GFP protein were selected after G418 screening. Under normal light microscopy, the HepG2 cells expressed GFP protein were the same as ordinary HepG2 cells in morphous. Under fluorescent light, cells appeared green demonstrating the cells expressed GFP protein and after 20 generation without G418 screening (data not shown).

The Nightowl molecular light imaging system was used for fluorescent detection of tumor after hypodermic inoculation 7 days. The fluorescence intensities defined as photon per second per millimeter squared (ph/s/mm²) in the tumor. A strong signal showed up on the images 7 days after hypodermic inoculation tumor structure, which suggested that the tumor cells grow rapidly. During the following 13 days, fluorescence upgraded rapidly in control group and it went up slightly in 10 mg/kg group. In the last day of treatment, the tumors in both control group and 10 mg/kg group seems to necrosis (Figure 3a). The fluorescence image of tumor *in vitro* showed that photons in all the three experiment groups were lower than the control group (Figures 3b and 3c).

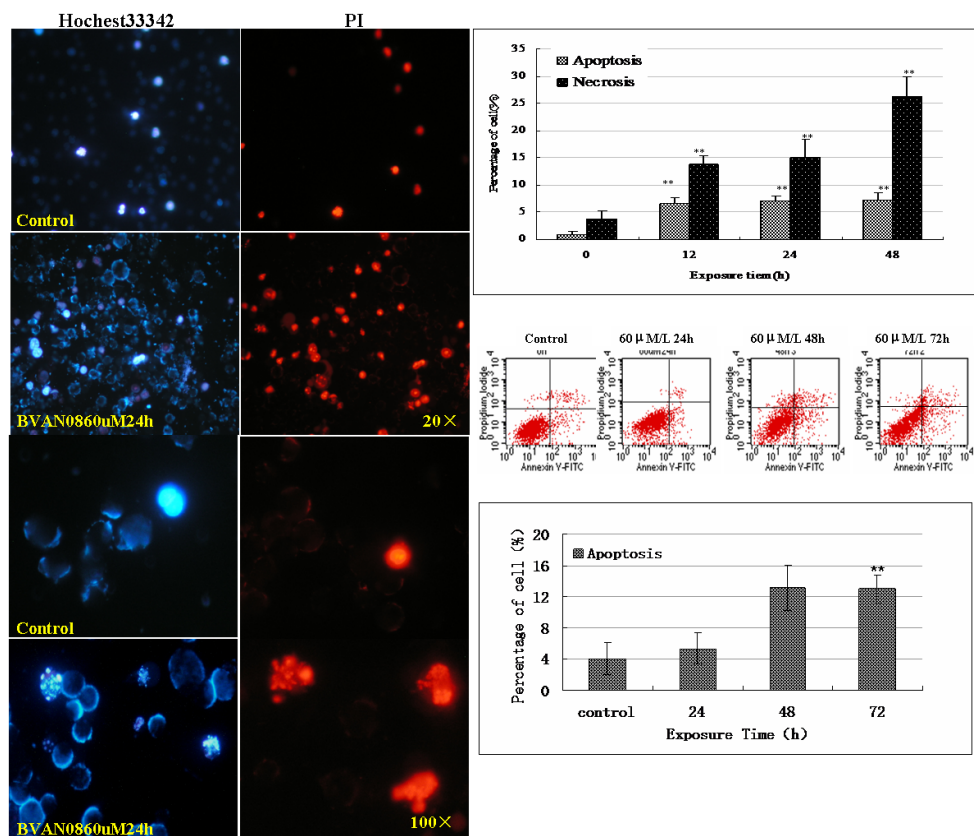


Figure 2: Induction of apoptosis by BVAN08 on HepG2 cells. a. Morphological characteristics of apoptosis displayed under fluorescence microscopy. b. The percentage of apoptosis and necrosis after 60 μ M BVAN08 treated for different times. c. Detection of BVAN08-induced apoptosis by flow cytometry. d. The percentage of apoptosis and necrosis after 60 μ M BVAN08 treated for different times.

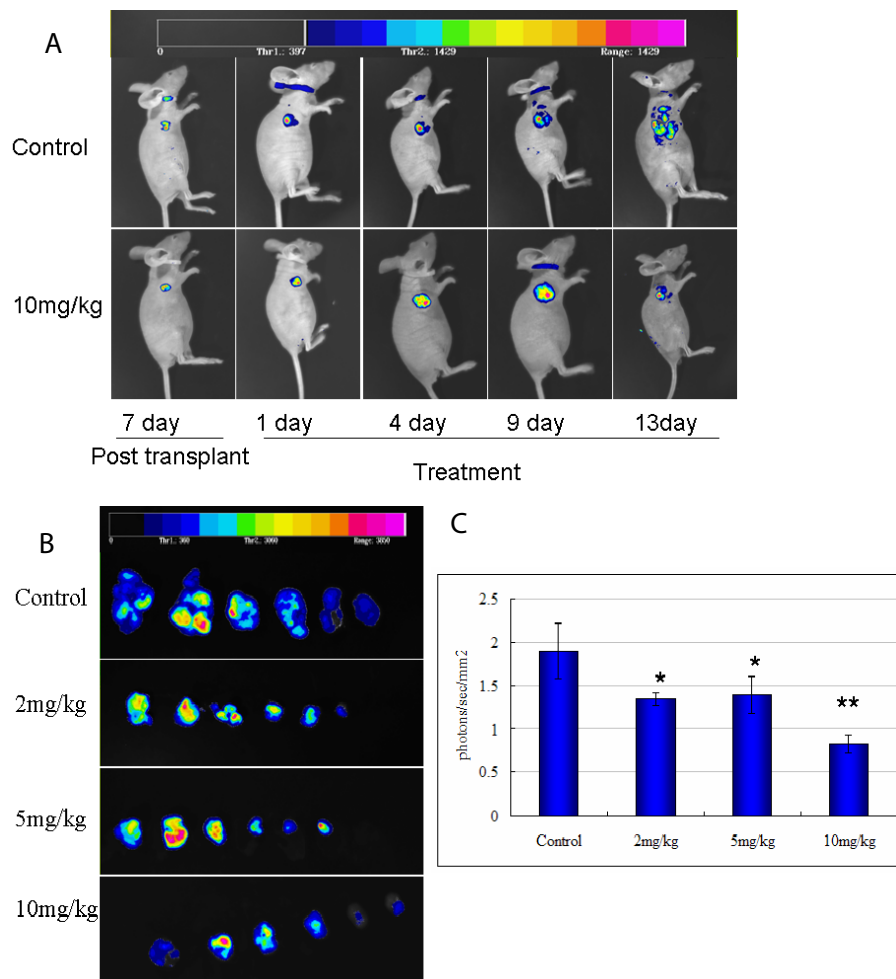


Figure 3: Molecular light imaging system to detected tumor. a. *In vivo* optical imaging of hypodermi tumor. b. *In vitro* optical imaging tumor. c. Quantitative of tumor photons.

BVAN08 inhibits tumor growth in nude mice

The results showed no significant variation in body weight after 15 days of treatment with BVAN08 (Figure 4a), suggesting that BVAN08 produced no significant non-tumor toxicity in tumor-bearing mice.

However, the tumor volume also significantly decreased when compared with non-treated with BVAN08 (Figure 4b). In addition, the average weights of tumors treated with BVAN08 at 2, 5, and 10 mg/kg were 220.4 mg, 191.0 mg, and 229.9 mg respectively, *versus* 531.9 mg of negative control tumors, which correspond to 58.5%, 64.0%, and 56.7% of inhibition (Figure 4c) ($p < 0.01$). The peripheral blood leucocyte had no difference in control group and BVAN08 treated group (Figure 4d). These results suggested that BVAN08 was efficiently capable of inhibiting the tumor growth *in vivo* and no obvious side effects were found.

HE staining showed the nucleus of cells was greatly different and morphous rugosity, nuclus divisions were abnormal indicated that the structures were malignant tumor.

One important requirement in order to ensure a successful therapy using BVAN08 is that DNA-PKcs is low expressed in tumor structure. IHC studies indicating that DNA-PKcs was mainly moderately positive expressed in BVAN08 treated group. However, strong expression was

found in control group, with highly positive expression of DNA-PKcs tumor structure (Figure 4e).

Discussion

The adriamycin is majorly used to cure liver cancer clinically but it has a lot of adverse effects, for this reason, it is important to develop new antineoplastic agents which kill tumor cells specificity and have no harm to normal hepatic cells [12]. The antiproliferative effect of BVAN08 on various cancer cell lines has been investigated [3] and we have previously reported that BVAN08 induced apoptosis of Human Jurkat leukemia cells [2]. In present study, we demonstrated that BVAN08 inhibited proliferation of HepG2 cells, and 40 μ M BVAN08 treated for 24 h could obviously inhibit the growth of HepG2 cells. In contrast, even 80 μ M BVAN08 did not have obvious effect after 48h incubation. The results of clone-formation also indicated that BVAN08 have antiproliferative effects on HepG2 cells but small marginal effects on LO2 cells. Although BVAN08 exhibited very potent cytotoxicity towards HepG2 cells by MTT and clon-formation, it did not exhibited very potent cytotoxicity towards normal human hepatocyte cells LO2, suggesting its tumor-selective growth inhibitory effect.

Mammalian cells initiate cell cycle arrest at different phases of the cell cycle in response to various forms of genotoxic stress to allow time

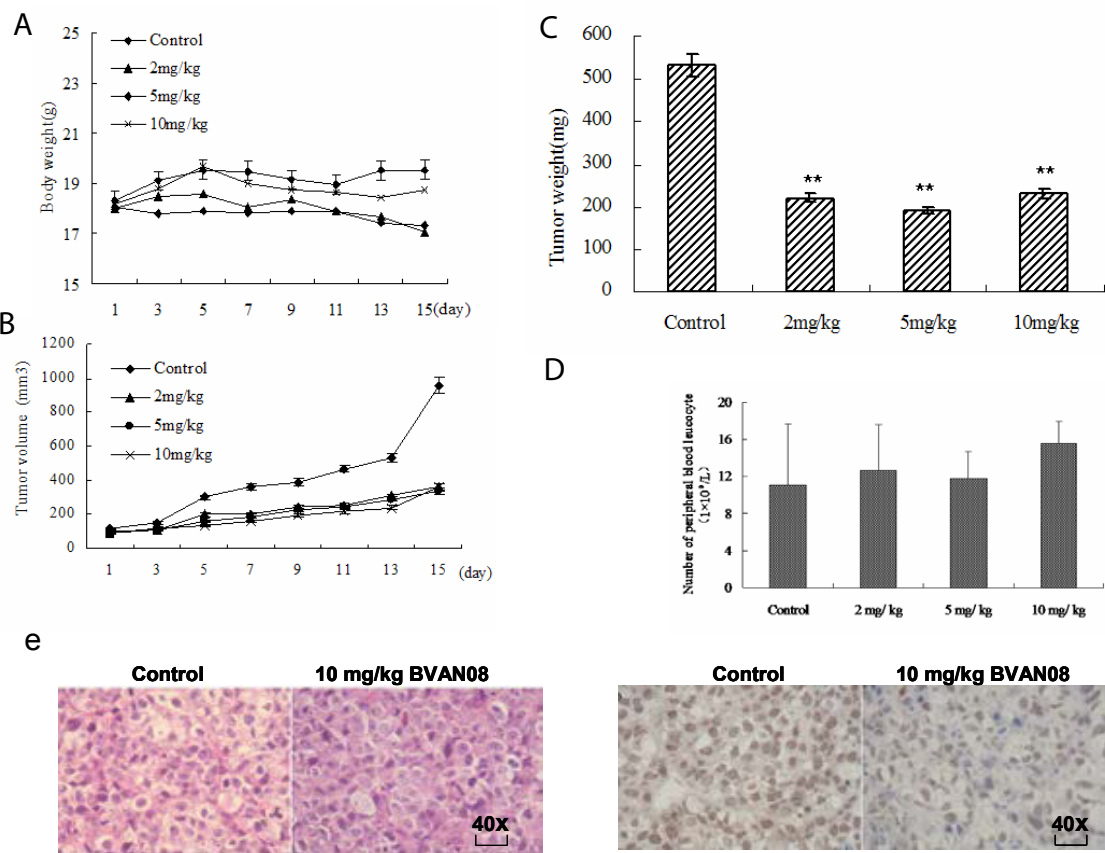


Figure 4: The *in vivo* anti-tumor effect of BVAN08 on athymic nude mice bearing HepG2 cells subcutaneously was studied. a. Body weight of control and BVAN08 treatment group. b. Tumor volume of control BVAN08 treatment groups. Tumor volume was measured once every 2 days. c. The weight of tumor of control BVA08 treatment groups. After nude mice were treated for 15 days, the tumors were isolated and weighed. *P<0.05 and **P<0.01 compared with control. d. The peripheral blood leucocyte of control and BVAN08 treatment group. e. H&E stain to identify tumor of HepG2 cell and immunohistochemical analysis of DNA-PKcs.

for DNA repair, and thus preserving their genomic integrity. In this study we examined the cell cycle by flow cytometry, and we observed a dramatic accumulation of G2/M population in BVAN08-treated HepG2 cells and LO2 cells. The G2/M arrest of HepG2 cells were decreased after BVAN08-treatment for 48 h, the main reason was that a number of cells died after so long time arrest, and accumulation of subG1 population of cells in HepG2 cells was higher than that of LO2 cells. Consequently, the BVAN08-induced HepG2 cells apoptosis were detected by means of fluorescent and flow cytometry, and apoptosis was characterized by Annexin-V staining. Our results showed that BVAN08 could dose-dependently induce apoptosis. Many researchers reported that some antitumor drugs induced a lot of kinds of cell death such as apoptotic, mitotic catastrophe, autophagy, and necrosis [13]. Our study showed not so much apoptosis in BVAN08-treated HepG2 cells, but the inhibition ration was up to 80%, so we supposed that BVAN08 may induce another type of cell death.

A super cooled charge coupled device (CCD) camera within the imaging systems is capable of detecting low quantities of light from beneath the tissue [14]. This technology provides an alternative method to histological evaluation to determine the location of tumor cells. Utilizing the fluorescence of EGFP expressed in the cells, we were able to demonstrate distribution of tumor cells. Quantification of cell growth was accomplished using the molecular light imaging system. These findings demonstrate that GFP-transfected cells may be useful

for imaging studies of superficial tumors where both excitation and emission wavelengths are able to penetrate tissue.

The fluorescence image of tumor *in vitro* also showed up that BVAN08 inhibit tumor growth, suggesting that BVAN08 had anti-cancer effect. The results from animal experiments also showed that after 15 days of treatment with BVAN08, no significant variation in body weight and peripheral blood leucocyte counts were observed, suggesting that BVAN08 did not produce significant non-tumor toxicity in tumor-bearing mice. We demonstrated the inhibition of proliferation of HepG2 cell growth *in vivo* with a corresponding reduction in the tumor volume and weight of xenografted tumor in nude nice with BVAN08 treatment. All these results show that BVAN08 is a potentially promising anti-cancer drug candidate with low toxicity and very little side effects. 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 [15]. The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) has a critical role in double-strand break repair *via* nonhomologous end-joining and regulates cellular responses to DNA double strand breaks [16]. In immunohistochemical studies, DAN-PKcs positive cells were detected at a high frequency in tumor tissues of control group, but low in 10 mg/kg treated group. These results suggested that BVAN08 inhibited tumor growth in tumor-bearing mice by down-regulating expression of DAN-PKcs protein expression in tumor tissues of nude mice. BVAN08

might decrease the adverse reaction and improve the efficacy of cancer treatment.

In summary, our results showed that BVAN08 could inhibit the process of tumorigenesis through DNA-PKcs, and indicated that BVAN08 remarkably exerted potent inhibitory effects on the HepG2 cell growth by inducing apoptosis. These results suggested that BVAN08 could be developed as a promising chemotherapeutic agent in liver cancer therapy in the future.

Acknowledgments

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