Original Paper

B4G2 Induces Mitochondrial Apoptosis by the ROS-Mediated Opening of Ca²⁺-Dependent Permeability Transition Pores

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Key Words

23-hydroxybetulinic acid derivative • HepG2 cells • ROS • PT pore • Mitochondrial pathway

Abstract

Background/Aims: Hepatocellular carcinoma (HCC) is the most common type of liver cancer. At present, only sorafenib is approved to treat HCC. In this study, we found that a 23-hydroxybetulinic acid derivative, B4G2, exhibited potent antiproliferative activity in HCC cell lines. **Methods:** We used four HCC cell lines (HepG2, HepG2/ADM, Hep3B and Bel-7402) to evaluate the anti-tumour activity and explore underlying mechanisms by which B4G2 induces apoptosis. **Results:** Among these cell lines, HepG2 showed the highest sensitivity to B4G2. HepG2 cells treated with B4G2 showed a depolarized mitochondrial membrane potential, released cytochrome c, activated caspase-9 and caspase-3 and cleaved poly ADP-ribose polymerase (PARP). However, Z-VAD-FMK, a pan-caspase inhibitor, did not attenuate B4G2-induced apoptosis, implying that the induction of mitochondrial apoptosis by B4G2 may be independent of caspases. Moreover, pre-treatment with MgCl₂, a blocker of Ca²⁺-dependent permeability transition (PT) pores, attenuated the depolarization of the mitochondrial potential and decreased the population of apoptotic cells, indicating that B4G2-induced apoptosis was partly dependent on the opening of the Ca²⁺-dependent PT pores. B4G2 also increased the levels of intracellular calcium and reactive oxygen species (ROS). Furthermore, an ROS scavenger, N-acetyl-cysteine (NAC), markedly decreased the accumulation of intracellular calcium and apoptosis. **Conclusion:** This is the first demonstration that B4G2 inhibits the growth of HCC cells and induces mitochondrial apoptosis in hepatocellular carcinoma cells by the ROS-mediated opening of Ca²⁺-dependent permeability transition pores.

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Introduction

Liver cancer is the fifth most common malignancy, affecting approximately one million people around the world every year and is ranked second among all cancer-related deaths, causing more than 690,000 deaths annually [1]. China has the highest liver cancer incidence in the world, accounting for 45% and 53% of the worldwide morbidity and mortality, respectively [2, 3]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis, which is mainly induced by alcoholism [4]. A number of classical anticancer drugs, interferons and hormone drugs as well as some cytotoxic compounds, such as adriamycin are currently used for the treatment of HCC. However, most of these drugs show poor treatment efficacies with strong toxic and side effects [5]. Currently, only sorafenib, a tyrosine kinase inhibitor that has been shown to significantly block tumour growth, is approved for patients with advanced HCC [6]. Therefore, the development of novel chemotherapeutic agents is urgently needed.

23-Hydroxybetulinic acid (23-HBA) is a lupane-type pentacyclic triterpene from the Chinese medicinal herb *Pulsatilla chinensis* and has been found to exhibit antitumour activity *in vitro* and *in vivo* [7-10]. Several reports have indicated that the antineoplastic mechanisms of 23-HBA may be associated with depolarizations of the mitochondrial membrane potential and subsequent cell apoptosis [11-13]. 23-HBA has been shown to induce apoptosis in HL-60 cells *via* the inhibition of Bcl-2 expression and telomerase activity [14]. Additionally, 23-HBA is known to induce autophagic cell death in HL-60 cells *via* the up regulation of Beclin-1 expression [15]. Recently, we reported that 23-HBA and its bipiperidinyl derivatives reverse ABCB1-mediated multidrug resistance by simultaneously increasing the accumulation and decreasing the efflux of anti-cancer drugs [16-18]. Until now, there have been no report on the anti-hepatoma activity of 23-HBA. Given that 23-HBA has weak anticancer activity, our effort to improve its antitumour activity by structural modification has yielded a series of derivatives with enhanced bioactivity [19-22]. B4G2, a derivative of 23-HBA, was previously synthesized by our group and showed a stronger anticancer activity than 23-HBA in four cancer cell lines in preliminary activity screening [20]. The present study evaluated the anti-hepatoma activity of B4G2 and its underlying molecular mechanisms. Our results demonstrated that B4G2 inhibits the growth of HCC cells and induces a reactive oxygen species (ROS)-mediated accumulation of intracellular calcium, which subsequently opens Ca^{2+} -dependent permeability transition (PT) pores and leads to mitochondrial apoptosis.

Materials and Methods

Reagents

B4G2 (purity >98%, Fig. 1A) was synthesized as previously described [19]. B4G2 was dissolved in DMSO to give a stock solution of 50 mM and stored at -20°C . An anti-caspase-8 antibody, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI) and 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride hydrate bisbenzimidazole (Hoechst 33258) were purchased from Sigma (St. Louis, MO, USA). An Annexin V-FITC/PI staining assay kit was obtained from Trevigen, Inc. (Gaithersburg, MD, USA). We purchased 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and N-(4-(6-((acetyloxy)methoxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)-2-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxyethyl)amino)-5-methylphenoxy)ethoxy)phenyl)-N-(2-((acetyloxy)methoxy)-2-oxyethyl)-(acetyloxy) methyl ester (Fluo-3-AM) from Invitrogen (Eugene, OR, USA). Z-Val-Ala-Asp-CH₂F (Z-VAD-FMK) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Antibodies against caspase-3 and caspase-9 were purchased from Cell Signalling Technology (Beverly, MA, USA). We purchased 2', 7'-dichlorofluorescein diacetate (H2DCFDA) and antibodies against Bcl-2, Bax and PARP from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against cytochrome c was obtained from Epitomics (Burlingame, CA, USA). N-acetyl-L-cysteine (NAC) was purchased from Qiyun Biotechnology Company (Guangzhou, Guangdong, China).

Cell lines and cell culture

The HCC cell lines HepG2 and Hep3B were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The HCC cell line Bel-7402 and the human hepatocyte cell line LO2 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Xuhui, Shanghai, China). The HCC multidrug-resistant cell line HepG2/ADM was kindly provided by Prof. Kwok-Pui Fung (The Chinese University of Hong Kong). The HepG2, HepG2/ADM, Hep3B and Bel-7402 cell lines were maintained in RPMI 1640 medium (Gibco, NY, USA) containing 10% (v/v) foetal bovine serum (FBS, Gibco, NY, USA) and 1% (v/v) penicillin-streptomycin (PS; 10,000 U/mL, Gibco, NY, USA) at 37°C in a 5% CO₂ atmosphere. The HepG2/ADM cell line was cultured with doxorubicin (1.2 µM) to maintain the multidrug resistance.

MTT assay in vitro

The cytotoxicity of B4G2 in the HCC cells (HepG2, HepG2/ADM, Hep3B and Bel-7402) was measured using the MTT assay as previously described [23]. Cells were seeded in 96-well microplates at a density of 5000 cells/well and cultured for 24 h. The cells were then treated with various concentrations of B4G2 for 24, 48 and 72 h. MTT (30 µL; 5 mg/mL) was then added to the cells, and the cells were incubated for an additional 4 h. The medium was removed and 100 µL of DMSO was added to each well. The optical density was measured at 595 nm using a DTX 880 microplate reader (Beckman Coulter, CA, USA).

Colony formation assay

HepG2 cells were seeded in 6-well microplates at a density of 300 cells/well and cultured for 24 h. The cells were then treated with various concentrations of B4G2 for 48 h and maintained in fresh medium in a humidified atmosphere of 5% CO₂ at 37°C for 10 days. The cells were then fixed in methanol at -20°C for 10 min and stained with 1% crystal violet for 20 min. After being washed twice with phosphate-buffered saline (PBS), visible colonies were manually counted.

Cell cycle analysis

Cell cycle distribution was analysed as described previously [24]. Briefly, HepG2 cells were treated with or without B4G2 at various concentrations, collected, washed twice with PBS and fixed in ice-cold 70% (v/v) ethanol at 4°C overnight. After centrifugation at 2500 rpm, cell pellets were resuspended in 1 mL PBS containing 0.02 mg/mL PI and 0.1 mg/mL RNase A, and the samples were incubated at 37°C for 30 min in darkness. DNA content was measured by Epics XL Flow Cytometry (Beckman Coulter) (Ex=488 nm and Em=620 nm).

Hoechst 33258 staining

HepG2 cells treated with or without B4G2 for 48 h were stained with Hoechst 33258 (10 mg/mL in PBS) for 30 min at 37°C in darkness and then washed twice with PBS. Cell morphology was observed using a KX41 fluorescence microscope (Olympus, Tokyo, Japan) (Ex=350 nm and Em=460 nm).

Ultrastructural observation by transmission electron microscopy

HepG2 cells treated with B4G2 were first fixed in ice-cold 4% glutaraldehyde at 4°C overnight. The cells were then washed twice with PBS, post-fixed in 1% osmium tetroxide for 1 h and dehydrated in a graded series of ethanol and acetone. The cells were then polymerized by epoxy resin. Ultra-thin sections were cut and stained with aqueous uranyl acetate and lead citrate. Finally, the ultrastructure of the cells was examined using a JEM 1400 transmission electron microscope (Philips, Eindhoven, Netherlands).

Annexin-V-FITC/PI staining assay

HepG2 cells were stained with 500 µL of labelling solution containing 5 µL Annexin-V-FITC and 10 µL PI for 15 min at room temperature in darkness. The fluorescence of the sample was detected by Epics XL Flow Cytometry (Beckman Coulter) (Ex=488 nm and Em=525 nm for Annexin V-FITC; Ex=488 nm and Em=620 nm for PI).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in the mitochondrial membrane potential were detected using JC-1, a fluorescent dye that accumulates in mitochondria, as previously described [23, 24]. Cells with high mitochondrial membrane

potential were indicated by red fluorescence emitted from J-aggregates, whereas cells with depolarized mitochondria were detected by the green fluorescence emitted from JC-1 monomers. HepG2 cells treated with or without B4G2 at various concentrations were collected by trypsinization, and then washed twice with PBS. After centrifugation at 2500 rpm, cell pellets were resuspended in 500 μ L of JC-1 solution (5 μ M JC-1 in PBS) and incubated for 15 min at 37°C in darkness. The mitochondrial membrane potential was measured by Epics XL Flow Cytometry (Beckman Coulter, USA) (Ex=488 nm and Em=575 nm for J-aggregates; Ex=488 nm and Em=525 nm for JC-1 monomers).

Measurement of intracellular calcium level and ROS

For the observation of intracellular calcium level, HepG2 cells were seeded in 6-well plates and incubated overnight for attachment. Cells were treated with B4G2 (4 μ M) for 0.5, 1 and 4 h. After being washed three times with PBS, cells were incubated with Fluo-3-AM (10 μ M) for 30 min at 37°C in darkness. The cells were then washed with PBS after the Fluo-3-AM was removed. The green fluorescence was observed using a KX41 fluorescence microscope (Olympus, Japan) (Ex=488 nm and Em=535 nm). For the quantification of intracellular calcium levels and ROS, HepG2 cells were seeded in black 96-well microplates (transparent bottom) at a density of 20,000 cells/well and cultured with B4G2 for specified time intervals. The cells were then treated with Fluo-3-AM (10 μ M) or H2DCFDA (10 μ M) for 30 min at 37°C in darkness. The intensity of the fluorescence was measured using a DTX 880 microplate reader (Beckman Coulter, USA) (Ex=488 nm and Em=535 nm).

Western blotting

The preparation of total protein lysates and western blot analyses were performed as previously described [24]. HepG2 cells were collected by trypsinization and washed twice with ice-cold PBS. Total cellular protein was extracted using RIPA lysis buffer (1% Nonidet-P40, 0.5% sodium deoxycholate and 0.1% SDS in PBS) with 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 $\times$ phosphatase inhibitor and 1 $\times$ protease inhibitor on ice for 30 min. The lysates were centrifuged at 14,000 rpm for 10 min to obtain supernatants. For cytosol fraction preparation, cells were treated with cytoplasmic protein lysis buffer (10 mM HEPES, pH=7.9; 10 mM KCl; 0.1 mM EDTA; and 0.4% Nonidet-P40) containing 1 mM PMSF, 1 $\times$ phosphatase inhibitor and 1 $\times$ protease inhibitor on ice for 30 min. After centrifugation at 14,000 rpm for 1 min at 4°C, the supernatant was collected as cytoplasmic protein. The protein concentration was quantified using a BCA assay kit (Thermo Scientific, USA). Proteins (30-50 μ g) were resolved on SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk or bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris and 150 mM NaCl, pH 7.6) with 0.05% Tween-20 (TBS-T) and probed overnight with primary antibodies at 4°C. The membranes were then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) at room temperature for 1 h. An enhanced chemiluminescence kit (Thermo Scientific, MA, USA) and an X-ray film processor (Kodak X-102, NY, USA) were used to visualize the bands.

Statistical analysis

Each experiment was performed at least three times, and the results are represented as the mean $\pm$ standard deviation (SD). GraphPad Prism 4.0 was used for statistical analyses. A difference was considered significant when $P < 0.05$.

Results

B4G2 inhibits the growth of HCC cells

Four HCC cell lines, HepG2, HepG2/ADM, Hep3B and Bel-7402, were used to evaluate the cytotoxicity of B4G2. As shown in Table 1, B4G2 significantly reduced the cell viability of the four HCC cell lines. HepG2 cells were sensitive to B4G2, which inhibited the survival of HepG2 cells in a concentration-dependent manner at 48 and 72 h (Fig. 1B). B4G2 also had an inhibitory effect on the viability of HepG2/ADM cells, in which doxorubicin was ineffective (Table 1). B4G2 showed lower toxicity to normal cell line LO2 than the human cancer cells (Table 1). The colony formation assay also confirmed that B4G2 inhibited the proliferation

Table 1. Cytotoxicity of B4G2 towards four hepatoma carcinoma cell lines and the normal cell line LO2 (72 h).^a23-HBA (23-hydroxy betulinic acid) was used as the positive control

	HepG2	HepG2/ADM	IC ₅₀ (μM) Hep3B	Bel-7402	LO2
B4G2	3.20 ± 0.43	3.40 ± 0.39	3.24 ± 0.25	3.31 ± 0.74	9.45 ± 0.99
23-HBA ^a	32.44 ± 3.76	45.84 ± 3.66	43.96 ± 3.36	38.16 ± 3.37	48.69 ± 3.62

Fig. 1. (A) The chemical structure of B4G2. (B) The inhibitory effect of B4G2 on the viability of HepG2 cells. HepG2 cells were treated with different concentrations of B4G2 for 24, 48 and 72 h. Cell viability was measured by MTT assay. (C) The inhibitory effect of B4G2 on the colony formation of HepG2 cells. HepG2 cells were treated with different concentrations of B4G2 for 48 h. Clonogenic survival of HepG2 cells after B4G2 treatment was measured by the number of clones capable of anchorage-dependent growth.

of HepG2 cells in a concentration-dependent manner (Fig. 1C). We next investigated the molecular mechanisms underlying B4G2-induced cell death in HepG2 cells.

B4G2 induces apoptosis in HepG2 cells

To determine whether B4G2-induced cell death was related to cell cycle arrest, a cell cycle analysis was performed. As shown in Fig. 2A, HepG2 cells treated with different concentrations of B4G2 resulted in the accumulation of cells in the sub-G₁ phase of the cell cycle in a concentration-dependent manner at 48 h. Because the sub-G₁ phase of cell cycle may be attributed to the induction of either apoptosis or necrosis, morphological changes were examined to determine whether B4G2 induced apoptotic cell death. After treatment with B4G2 for 48 h, HepG2 cells showed both cell shrinkage and chromatin condensation as indicated by strong bright blue fluorescence, whereas control cells showed normal morphologies with weak blue fluorescence (Fig. 2B). Additionally, a few specific apoptotic characteristics, including cell volume reduction, plasma membrane blebbing, nuclear membrane shrinkage, chromatin condensation, chromatin margination and nuclear fragmentation, were observed by transmission electron microscopy in B4G2-treated HepG2 cells (Fig. 2C). The quantification of apoptotic cells by the Annexin V/PI double staining assay also showed that the B4G2 treatment led to significant increases in cell populations in the early apoptotic phases, ranging from 25.0% to 34.5% compared with a 3.8% increase in the vehicle-treated control (Fig. 2D). Collectively, these data indicated that B4G2 induces apoptotic cell death in HepG2 cells.

Fig. 2. B4G2 induces apoptosis in HepG2 cells. (A) B4G2 triggers the accumulation of HepG2 cells in the sub-G1 phase of the cell cycle. Cells were stained with PI (0.02 mg/mL). Cell cycle distribution was determined by flow cytometry. (B) Morphological changes of HepG2 cells after B4G2 treatment for 48 h. Cells were stained with Hoechst 33528 (10 mg/mL) and then observed under a fluorescence microscope. Apoptotic cells with strong blue fluorescence were observed (as indicated by the arrow). Original magnifications: $\times 100$; scale bar: 200 μ m. (C) Ultrastructural observation of HepG2 cells by transmission electron microscopy after B4G2 treatment for 48 h. Original magnifications (from left to right): $\times 8900$, $\times 8900$ and $\times 8900$; scale bar (from left to right): 2 μ m, 5 μ m and 2 μ m. (D) Apoptotic cells as a result of B4G2 treatment were quantified by the Annexin V/PI assay. Cells were stained using an Annexin V/PI staining kit and then detected by flow cytometry.

B4G2 induces apoptosis through the mitochondrial apoptotic pathway

It is well-known that apoptosis is primarily triggered through two signalling pathways: the death receptor pathway and the mitochondrial pathway. To explore the mechanisms of apoptosis induction by B4G2, several key molecules of the two apoptotic pathways were investigated. The activation of caspase-8, which occurs when apoptosis is triggered by death receptors, was not observed in B4G2-treated HepG2 cells (Fig. 3A). Instead, the treatment with B4G2 led to the activations of caspase-9 and caspase-3, as evidenced by the

Fig. 3. B4G2-induced apoptosis is mediated by the caspase-independent mitochondrial apoptotic pathway. (A) Effect of B4G2 on caspase-3, caspase-9, caspase-8 and PARP expression levels. Total cell lysate from HepG2 cells treated with B4G2 at the indicated concentrations for 48 h was evaluated by western blotting. β -Actin was used as a loading control. (B) B4G2-induced apoptosis is caspase-independent. HepG2 cells were cultured with various concentrations of B4G2 in the presence or absence of 10 μ M zVAD-fmk. Cell viability was measured by the MTT assay. *** $P \leq 0.001$ vs. control. (C) B4G2 induces mitochondrial membrane potential ($\Delta\Psi_m$) depolarization in HepG2 cells. Cells were stained with the JC-1 fluorescence dye, and changes of $\Delta\Psi_m$ were determined by flow cytometry. (D) B4G2 triggers the release of Cyto c. Cytosolic lysates from HepG2 cells treated with B4G2 at the indicated concentrations for 48 h were evaluated by western blotting. β -Actin was used as a loading control. One-way ANOVA, *post hoc* comparisons and Tukey's test were used for statistical analyses. Columns, mean; error bars, SD.

decreases of pro-caspase-9 and pro-caspase-3 expression levels (Fig. 3A). PARP, which is a protein associated with DNA repair and considered as a hallmark of apoptosis, was also found to be cleaved in response to the activation of caspase-3 (Fig. 3A). However, a pre-treatment with Z-VAD-FMK, a broad spectrum inhibitor of caspases, did not rescue cells from apoptosis caused by B4G2 (Fig. 3B). These results indicated that the mitochondrial pathway, rather than the death receptor pathway, may be involved in a caspase-independent apoptosis mechanism induced by B4G2. To further confirm this hypothesis, changes in the mitochondrial membrane potential and cytosolic levels of cytochrome c (Cyto c), which are

Fig. 4. Opening of the PT pore is associated with the B4G2-induced mitochondrial apoptotic pathway. Cells were treated with B4G2 in the presence or absence of MgCl₂ (5 mM) for 48 h and stained with JC-1 or Annexin V/PI fluorescent dyes. The percentage of apoptotic cells (A) and changes of $\Delta\Psi_m$ (B) were determined by flow cytometry. *** $P \leq 0.001$ vs. control, ## $P \leq 0.01$ and && $P \leq 0.01$ vs. B4G2 treatment alone at the same conditions. (C) The expressions of Bcl-2 and Bax changed after B4G2 treatment. Total cell lysate from HepG2 cells treated with B4G2 at the indicated concentrations for 48 h was evaluated by western blotting. β -Actin was used as a loading control. One-way ANOVA, *post hoc* comparisons and Tukey's test were used for statistical analyses. Columns, mean; error bars, SD.

two important events in the mitochondrial apoptotic pathway, were studied. Results from the JC-1 staining assay showed that after B4G2 treatment, the percentage of cells with depolarized mitochondrial membrane potentials increased in a concentration-dependent manner (from 37.4% to 67.3%; Fig. 3C). B4G2 treatment also significantly increased the level of cytosolic Cyto *c* in a concentration-dependent manner (Fig. 3D). Taken together, our results suggested that B4G2 induces apoptosis through the mitochondrial apoptotic pathway.

B4G2-induced mitochondrial apoptosis is dependent on the opening of the PT pore

The opening of the PT pores is one of the most important events leading to the mitochondrial apoptotic pathway [25]. Therefore, we evaluated the role of the PT pore in the induction of mitochondrial-mediated apoptosis caused by B4G2. Pre-treatments with

Fig. 5. B4G2 induces the generation of ROS and, in turn, increases intracellular calcium levels. (A) B4G2 increases the level of cytosolic calcium. HepG2 cells stained with Fluo-3-AM (10 μM) after B4G2 treatment were observed by fluorescence microscopy (left panel, Original magnifications: × 100; scale bar: 200 μm), and the fluorescence intensity was further measured by a microplate reader (right panel). *** $P \leq 0.001$ vs. control. (B) B4G2 induces the generation of ROS in the cytoplasmic fraction of HepG2 cells. HepG2 cells were exposed to B4G2 at different times and stained with H2DCFDA (10 μM). Fluorescence intensity was measured by a microplate reader. ** $P \leq 0.01$ vs. control, *** $P \leq 0.001$ vs. control. (C) The release of ROS is abrogated in the presence of NAC. HepG2 cells treated with a combination of B4G2 and NAC (20 mM) were stained by H2DCFDA (10 μM). Fluorescence intensity was measured by a microplate reader. *** $P \leq 0.001$ vs. control, ### $P \leq 0.001$ vs. B4G2 (4 μM) treatment alone. (D) The intracellular calcium level increases in a ROS-dependent manner. HepG2 cells exposed to B4G2 with or without NAC (20 mM) at different times were stained with Fluo-3-AM (10 μM). Fluorescence intensity was measured by a microplate reader. ** $P \leq 0.01$, *** $P \leq 0.001$ vs. control, ## $P \leq 0.01$, &&& $P \leq 0.001$ and \$\$\$ $P \leq 0.001$ vs. B4G2 treatment alone at the same conditions. One-way ANOVA, *post hoc* comparisons and Tukey's test were used for statistical analyses. Columns, mean; error bars, SD.

MgCl₂, an inhibitor of the PT pore, markedly decreased the collapse of the mitochondrial potential and the population of Annexin V-positive cells induced by B4G2 (Fig. 4A and B). This

Fig. 6. Blockage of ROS attenuates B4G2-induced apoptosis. (A) Inhibition of ROS increases the cell viability of HepG2 cells. HepG2 cells were cultured with various concentrations of B4G2 in the presence or absence of NAC (20 mM). Cell viability was measured by the MTT assay. *** $P \leq 0.001$ vs. control, ### $P \leq 0.001$ and &&& $P \leq 0.001$ vs. B4G2 treatment alone at the same conditions. (B) Blockage of ROS decreases B4G2-induced apoptotic cells. HepG2 cells were exposed to B4G2 with or without of NAC (20 mM) for 48 h. Proportion of apoptotic cells was measured by flow cytometry. *** $P \leq 0.001$ vs. control, ## $P \leq 0.01$ and && $P \leq 0.01$ vs. B4G2 treatment alone at the same conditions. (C) Pretreatment with NAC decreases B4G2-induced collapse of mitochondrial membrane potential. HepG2 cells were treated with B4G2 in the presence or absence of NAC (20 mM) for 48 h and then $\Delta\Psi_m$ were measured by flow cytometry. (D) NAC pretreatment attenuates B4G2-induced cleavage of PARP. Total cell lysate from HepG2 cells treated with a combination of B4G2 and NAC (20 mM) for 48 h was evaluated by western blotting. β -Actin was used as a loading control. One-way ANOVA, *post hoc* comparisons and Tukey's test were used for statistical analyses. Columns, mean; error bars, SD.

observation suggested that B4G2-induced mitochondrial apoptosis is partially promoted by the opening of the PT pore. Moreover, the expression levels of Bcl-2 and Bax, which play important roles in the induction of the PT pore, exhibited significant changes after the B4G2 treatment, as shown in Fig. 4C.

B4G2 induces the generation of ROS to mediate the increase of intracellular calcium levels

Given the disruption of the mitochondrial potential induced by B4G2, the intracellular calcium levels, which regulate mitochondrial function, were examined. The occurrence and intensity of green fluorescence were both markedly increased by extending the duration of B4G2 treatment from 0.5 to 4 h, which indicated an overloading of calcium in the cytoplasmic fraction (Fig. 5A). ROS, important markers of mitochondrial damage, were also detected. As indicated in Fig. 5B and C, the exposure to B4G2 also induced an increase of intracellular ROS as early as 0.5 h, which was abrogated in the presence of the NAC, a ROS scavenger. Moreover, pre-

treatment with NAC decreased the B4G2-induced accumulation of intracellular calcium (Fig. 5D), indicating that the release of ROS elevated the intracellular calcium levels.

Fig. 7. A proposed model of interaction to delineate the action mechanism of B4G2.

Blockage of ROS attenuates B4G2-induced mitochondrial apoptosis

To further confirm the role of ROS as an initiator in B4G2-induced apoptosis, cells were preincubated with 20 mM NAC prior to B4G2 treatment. As shown in Fig. 6, NAC pre-treatment markedly increased cell viability compared with B4G2 treatment alone. NAC pre-treatment also attenuated the B4G2-induced collapse of the mitochondrial membrane potential, increased the population of Annexin V-positive cells and increased the specific cleavage of PARP. These results suggested that ROS initiate the B4G2-induced mitochondrial apoptosis in HepG2 cells.

Discussion

Natural products and their derivatives have been considered major sources of chemotherapeutic drugs and leading drugs [26]. 23-HBA, a derivative of the Chinese medicinal herb *Pulsatilla chinensis*, has weak antitumour activity *in vitro* and *in vivo* [8, 9, 11-14]. Therefore, our group chemically modified 23-HBA by introducing a nitrogenic side chain at the C28 position to obtain B4G2, which was shown to have remarkably improved anticancer activity [10]. In the present study, we report the effects of B4G2 on HBV-infected HCC cells, *i.e.*, HepG2, Bel-7402 and Hep3B cells, and on multidrug resistant HepG2/ADM cells. The results indicated the potential of B4G2 in the treatment of various types of HCC. Based on the present data, an *in vivo* study will be performed to determine whether B4G2 can inhibit tumour growth in a nude mouse xenograft model. Additionally, the *in vivo* toxicity of B4G2 towards normal tissues (*e.g.*, heart, lung, liver, kidney and spleen) should be determined to further develop B4G2 as a chemotherapeutic agent for preclinical studies. Many studies have shown that the induction of apoptosis in cancer cells is crucial to the killing mechanisms of most anti-cancer treatments including chemotherapy, immunotherapy and cytokine therapy [27, 28]. At least two main pathways have been identified that lead to apoptosis, namely the death receptor pathway and the mitochondrial pathway [27]. Abundant studies have demonstrated that betulinic acid (BA) induces apoptosis through the mitochondrial pathway, as evidenced by mitochondrial membrane potential depolarizations, Cyto c release from mitochondria, Smac release from mitochondria and increased levels of proteolytically cleaved caspase-3 and PARP [29]. However, Liby reported that TP-295 and TP-296 (BA derivatives that contain a methoxycarbonyl group in ring A) activate apoptosis through the

extrinsic death receptor-mediated pathway [30] in Bax/Bak^{-/-} cells. In our research, typical apoptotic morphological characteristics, including an increase in the sub-G1 phase of the cell cycle, the appearance of Annexin V-positive cells and the cleavage of PARP, were found to be responses to the effects of B4G2, which suggested that B4G2 decreases the viability of HepG2 cells through the induction of apoptosis. Moreover, in B4G2-treated HepG2 cells, B4G2 activated the mitochondrial apoptotic pathway, as supported by the decrease in the mitochondrial membrane potential, the release of Cyto *c*, and the activations of caspase-9 and caspase-3. However, caspase-8 expression remained unchanged, and the pan-caspase inhibitor had no effect on B4G2-induced cell death. These results indicated that caspase-independent mechanisms may be involved, such as the release of apoptosis-inducing factor and endonuclease G from mitochondria. Interestingly, these events are observed following increases to the mitochondrial membrane permeability. These mechanisms require further study.

Mitochondrial membrane permeability transition plays a critical role in apoptosis; this transition occurs after the opening of the PT pore [31]. The PT pore is a multi-protein complex formed between the inner and outer mitochondrial membranes. The PT pore permits water, ions and low molecular weight proteins up to 1.5 kDa to cross the mitochondrial matrix and cytosol. The pore is composed of adenine nucleotide translocase (ANT) and cyclophilin D (CyPD), which are located in the inner membrane, as well as VDAC and the recently discovered mitochondrial phosphate carrier, which are located in the outer membrane [25]. Arsenic trioxide may activate the PT pore through a direct interaction with ANT in the treatment of acute promyelocytic leukaemia [32]. This finding suggests that the regulation of the PT pore is an attractive target for cancer therapeutics. Recent research has indicated that Bak and Bax interact with ANT and/or VDAC, and this interaction results in a sudden permeability increase in the inner mitochondrial membrane. Subsequently, mitochondrial functions are disrupted and proapoptotic proteins are released, including Cyto *c*, apoptosis-inducing factor and endonuclease G, leading to apoptosis [33]. Moreover, anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, could block the amplification in mitochondrial membrane permeability by directly inhibiting VDAC or heterodimerizing pro-apoptotic Bcl-2 family members. Other Bcl-2 proteins may also form autonomous channels independent of the PT pore due to their ability to oligomerize into ion channel structures that increase the depolarizing effect induced by PT [25]. Fulda found that the overexpression of the anti-apoptotic proteins, Bcl-2 or Bcl-xL, counteracted BA-induced apoptosis [34]. Moreover, Mullauer reported that BA altered the levels of Bax and Bcl-2 proteins but did not alter the levels of Bcl-xS, Bcl-xL, Bax and Bak [35]. Mullauer further reported that BA affected mitochondria and induced the release of Cyto *c* directly *via* the PT pore. In our study, we found that the expressions Bcl-2 and Bax significantly changed after B4G2 treatment, suggesting that Bcl-2 and Bax are involved in the regulation of the mitochondrial membrane permeability. Additionally, the roles of the other Bcl-2 family members, such as Bak or Bcl-xL, in the formation of the PT pores remain unknown. As an important modulator of PT pores, Ca²⁺ induces a conformational change in the inner mitochondrial membrane by binding to the membrane and also alters the activity of mitochondrial membrane protein thiol groups. Thus, a cytosolic Ca²⁺ overload can induce excessive mitochondrial Ca²⁺ sequestration and can subsequently induce the opening of the PT pores [25]. In our study, B4G2 induced Ca²⁺ overloading, suggesting that it may have resulted in the opening of the PT pores. Until now, the Ca²⁺-based mechanism behind the regulation of the PT pore remained undetermined [25]. The common pharmacological inhibitors of the Ca²⁺-dependent PT pore are the competitive inhibitors of Ca²⁺, such as Mg²⁺, Mn²⁺ and Sr²⁺ [25]. We further found that pre-treatment with MgCl₂, which inhibits the PT pore by competing with Ca²⁺ for mitochondrial membrane binding sites [36], inhibited the depolarization of the mitochondrial membrane potential and protected cells from apoptotic cell death. Thus, these data demonstrated that the PT pore plays a pivotal role in B4G2-induced apoptosis. Based on the inhibition caused by MgCl₂, it can be concluded that B4G2 can induce the opening of the Ca²⁺-dependent PT pore. In addition to Ca²⁺, many other signals, such as increased cytosolic ROS and low

ATP concentration [37], can also trigger the opening of the PT pore by inducing several mitochondrial dysfunctions. These dysfunctions include oxidative damage to mitochondrial proteins, mitochondrial respiratory chain uncoupling, inner mitochondrial membrane reorganization and outer mitochondrial membrane permeabilization. These factors could all potentially lead to the activation of the mitochondrial apoptosis pathway [38]. Among these signals, ROS act by oxidizing the thiol groups of mitochondrial membrane proteins, resulting in the alterations of structural proteins and thus the formation of the PT pore [25]. In our study, B4G2 induced ROS generation, suggesting that ROS may be associated with the opening of the PT pore. Indeed, the effects of Ca^{2+} and ROS are often dependent on each other in the induction of the PT pore [39]. Mitochondrial Ca^{2+} uptake induces ROS formation by enhancing ATP production and interacting with some forms of ROS in the opening of the PT pore. Conversely, the release of Ca^{2+} from the endoplasmic/sarcoplasmic reticula (ER/SR) is regulated by ROS through the regulation of ryanodine receptors and 1, 4, 5-inositol-triphosphate receptors [40]. The transfer of Ca^{2+} between the ER/SR and mitochondria leads to increased Ca^{2+} levels in mitochondria. Moreover, Ca^{2+} and ROS can be both stimulatory and inhibitory, depending on the type of target proteins, chemical dose and cell type [41]. In this study, B4G2 increased the level of Ca^{2+} , which was completely blocked by NAC, suggesting that the induction of ROS is necessary for the release of Ca^{2+} in B4G2-treated cells. Furthermore, the B4G2-induced mitochondrial potential collapse and apoptosis were partially blocked by NAC, which implied that the B4G2-induced opening of the PT pore and apoptosis are dependent on the generation of ROS. However, the identity of membrane proteins of the PT pore complex involved in the ROS-induced opening of the pore remains unknown. It should also be noted that oxidative stress and ER stress are closely correlated and that the ER/SR is the main Ca^{2+} pool [42]. Hence, the relationship among ER stress, ROS generation and Ca^{2+} release as well as the crosstalk between the ER/SR and mitochondria in B4G2-induced apoptosis are interesting issues that require further study.

In summary, we demonstrated that B4G2 significantly inhibits the growth of HCC cells and revealed that ROS play a pivotal role in the anti-hepatoma effect of B4G2. B4G2 increases the accumulation of cytosolic Ca^{2+} and subsequently opens the PT pores, leading to the induction of apoptosis through the mitochondrial pathway (Fig. 7).

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Disclosure Statement

The authors have not conflict of interest to disclose.

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