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A novel bis-benzylidenecyclopentanone derivative, BPR0Y007, inducing a rapid caspase activation involving upregulation of Fas (CD95/APO-1) and wild-type p53 in human oral epidermoid carcinoma cells

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Abstract

BPR0Y007, a bis-benzylidenecyclopentanone derivative (2,5-bis- (4-hydroxy-3-methoxybenzylidene) cyclopentanone), was identified in our laboratory as a novel antineoplastic agent with a broad spectrum of antitumor activity against many human cancer cells. A previous study showed that BPR0Y007 inhibited DNA topoisomerase I (Top 1) activity and prevented tubulin polymerization. Notably, no cross-resistance with BPR0Y007 was observed in camptothecin-, VP-16- or vincristine-resistant cell lines. In this study, we further investigated the cellular and molecular events underlying the antitumoral function of this compound in human oral epidermoid carcinoma KB cells, focusing on the early cytotoxic effect. Treatment of KB cells with BPR0Y007-induced G_2/M phase arrest followed by sub-G_1 phase accumulation. Annexin-V–propidium iodide (PI) binding assay and DNA fragmentation assay further indicated that BPR0Y007-induced cell death proceeded through an apoptotic pathway as opposed to via necrosis. This compound produced a time-dependent activation of caspases-3 and -8, however, another caspase-3 initiator, caspase-9, was only marginally activated at later time point. We further demonstrated that the activation of the caspases cascade and nuclear fragmentation was not associated with inactivated Bcl-2 and perturbed mitochondrial membrane potential by BPR0Y007. The finding that BPR0Y007-induced apoptosis through a membrane-mediated mechanism was supported by up-regulated expression of Fas (CD95/APO-1), but not Fas-L. Furthermore, up-regulation of p53 and its affected gene, MDM2, in KB cells was found after BPR0Y007 exposure. Overall, our results demonstrated that the BPR0Y007 could induce an early cytotoxic apoptosis through a caspase-8-dependent but mitochondrial-caspase-9 independent pathway, and involving upregulation of p53.

Keywords: bis-Benzylidenecyclopentanone derivative; Antitumoral; Apoptosis; Caspase; Fas (CD95/APO-1); p53

1. Introduction

The novel synthetic bis-benzylidenecyclopentanone derivative, 2,5-bis-(4-hydroxy-3-methoxybenzylidene)-cyclopentanone (BPR0Y007) (Fig. 1), was identified as a dual inhibitor of DNA topoisomerase I (Top 1)s and microtubule in our laboratory [1]. Notably, we showed that BPR0Y007 possessed equally effective activity against parental- and in vitro-derived drug-resistant cells that were generated by vincristine, VP-16, or camptothecin-driven selection [1]. Our previous study demonstrated that BPR0Y007 is a potent inducer of apoptosis in cancer cells. However, the mode of action of BPR0Y007-induced apoptosis is not yet fully understood.

Apoptosis is an active cell suicide mechanism consisting of an evolutionarily conserved cascade that includes characteristic features such as cell shrinkage, condensation of chromatin, and formation of specific oligonucleotide fragments. All apoptotic pathways converge on a family of cysteine-aspartases, named caspases [2]. To date, two major caspases pathways, led by caspases-8 and -9 have

Abbreviations: BPR0Y007, 2,5-bis-(4-hydroxy-3-methoxybenzylidene) cyclopentanone; $DiOC_6(3)$, 3,3′-Dihexyloxacarbocyanine iodide; MPT, mitochondrial potential transition; PBS, phosphate buffered saline; PI, propidium iodide; Top 1, DNA topoisomerase I

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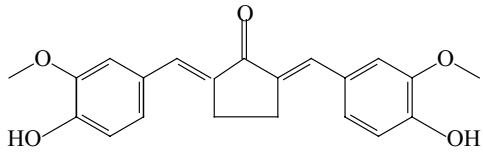


Fig. 1. Chemical structure of BPR0Y007.

been described and shown to mediate distinct sets of signals [3–5]. The death receptor-mediated pathway is triggered by rapid formation of death-inducing signaling complex (DISC) [6] by Fas, tumor necrosis factor (TNF), or TNF-α-related apoptosis-inducing ligand (TRAIL). On activation, these receptors recruit Fas (CD95/APO-1)-associated death domain (FADD), which in turn binds to procaspase-8 and cleaves into its active form. The stress-induced mitochondrial-dependent pathway is triggered by the cytochrome *c* release from mitochondria, leading to the formation of a complex with Apaf-1 that, via its caspase-recruitment domain, binds to procaspase-9. This in turn activates effector caspases [7].

It is well known that antitumor agents induce apoptosis through various signaling pathways in tumor cells [8]. In the present study, we investigated the contribution of the major apoptotic pathways to the cytotoxic effect of BPR0Y007 in human oral epidermoid carcinoma KB cells. We showed that BPR0Y007-induced apoptosis involves the induction of Fas (CD95/APO-1), and activation of p53, caspase-8, and downstream caspases. We further demonstrated that the activation of the caspase cascade was not associated with perturbed mitochondrial membrane potential by BPR0Y007. Triggering of this signaling route can explain the rapid and potent proapoptotic activity of BPR0Y007. These data suggest that BPR0Y007 may be a promising candidate in the treatment of neoplastic disease.

2. Materials and methods

2.1. Synthesis of BPR0Y007

BPR0Y007 was synthesized at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taipei, Taiwan, Republic of China, according to the procedure described by Sardjiman et al. [9]. Briefly, vanillin and cyclopentanone were heated in a water bath (45–50 °C) until a clear solution was obtained; concentrated hydrochloric acid was then added followed by 2 h of stirring. After standing overnight, the mixture was treated with cold acetic acid/water (1:1) and filtered. The solid material was washed first with cold ethanol, then with hot water, and dried under a vacuum. The yellow substance was recrystallized from ethanol. The structure of BPR0Y007 was confirmed by ^{1}H NMR and EI–MS.

2.2. Reagents

Monoclonal antibodies to Bcl-2, Fas (CD95/APO-1), MDM2, p53, PARP, as well as horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody to Fas-L was purchased from Oncogene Research Products (Darmstadt, Germany). Monoclonal antibody for α-tubulin was purchased from Sigma Chemical (St. Louis, MO). Caspase inhibitor Z-DEVE-fmk, Z-IETD-fmk, and Z-LEHD-fmk were purchased from Calbiochem-Novabiochem Co (La Jolla, CA). Cell culture reagents were obtained from Gibco-BRL Life Technologies (Gaitherburg, MD). 3,3′-Dihexyloxacarbocyanine iodide [($DiOC_6$)(3)] was purchased from Molecular Probes Inc (Eugene, OR). Acrylamide, bisacryamide, ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetramethylethylene diamine were from Bio-Rad (Richmond, CA). Western blot chemilluminescent reagent was purchased from Perkin-Elmer Life Sciences (Boston, MA). All of the other chemicals were from E. Merck Co. (Darmstadt, Germany) or Sigma Chemical (St. Louis, MO), and were standard analytic grade or higher.

2.3. Cell line and growth inhibitory assay

The human oral epidermoid carcinoma KB cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium supplied with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. For cytotoxic assay, cells in logarithmic phase were cultured at a density of 5000 cells/well in a 24-well plate. The cells were exposed to various concentrations of BPR0Y007 for indicated times. The methylene blue dye assay was used to evaluate the effects of the test compound on cell growth, as described previously [10], to determine the concentration of BPR0Y007 that inhibited 50% of cell growth (IC_{50}).

2.4. Flow cytometric analysis

Flow cytometry was performed on a single-cell suspension on adherent as well as floating cells after pooling them. For cell cycle analysis, the exponentially growing cells were incubated with BPR0Y007-containing medium for the selected treatment duration; then cells were fixed and stained with 50 μg/mL of propidium iodide (PI). DNA content was evaluated using FACSVantage (Becton Dickinson Labware, Franklin Lakes, NJ) and the percentage of cells in various cell cycle phases were determined using the ModFit LT software (Verity Software House, Topsham, ME). For each analysis, 10,000 events were recorded. For Annexin-V–PI binding assay, Annexin-V-FLUOS Staining Kit (Roche Applied Science, Mannheim, Germany) was used according the manufacturer's instruction. Briefly, the

BPR0Y007-treated cells were trypsinized and collected with in phosphate buffered saline (PBS) by centrifugation and then resuspended in 100 μl of Annexin-V-FLUOS labeling solution. After incubation at room temperature for 15 min in dark, the cells were analyzed by flow cytometry (FACSVantage, Becton Dickinson Labware, Franklin Lakes, NJ). Control cells stained with Annexin-V or PI alone was used to compensate for the flow cytometric analysis. Annexin-V and PI double-negative cells are defined as live cells; Annexin-V-positive, PI-negative cells are defined as early apoptotic cells; and annexin-V and PI double-positive cells are defined as late apoptotic and necrotic cells. For mitochondrial membrane potential assay, mitochondrial potential transition (MPT; $\Delta\psi_m$) was determined as proportional to the retention of the dye $DiOC_6(3)$. Cells were treated with 5 μM BPR0Y007 for the selected treatment duration. Thirty minutes prior to the conclusion of each treatment duration, in vitro labeling of cells was performed with 40 nM $DiOC_6(3)$, and cells were incubated at 37 °C in a humidified atmosphere at 95% O_2 and 5% CO_2 following the instructions of the manufacturer. After removal of the medium supernatant and rinsing of the cell dish with PBS, cells were harvested and suspended in PBS. Measurement of the retained $DiOC_6(3)$ in 10,000 cells of each sample was performed in a flow cytometer (FACSVantage, Becton Dickinson Labware, Franklin Lakes, NJ).

2.5. Nuclear fragmentation assay

Cells were initially seeded at 1×10^6 cells in 100 mm^2 dishes overnight, and BPR0Y007 was added to the final concentration of 5 μM. At predetermined time intervals, the non-adherent cells were collected by aspiration, and adherent cells were harvested using 0.25% trypsin/0.05% EDTA. DNA was extracted from adherent and non-adherent cells using Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the instructions of the manufacturer. The isolated DNA samples were fractionated by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining (0.5 μg/mL) under ultraviolet transillumination.

Determination of Caspase Activity. The activity of caspases-3, -8 and -9 on BPR0Y007-treated cells were determined by using Caspase Fluorometric Assay kits (R&D System Inc., Minneapolis, MN) or CaspACE Assay System-Fluorometric (Promega Corporation, Madison, WI) for the cleavage of respectively specific fluorogenic peptide substrate according to the instruction of the manufacturer. Briefly, 3×10^6 cells were collected, lysed in a lysis buffer, and clarified by centrifugation. Equal amounts of the extract supernatants were incubated with 50 μM of fluorescent substrate. After 1 h of incubation at 37 °C, fluorescence of the cleaved substrate was determined using the Victor 1420 Multilable Counter (Wallac, Turku, Finland), and caspase activity was calculated by subtracting the value obtained in the untreated sample.

2.6. Caspases inhibitory assay

In the caspase inhibitory experiments, the cell-permeable and specific irreversible inhibitors of caspases-3, -8, or -9 were added to the medium 1 h prior to BPR0Y007 administration. Cell survival was determined by the methylene blue dye assay to evaluate the effects of the test compound on cell growth as described previously [10]. The stock solution of caspase inhibitors was prepared in DMSO and all additions were 0.5% (v/v) or less.

2.7. Preparation of cell lysates and western blot analysis

Cells were initially seeded at 1×10^6 cells in 100 mm^2 dishes. After treatment for the indicated time with various concentrations of BPR0Y007, adherent cells were washed twice with PBS, gently scraped from the dishes, then centrifuged, lysed in ice-cold lysis buffer (50 mM Tris [pH 7.4], 0.8 M NaCl, 5 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 μg/mL pepstatin, and 50 μg/mL leupeptin), and cleared by microcentrifugation. Protein concentrations of lysates were determined using the BCA Protein Assay Reagent (PIERCE Biotechnology, Rockford, IL). Equivalent protein amounts of each sample were separated on SDS-PAGE gels and blotted to nitrocellulose membrane. After soaking in a blocking solution containing PBS, 0.05% Tween 20, and 5% skim milk, the blot was incubated with the primary antibody at 1–2 μg/mL, and antibody binding was detected using the appropriate secondary antibody coupled with horseradish peroxidase according to the instructions of manufacturer. Enhanced chemiluminescence was used to detect the relevant proteins following protocols suggested by the manufacturer.

2.8. p53 transactivation assay

p53 status was studied by transactivation assay. KB cells were seeded 24 h before transiently transfected with 2 μg of p53-Luc reporter plasmid (Stratagene, La Jolla, CA) using the electropration method following the manufacturer's instructions (Cellject Pro, Thermo Hybaid, MA). Sixteen hours after transfection, cells were cultured in the presence of 5 μM BPR0Y007 for the selected treatment duration. After incubation, cells were collected and lysed with the reported lysis buffer (Promega, Madison, WI). Luciferase activity was then determined on a Victor 1420 Multilable Counter (Wallac, Turku, Finland) following the manufacturer's instructions.

2.9. Statistical analysis

All assays were carried out in triplicate. Data were expressed as means with standard deviations. Student's *t*-test ($P < 0.05$) was calculated to compare the mean of each group with that of the control group.

3. Results

3.1. Treatment with BPR0Y007 caused concentration- and time-dependent growth inhibition in human oral epidermoid carcinoma KB cells

The growth inhibitory effect of BPR0Y007 was examined at doses between 0.5 and 10 μM for the selected treatment duration. Cell growth was markedly inhibited by BPR0Y007 treatment in a concentration- and time-dependent manner (Fig. 2). Approximately 60, 76, and 93% growth inhibition were detected after 24, 48, and 72 h BPR0Y007 treatment, respectively. Thus, we chose 5 μM BPR0Y007 to detect changes in molecular events in the following experiments.

3.2. Induction of G_2/M phase arrest and apoptosis by BPR0Y007

To examine the effect of BPR0Y007 on cell cycle progression, KB cells were treated with 5 μM BPR0Y007 for the selected treatment duration and the cell cycle distribution was analyzed by flow cytometry. As shown in Fig. 3A, treatment with BPR0Y007 resulted in accumulation of cells in the G_2/M phase starting from 2 h of exposure, with a maximum accumulation being observed at 12 h of treatment (70% in the drug-treated cells versus 32% in the control cells) with a concomitant loss of the G_0/G_1 phase. The hypodiploid population of KB cells increased from approximately 2% in untreated control cells to approximately 18% in 24 h in BPR0Y007-treated cells. To further assess the hypodiploid population in the BPR0Y007-treated KB cells, we performed the Annexin-V–PI binding assay. A significant increase from 2.9 to 5 and 12.8% of apoptotic cells (Annexin-V positive/PI-negative) were found in the 12 and 18 h-BPR0Y007 treated KB cells, respectively (Fig. 3B). Furthermore, the DNA fragmentation assay revealed a typical laddering of the DNA in KB cells exposed to BPR0Y007 as early as 12 h, and a strong laddering signal was found after 24 h (Fig. 3C). These results demonstrated that BPR0Y007 inhibited KB cells by arresting the cells in the G_2/M phase, and this, in turn, activated the apoptotic signaling pathway.

3.3. Activation of caspase-3 and cleavage of PARP by BPR0Y007

To gain insights into the mechanism by which BPR0Y007 induces apoptosis, we investigated the effects of BPR0Y007 on caspase. Of the many caspases involved in apoptosis, we first examined the in vivo effect of BPR0Y007 on caspase-3, which is activated by a number of apoptotic signals [11]. As shown in Fig. 4A, incubation of KB cells with BPR0Y007 resulted in the activation of caspase-3 in a time-dependent manner; this effect started from 4 h of exposure (1.56-fold increase compared with control cells), with a maximum of 3.64-fold induction being observed by 12 h of treatment. Activation of caspase-3 during BPR0Y007-induced apoptosis was also confirmed by examining PARP, a known endogenous substrate for caspase-3 and an early marker of apoptosis [12,13]. As shown in Fig. 4B, caspase-3 activation was accompanied by the cleavage of PARP (116 kDa) into an 85-kDa C-terminal fragment that became evident 12 h after BPR0Y007 treatment.

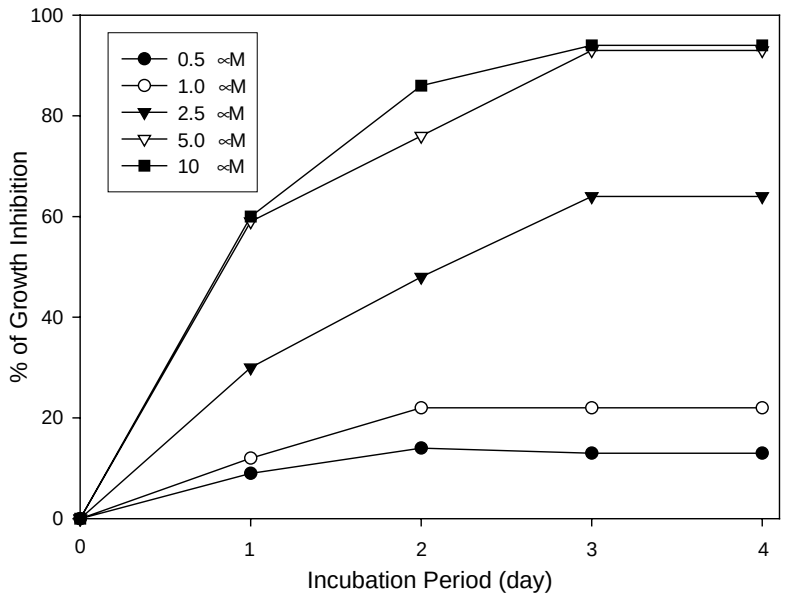
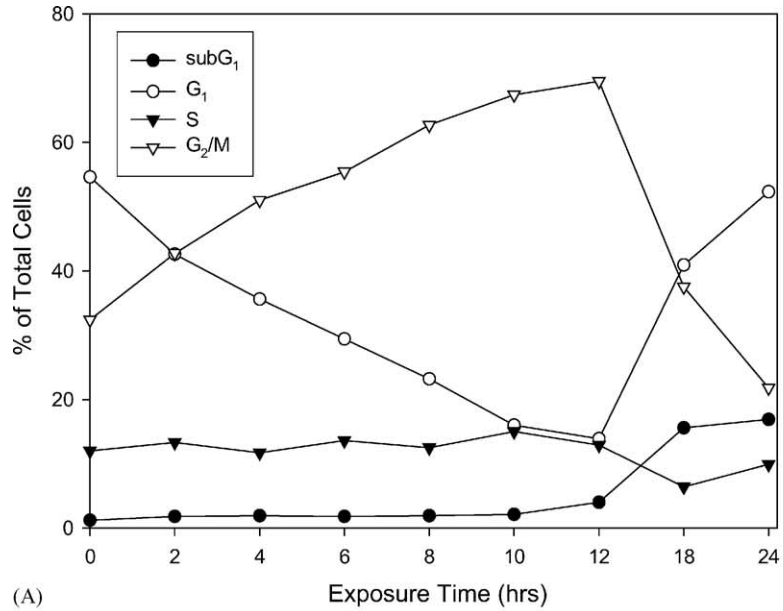


Fig. 2. BPR0Y007-induced growth inhibition in human epidermoid carcinoma KB cells. Cell were treated with various concentrations of BPR0Y007 at 37 °C for the indicated times. Growth inhibition of cells was determined by methylene blue assay as described under Section 2.



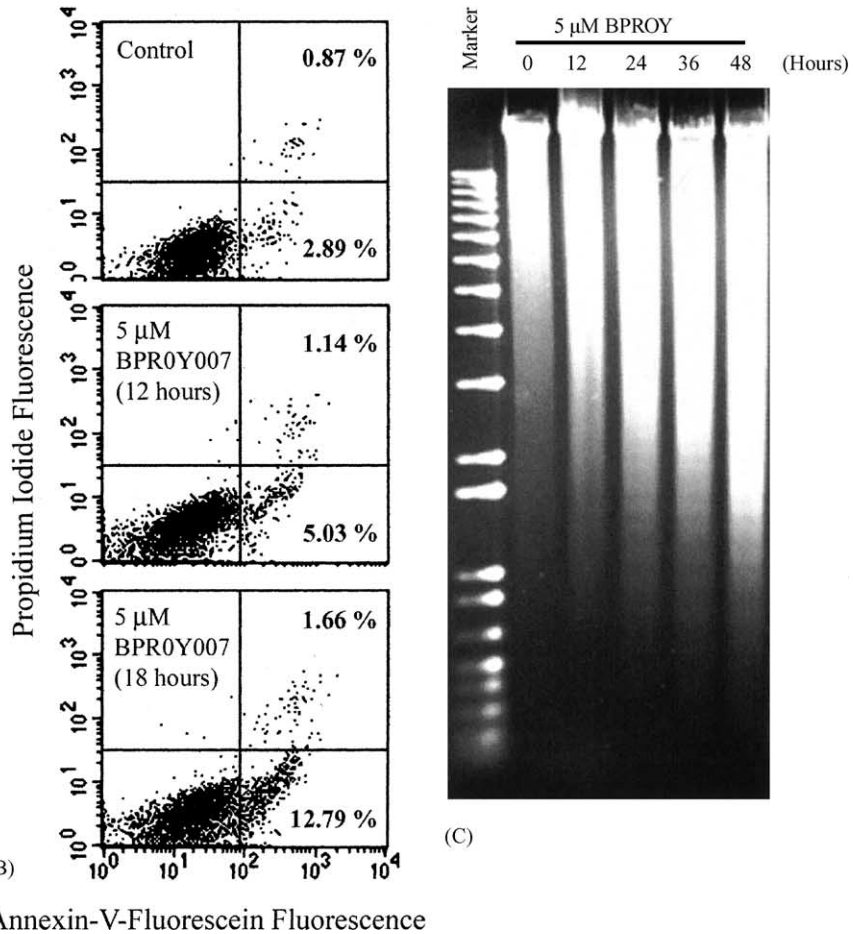


Fig. 3. Effect of BPR0Y007 on cell cycle progression and apoptosis in oral epidermoid carcinoma KB cells. (A) Time effect of BPR0Y007 on cell cycle profile in KB cells. Cells were treated with 5 μM BPR0Y007 for the indicated time and analyzed for PI-stained DNA content by flow cytometry. (B) BPR0Y007-induced cells underwent apoptosis as demonstrated by Annexin-V positivity. KB cells were treated with 5 μM BPR0Y007 for the indicated times then incubated with Annexin-V and PI and subjected to flow cytometric analysis. (C) BPR0Y007 induces DNA laddering fragmentation. KB cells were treated with indicated concentrations of BPR0Y007 for the selected treatment duration. After treatment, cells were lysed with lysis buffer, and DNA was extracted, then dissolved in TE buffer, and subjected to electrophoresis in a 1% agarose gel. DNA laddering was visualized by ultraviolet transillumination.

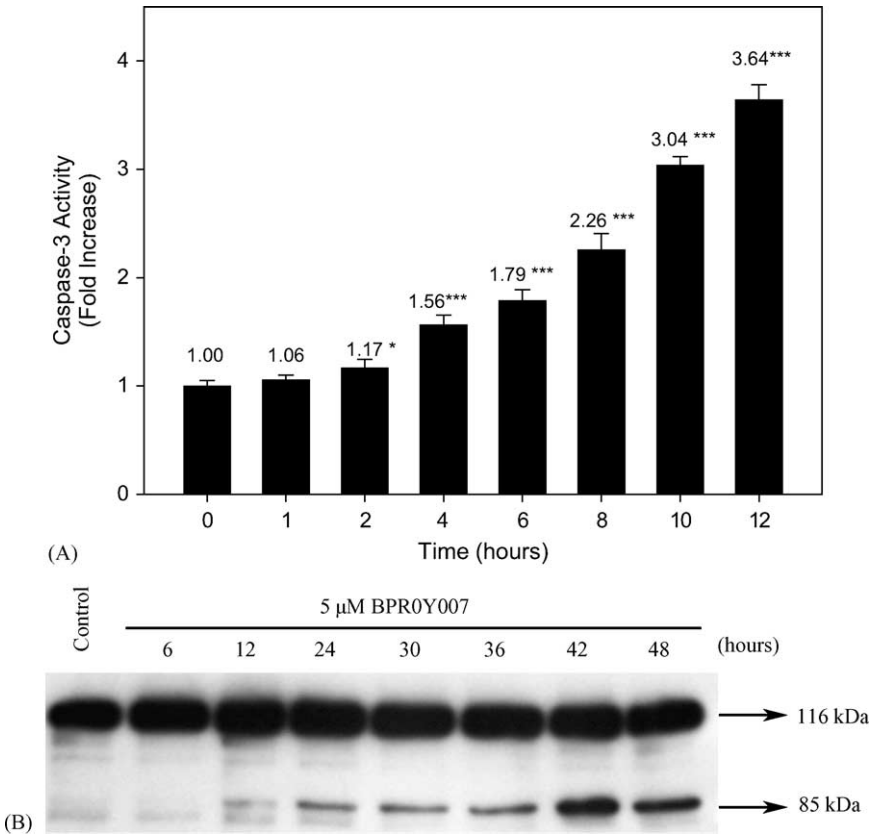


Fig. 4. Activation of caspase-3 and proteolytic cleavage of PARP induced by BPR0Y007 in oral epidermoid carcinoma KB cells. KB cells were culture in control medium only, or 5 μM BPR0Y007 for different time intervals as indicated. (A) Caspase-3 activity was determined as described in Section 2 using caspase-3 fluorogenic peptide substrate DEVD-AMC. Values are the means of triplicate analysis. Error bars show the standard deviations. (∗), (∗∗), (∗∗∗), significantly different form the control at $P < 0.05$, <0.01, <0.001, respectively, with Student's *t*-test. (B) The cleavage of PARP was examined by Western blot analysis as described in Section 2.

3.4. Apoptosis in BPR0Y007-treated cells is associated with activation of caspase-8, but not caspase-9

To understand the mechanism by which caspase-3 is activated by BPR0Y007, we further investigated the effect of BPR0Y007 on the activity of caspases-8 and -9, upstream activators of caspase-3. Upon treatment of KB cells with BPR0Y007, a strong activity toward the caspase-8 specific IETD-AFC fluorogenic tetrapeptide substrate were detected as early as 4 h after treatment with a further increase with time (Fig. 5A). In contrast to the high level of caspase-8-specific activity, weak caspase-9-specific activity was detected (Fig. 5A), with only 1.37-fold of caspase-9 activity being observed in cells treated with BPR0Y007 for 24 h. In addition, BPR0Y007-induced caspase-8 activation showed similar time courses compared with caspase-3 activation. Thus, we propose that BPR0Y007 induces apoptosis through a caspase-8 mediated pathway.

3.5. Inhibition of caspase-3 or caspases-8 activity suppresses BPR0Y007-induced apoptosis

To further examine the relationship between caspase activation and BPR0Y007-induced cytotoxicity, we pretreated KB cells with membrane-permeable irreversible inhibitor of caspases-3, -8, or -9 and measured the cell growth under the influence of BPR0Y007. Results demonstrated that in the presence of caspase-8-specific inhibitor Z-IETD-fmk and the caspase-3 inhibitor Z-DEVD-fmk, BPR0Y007-induced apoptosis is significantly reduced from 50 to 27 and 22%, respectively at 24 h (Fig. 5B). In contrast to caspases-3 and -8, cells pretreated with the caspase-9-specific inhibitor, Z-LEHD-fmk, did not prevent BPR0Y007-induced cell death (Fig. 5B), indicating that the activation of caspase-9 might not involve BPR0Y007-induced apoptosis.

3.6. Apoptosis in BPR0Y007-treated cells is not related to mitochondria-mediated caspase-9 pathway

Because weak caspase-9-specific activity was detected until treatment with 5 μM BPR0Y007 for 24 h, and because a previous report by Ferreira et al. [14] showed that caspase-8 can be activated in a mitochondria-controlled, caspase-9 independent manner. Therefore, we examined whether mitochondria were involved in initiating BPR0Y007-induced apoptosis in KB cells. We compared BPR0Y007-treated and -untreated KB cells by

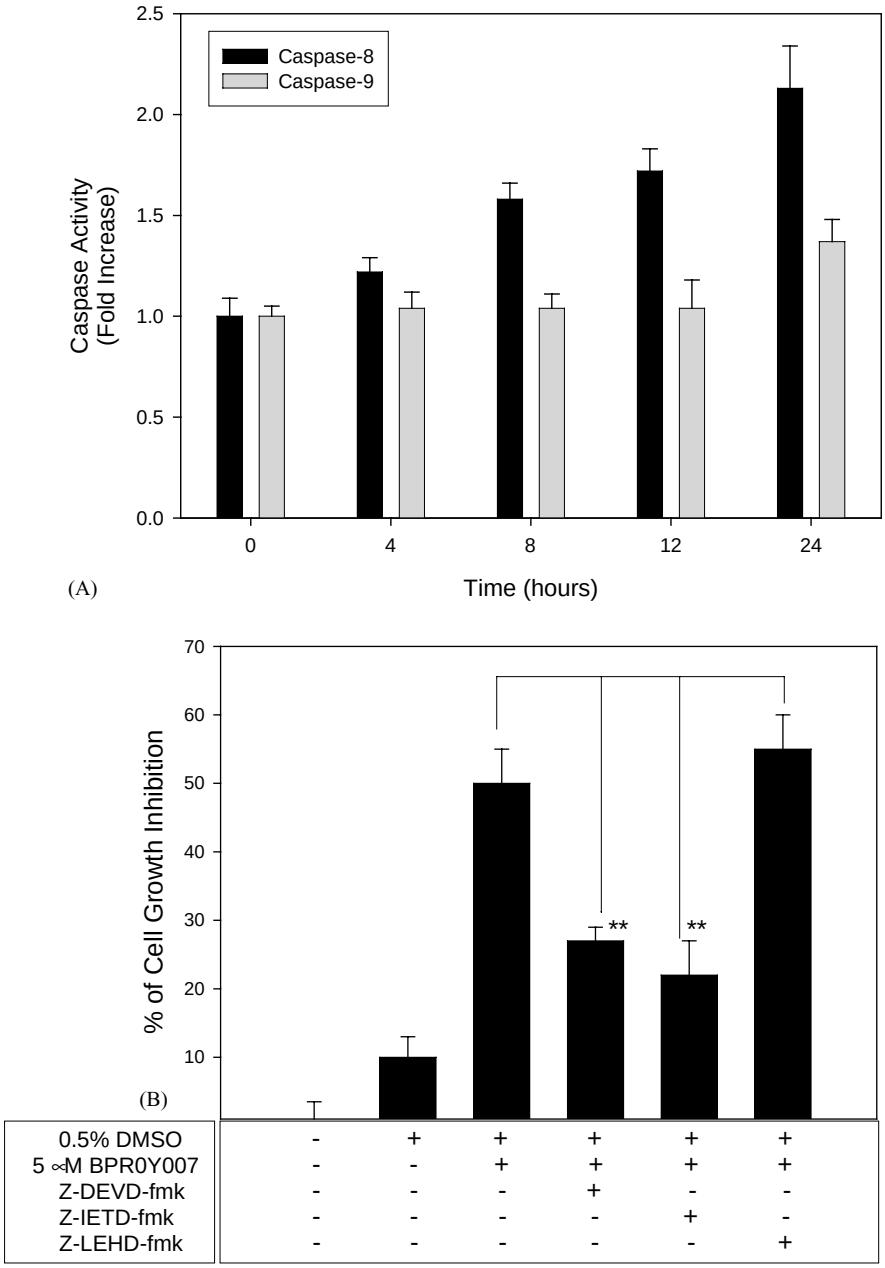


Fig. 5. (A) Effect of BPR0Y007 on caspase-8 and caspase-9 activity in oral epidermoid carcinoma KB cells. Cells were cultured in control medium only, or 5 μM BPR0Y007 for different time intervals as indicated. Caspases-8 and -9 activities were determined as described in Section 2 using fluorogenic peptide substrates IETD-AFC and LEHD-AFC, respectively. (B) BPR0Y007-induced cell death in KB cells is reduced with caspase inhibitors. KB cells were pretreated with 100 μM of the caspase inhibitors (Z-DEVD-fmk, caspase-3 inhibitor; Z-IETD-fmk, caspase-8 inhibitor; Z-LEHD-fmk, caspase-9 inhibitor) for 1 h, then treated with 5 μM BPR0Y007 for 24 h. The cell survival was determined by the methylene blue dye assay. Values are the means of triplicate analysis. Error bars show the standard deviations. (∗), (∗∗), (∗∗), significantly different form the control at $P < 0.05$, <0.01, <0.001, respectively, with Student's *t*-test.

staining mitochondria with fluorochrome $DiOC_6(3)$ and determined MPT by measuring the retained $DiOC_6(3)$ using a flow cytometer. Treatment with carbamyl cyanide *m*-chlorophenyl hydrazine (mCCCP), a direct mitochondrial activator that can completely abrogate the retention of $DiOC_6(3)$ in the cell, was used as a positive control. The results are presented in Fig. 6A, MPT remains unchanged after KB cells are treated for 24 h with BPR0Y007. However, MPT only showed a slight increase until 48 h of 5 μM BPR0Y007 treatment. Furthermore, we then examined the effect of BPR0Y007 in antiapoptotic protein, Bcl-2 [15,16]. The immunoblot analysis presented in Fig. 6B indicates that KB cells treated with 5 μM BPR0Y007 remained unchanged on Bcl-2 expression within the first 20 h of treatment. However, very faint slower migration form of Bcl-2 band was observed at 24 h of treatment.

In comparison with caspase-3 activation, PARP cleavage and DNA ladder were observed in the first 12 h of

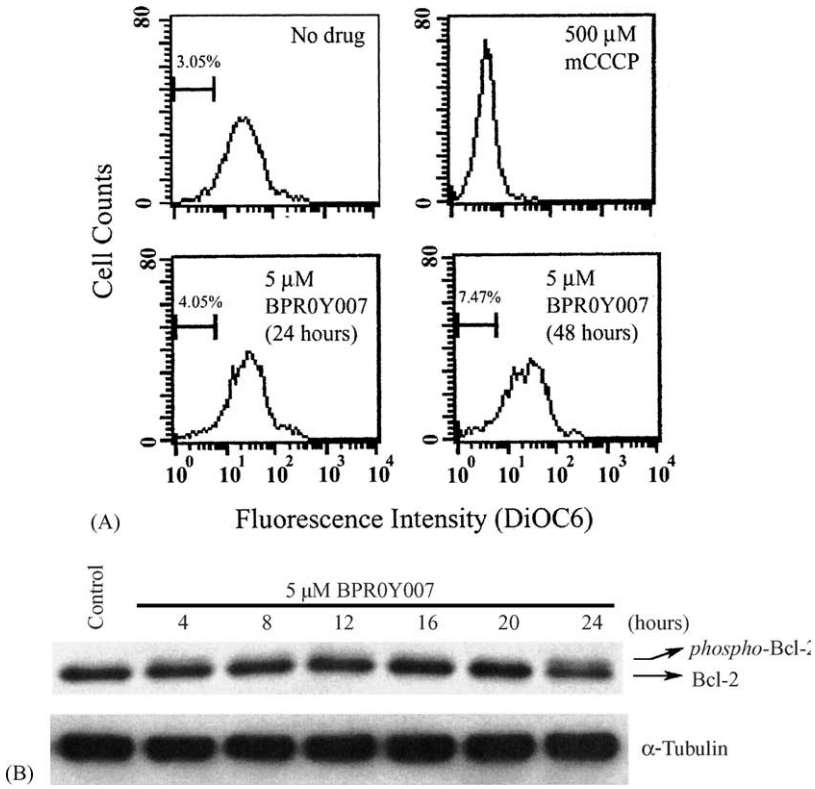


Fig. 6. Effect of BPR0Y007 on mitochondrial potential transition and Bcl-2 expression in oral epidermoid carcinoma KB cells. KB cells were treated with 5 μM BPR0Y007 for the selected treatment duration. (A) Mitochondrial potential transition was detected by used $DiOC_6$ assay as described in Section 2. (B) Bcl-2 expression was examined by Western blot analysis as described in Section 2.

treatment. These results suggest that mitochondria-mediated caspase-9 activation and Bcl-2 hyperphosphorylation are not the major and critical steps in the early induction of apoptosis in KB cells.

3.7. Induction of apoptosis by BPR0Y007 is mediated by an increase in expression of Fas (CD95/APO-1) but Not Fas-L in KB cells

The results obtained thus far suggest that caspase-8 may function as an apical caspase in the pathway that triggers apoptosis in KB cells. Caspase-8 has been shown to be the classical initiator caspase of the Fas (CD95/APO-1) pathway [17,18]. The Fas (CD95/APO-1) pathway has also been shown to contribute to chemotherapeutic agents-induced apoptosis in various cellular systems [19–21], therefore, the expression of Fas (CD95/APO-1) was assessed by immunoblotting. As shown in Fig. 7, BPR0Y007 significantly induced the level of Fas (CD95/APO-1) after 4 h treatment. In contrast to Fas upregulation, no appreciable change in the expression of Fas-L in KB cells treated with the same concentration of BPR0Y007 from 4 to 24 h was seen (Fig. 7). This finding suggests that BPR0Y007-induced Fas (CD95/APO-1) expression is independent of its ligand.

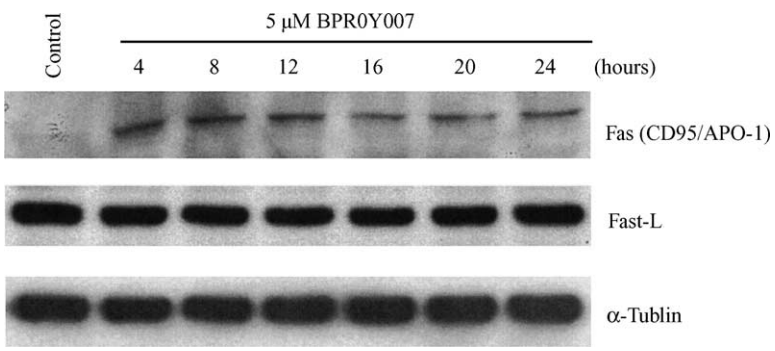


Fig. 7. Effect of BPR0Y007 on Fas (CD95/APO-1) and Fas-L expression on oral epidermoid carcinoma KB cells. KB cells were treated with 5 μM BPR0Y007 for the selected treatment duration. Cellular extracts were prepared and analyzed by SDS-PAGE and Western blot analysis as described in Section 2.

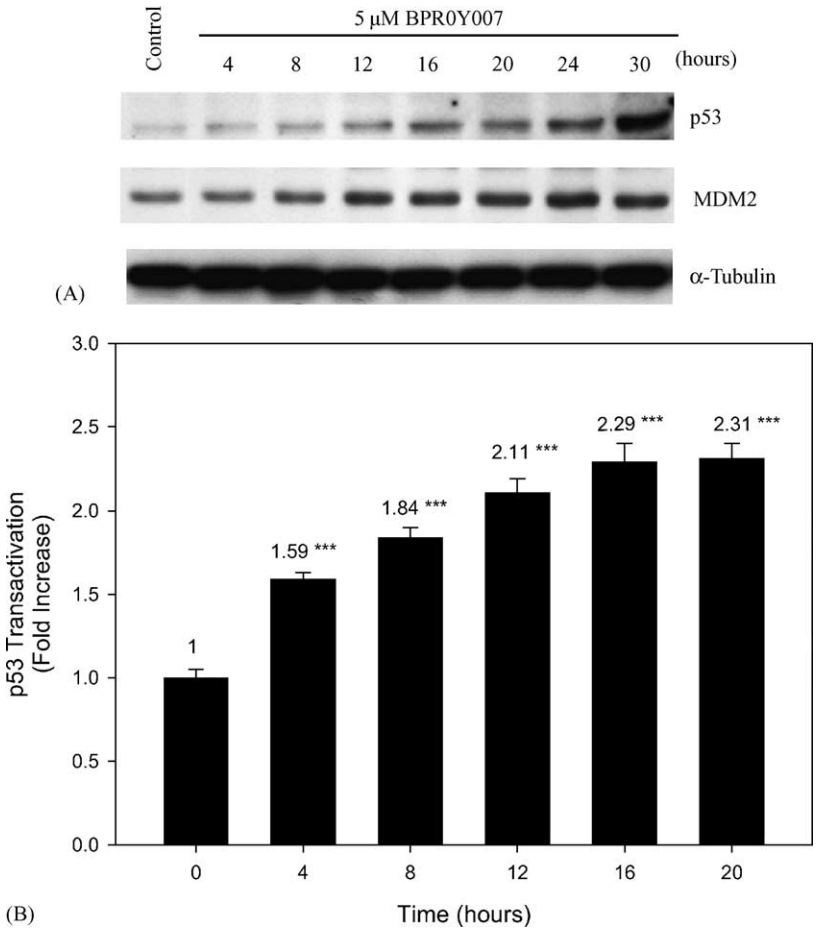


Fig. 8. BPR0Y007-induced apoptosis involving upregulation of p53 in KB cells. (A) BPR0Y007 increased protein levels of p53 and MDM2. KB cells were treated with 5 μM BPR0Y007 for the selected treatment duration. Cellular extracts were prepared and analyzed by SDS-PAGE and Western blot analysis as described in Section 2. (B) Enhanced p53 transcriptional transactivating activity after BPR0Y007 treatment. KB cells were transiently transfected with a p53-responsive (p53-Luc) luciferase indicator construct. Luciferase activities in cell lysates were determined on a Victor 1420 Multiable Counter. Values are the means of triplicate analysis. Error bars show the standard deviations. (∗), (∗∗), (∗∗∗), significantly different form the control at $P < 0.05$, <0.01, <0.001, respectively, with Student's *t*-test.

3.8. BPR0Y007 up-regulates wild-type p53

Because Fas (CD95/APO-1) is a p53-regulated gene, we further determined the expression and activity of p53 in KB cells in response to BPR0Y007 exposure. As shown in Fig. 8A, BPR0Y007 significantly increased the protein levels of p53 and its affected gene, MDM2, in a time-dependent manner. The 1.8-fold of p53 expression has been increased after 4 h treatment, with a maximum of 4.8-fold induction being observed by 30 h of treatment. In addition, by transiently transfecting a reporter gene under the control of a p53 response element, increases in the level of p53 transactivation were observed in a time-dependent manner after BPR0Y007 treatment (Fig. 8B).

4. Discussion

The novel synthetic BPR0Y007 is efficacious in suppressing cell growth in a variety of human cancer cells despite their P-gp170/MDR or MRP status, and acts as a dual inhibitor of Top 1 and microtubule [1]. In the present report, we describe the cellular and molecular events underlying the growth inhibitory effect of this compound in human oral epidermoid carcinoma KB cells, focusing on early cytotoxic characteristics.

Our data showed that treatment with BPR0Y007 results in a rapid accumulation of the G_2/M phase starting from 2 h of exposure, with a maximum accumulation being observed at 12 h of treatment. The typical apoptotic characteristics, the hypodiploid DNA content peak (sub-G_1), Annexin-V-positive/PI -negative pattern and DNA laddering can be detected with treatment duration over 12 h (Fig. 3). These results demonstrate that BPR0Y007-induced cells cause an arrest in the G_2/M phase before apoptotic cell death. The percentage of G_2/M cells decreases and the percentage of cells in G_0/G_1 goes back up to a level similar the one observed at 0 h after BPR0Y007-treated for 12 h which indicated that KB cell might escape the BPR0Y007-induced G_2/M arrest, however, the detail mechanism is under active investigation.

Caspases are at the heart of the apoptotic machinery [22]. Several caspases have been shown to be key executioners of apoptosis, mediated by various inducers including antitumor agents [23]. Recently data have suggested that caspase-3 is involved in Top 1 and microtubule inhibitors-induced apoptosis in tumor cells [24,25]. As shown in Fig. 4, caspase-3 activation was accompanied by the cleavage of PARP (116 kDa) into an 85-kDa C-terminal fragment in the BPR0Y007-treated KB cells. Questions remain as to how caspase-3 is activated by BPR0Y007. Previous reports have demonstrated that caspases-8 and -9 are upstream initiator caspases that can directly activate caspase-3 [3].

The Bcl-2 located on the outer mitochondrial membrane is important for the suppression of apoptosis and mitochondrial manifestations of apoptosis [15]. Bcl-2 prevents the initiation of the cellular program by stabilizing the mitochondrial permeability and avoiding the subsequent release of cytochrome *c* to prevent caspase activation [16]. Several microtubule-disrupting drugs that induce phosphorylation and inactivate Bcl-2 lead to accumulation in the G_2/M phase and trigger the caspase-9/mitochondrial pathway [26–33]. However, our results showed that caspase-9 was not activated in response to BPR0Y007 treatment (Fig. 5) and loss of mitochondrial transmembrane potential did not appear (Fig. 6A). Furthermore, our results show that BPR0Y007 did not affect Bcl-2 expression in the first 20 h of treatment when apoptosis was clearly observed, the Bcl-2 was hyperphophorylated and lost its protective function against apoptosis only after a prolonged BPR0Y007 treatment (Fig. 6B). These findings indicate that mitochondrial perturbation and Bcl-2 inactivation might not be involved in rapid activation of the caspase cascade by BPR0Y007 treatment. These data suggest that BPR0Y007-induced apoptosis is different from that of microtubule-disrupting agents.

Using IETD-AFC as substrates to measure caspase-8 activity, we observed that the activity of caspase-8 was strongly increased in KB cells exposed to BPR0Y007. In addition, BPR0Y007-induced caspase-8 activation showed similar time courses compared with caspase-3 activation (Figs. 4A and 5A). These results demonstrate that BPR0Y007-triggered apoptosis is crucially dependent on activation of the caspase-8 cascade. Caspase-8 is the most apical caspase in Fas (CD95/APO-1)-mediated apoptosis [34], and the changes in Fas (CD95/APO-1) expression were assessed by immunoblotting. Results showed that Fas (CD95/APO-1) expression was induced in KB cells after PBR0Y007 treatment (Fig. 7). However, we did not observe modulation of Fas-L expression, indicating that BPR0Y007 selectively induces Fas (CD95/APO-1) expression in KB cells.

One mechanism of Fas (CD95/APO-1) up-regulation involves transactivation by p53 through p53 binding sites in the promoter and first intron of the *Fas* gene [21,35,36]. We examined the effect of BPR0Y007 on the expression and activity of p53 in KB cells. Results, illustrated in Fig. 8, show that p53 was activated in response to BPR0Y007. To the best of our knowledge, this is the first report that a bisbenzylidenecyclopentanone compound could up-regulate both Fas (CD95/APO-1) and p53 expression in human cancer cells.

In conclusion, our results provide evidence that BPR0Y007 induces early apoptotic cell death via a Fas/caspase-8 dependent but mitochondria/caspase-9 independent pathway. Our data suggest that upregulation of Fas (CD95/APO-1) and p53 may account for the early cytotoxicity of BPR0Y007 in the oral epidermoid carcinoma cell line. Because p53 has been reported to up-regulate transcription of Fas (CD95/APO-1) [35], the detailed molecular mechanism between BPR0Y007-induced activation of p53 and the Fas (CD95/APO-1)/Fas-L system observed in KB cells is currently being investigated.

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