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ORIGINAL ARTICLE

Quinoline-Based Compound BPIQ Exerts Anti-Proliferative Effects on Human Retinoblastoma Cells via Modulating Intracellular Reactive Oxygen Species

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Abstract Retinoblastoma (Rb) is the most common primary intraocular malignant tumor of childhood. It is important to develop the strategy for Rb treatment. We have tested a quinolone derivative 2,9-bis[2-(pyrrolidin-1-yl)ethoxy]-6-{4-[2-(pyrrolidin-1-yl)ethoxy]phenyl}-11H-indeno[1,2-c]quinolin-11-one (BPIQ) for its anti-cancer effects against Rb via cultured human Rb cell line Y79. Our results showed that BPIQ significantly inhibits cell growth of Y79. Furthermore, the flow cytometer-based assays and Western blotting showed that BPIQ induces the apoptosis of Y79 via increasing the level of reactive oxygen species (ROS). Besides, the activation of γH2AX, a DNA damage sensor in human Y79 cells was also observed, indicating the potential of BPIQ for causing DNA damage of Rb cells. On the contrary, BPIQ-induced apoptosis of Y79 cells was attenuated significantly by *N*-acetyl-L-cysteine (NAC), an ROS scavenger. The results of Western blot showed that BPIQ down-regulates the levels of anti-apoptotic proteins Bcl-2, survivin and XIAP while up-regulates the pro-apoptotic proteins Bad, Bax and Bid. Our present study demonstrated the anti-proliferative effect of BPIQ in human Y79 cells. The inhibitory effect of BPIQ on the proliferation of Y79 cells is, at least, partly mediated by the regulation of ROS and DNA damage pathway. In conclusion, BPIQ may provide an alternative option in the chemotherapeutics or chemoprevention on the Rb therapy in the future.

Keywords BPIQ · Quinoline · Retinoblastoma · Reactive oxygen species · DNA damage · Apoptosis

Introduction

Retinoblastoma (Rb) is a most common intraocular malignant tumor of childhood. Retinoblastoma accounts for approximately 4 % of pediatric cancer cases and about

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5000 Rb cases are diagnosed in the world yearly (Moll et al. 2003). Among these Rb patients, 50 % of children patient survive after the malignancy treatment. Despite enucleation, the surgical removal of the eyeball, successfully cures unilateral Rb with approximately 95 % patients (Beck et al. 2000); however, this may cause loss of the vision and the unsatisfactory appearance. Furthermore, like most metastatic V, advanced Rb usually are incurable in diagnosed people due to insensitive to most systemic treatments (Chantada et al. 2012). Therefore, it is urgent to search potential therapeutic drugs for Rb chemotherapy. However, due to the rare incidence of Rb compared to that of lung cancer and liver cancer, fewer research studies were reported regarding chemotherapy of Rb. Additionally, the metastatic Rb metastasis makes the poor prognosis of Rb, and no effective chemotherapies are available now.

Quinoline-based derivatives have been shown to exert a variety of pharmacological activities, including antimicrobial (Afzal et al. 2015; El-Gamal et al. 2014), anti-cancer, antiviral (Loddo et al. 2014), anti-convulsant (Deng et al. 2014), anti-inflammatory (Chen et al. 2004; El-Sayed et al. 2004) and cardiovascular activities (Tukulula et al. 2012; Yin et al. 2013). Therefore, many synthetic quinoline-based compounds have attracted tremendous research interests for the evaluation of their possible biological activities (Kim et al. 2014; Lu et al. 2014; Saleh et al. 2014). Camptothecin (CPT) is a well-known quinoline derivative isolated from a tree *Camptotheca acuminata*, which has been shown to exert cytotoxicity in many cancers. Several CPT derivatives such as topotecan and irinotecan are currently used as clinical anti-cancer drugs (Tseng et al. 2008).

Quinoline derivatives have been reported as potent agents in anti-cancer proliferative activities by means of in vitro test, using glioblastoma SNB-75 (Chen et al. 2002), breast (T-47D) (Ahsan et al. 2014), and lung cancer cell lines (Perin et al. 2014), and of in vivo tests, using both ascites and solid forms of Ehrlich mouse carcinoma (Sakai et al. 1955).

2,9-Bis[2-(pyrrolidin-1-yl)ethoxy]-6-{4-[2-(pyrrolidin-1-yl)ethoxy]phenyl}-11*H*-indeno (Barot et al. 2014) quinolin-11-one (BPIQ) is a novel synthetic quinolone derivative of 6-arylindeno[1,2-*c*]quinoline, which exhibits anti-cancer effects (Tseng et al. 2011). In this study, we investigated the effect of BPIQ on proliferation and cell death of Rb cancer cells using cell model. Our results demonstrated that the anti-tumor effect of BPIQ on human Rb cells Y79. Besides, the mechanism of BPIQ-induced anti-growth and apoptosis was also investigated.

Materials and Methods

Preparation of BPIQ

BPIQ was synthesized as our previously described (Barot et al. 2014). BPIQ was dissolved in 0.01 % dimethyl sulphoxide (DMSO) freshly prior to assays.

Reagents

Medium RPMI 1640 (Life Technologies, NY,USA), fetal bovine serum (FBS), trypan blue, penicillin G and streptomycin were obtained from Gibco (Gaithersburg, MD, USA). DMSO, *N*-acetyl-L-cysteine (NAC, #A9165), ribonuclease A (RNase A) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cytochrome *c*, Bcl-2, survivin, XIAP, Bax, Bad, PARP and ß-Actin were purchased from Santa Cruz, CA, USA. Antibodies against cleaved caspase-3 and caspase-9 were punched from Anaspec (San Jose, CA, USA). Anti-mouse antibodies were purchased from Pierce (Rockford, IL, USA) secondary antibodies were purchased from Pierce (Rockford, IL, USA). Annexin V kit was purchased from Strong Biotech (Taipei, Taiwan).

Cell Culture

Human Rb cell Y79 was purchased from Bioresource Collection and Research Center (#60422, BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Y79 cells were incubated in a 10 cm^2 dish with RPMI 1640 medium supplemented with 15 % non-heat inactivated FBS, 1 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 90 % air and 10 % CO_2.

Morphological Observation

Cells were treated with BPIQ for indicated time courses (24–72 h), and then detected by the morphological changes of cell membrane, organelles by an inverted microscope (TE2000-U; Nikon, Tokyo, Japan).

Cell Proliferation Assessment by XTT-Based Colorimetric Assay

Y79 cell were seeded in 96-well plate at a density of 5×10^3 cells/well (100 µL medium/well) and cultured for 24 h. Afterward, added 100 µL BPIQ to each well with the final drug concentrations (0.5–10 µM) and the plate was

incubated for indicated time courses (24–96 h). To detect the cell proliferation, using 50 mg XTT in 50 ml RPMI 1640 medium) and phenazine methylsulfate (PMS) (19.13 mg in 50 mL ddH_2O) stock solutions were stored at 4 °C for no longer than 10 days. XTT-PMS solution was composed of 5 mL of XTT and 100 µL of PMS stock solutions. The mixture was added 100 µL in a 96 well plate and incubated for indicated time courses (24–96 h). OD values were obtained through reading the plate at 450 nm with a 96-well micro test spectrophotometer.

Assessment of Endogenous Reactive Oxygen Species

The endogenous reactive oxygen species (ROS) levels were detected using a fluorescent indicator 2′,7′-dichlorofluorescin diacetate (DCFDA; Sigma, Lenexa, USA). A total of 1×10^5 Y79 cells were seeded onto a 6-well plate, treated with BPIQ or DMSO, respectively. Y79 cells in suspension (1×10^6 cells/mL) were stained with 100 nM DCFDA for 30 min at 37 °C in phosphate buffered saline (PBS). Then, cells were washed twice with PBS. The levels of endogenous ROS were measured by flow cytometry. The excitation wavelength of DCFDA is 485 nm, and the emission wavelength is 530 nm.

Apoptosis Assay

The apoptosis of Y79 cells was detected by flow cytometry after concurrent staining with Annexin V-FITC apoptosis detection kit (BioVision Research Products, Mountain View, CA 94043 USA). Y79 cells were incubated with indicated concentrations (0.5–10 µM) of BPIQ for 24 and 48 h, respectively. Following incubation, cells were resuspended in 200 µL annexin V-PI binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Afterwards, cells were incubated with 5 µL of annexin-v conjugated with FITC and 5 µL of PI for 20 min at room temperature in the dark. Finally, cells were added with 500 µL cold-PBS and subjected to flow cytometry analysis on FACScan (Becton Dickson, Franklin Lakes, NL, USA).

Cell Cycle Distribution

To analyze the cell cycle, cells of Rb cell line Y79 were adjusted to 5×10^5 cells/mL in a 6 cm Petri dish at 37 °C with 5 % CO_2. The cells were incubated for 24 and 48 h, respectively, and collected by washing three times with sterile PBS. The distributions of the cell cycle were measured by flow cytometry (cell cytometer FACS caliber, American BD Biosciences, San Jose, CA, USA). Cells were harvested by centrifugation, washed with PBS, and fixed with 70 % ethanol overnight at −20 °C. Cells then were washed with PBS and collected by centrifugation. Cells were stained with A 50 µL propidium iodide (50 µg/mL) mixed with 100 µL RNase A (100 µg/mL) at 37 °C for 30 min. Flow cytometric analysis was performed using the FACS caliber with CellQuest™ software (BD Biosciences, San Diego, CA, USA). 10,000 cells were analyzed in each experiment.

Western Blotting

Total cell extracts were made in RadioImmunoPrecipitation Assay (RIPA) lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5 % Sodium deoxycholate, 1 % NP-40 and 0.1 % SDS) stored at 4 °C. The protease/phosphatase inhibitors were added prior to the use of RIPA buffer. Equal amounts of total protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with Tris-based saline-0.5 % Tween 20 (TBST) buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1 % Tween-20) containing 5 % fat-free dried milk for 1 h at 37 °C and incubated with primary antibodies overnight at 4 °C. Membranes were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The nitrocellulose membrane underwent additional washing, and the immunoreactive proteins were visualized using the chemiluminescent reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol.

Statistical Analysis

Data are shown as mean ± SD. All results were analyzed using the Student's t test. Statistical significance was set at $p < 0.05$.

Results

The Inhibitory Effects of BPIQ on the Proliferation of Rb Cells

Our previous works demonstrated the anti-proliferation of BPIQ on several cancer cells, including lung cancer cells, and hepatocellular carcinoma cells (Tseng et al. 2008, 2010). CPT and its quinoline derivatives have been shown to induce apoptosis of human Rb cells Y79 (Han and Wei 2011). We performed the proliferation assay to make a comparison of CPT and BPIQ. Both treatment of BPIQ and CPT in 5 µM slightly decreased cell viability to 87.04 ± 1.21 %; 79.00 ± 2.36 % and to 47.10 ± 1.26 %. CPT also has the same inhibitory effect to 77.71 ± 1.36 %, 77.33 ± 2.58 and 52.95 % ± 1.47 after 24, 48 and 72 h, respectively. The anti-proliferative effect of BPIQ and CPT

on Y79 cells were both dose-responsive and time-dependent. Additionally, IC_{50} values of BPIQ were 18.368, 13.642 and 5.983 μM after 24, 48 and 72 h, respectively. The results showed that both CPT and BPIQ exerted anti-proliferative effect on Y79 cells and there was no significant difference between the two quinoline derivatives. The results suggest the promising potential of BPIQ in Rb treatment (Fig. 1).

BPIQ Significantly Induces Apoptosis of Rb Cell Lines

To determine whether BPIQ-induced anti-growth of Y79 cells by inducing apoptosis, the cytometer-based Annexin V/PI double staining assay was conducted. Y79 cells were inoculated with indicated concentrations of BPIQ for 36 h. Afterwards, cells were stained with annexin V/PI for detecting the externalization of phosphatidylserine from the inner plasma membrane. After treatment with control, 0.5, 1 and 5 μM of BPIQ, the percentages of early-/late-apoptotic cells were 4.92/8.15, 6.34/16.4, 21.96/13.61, and 18.49 %/35.6 %, respectively (Fig. 2). The results showed that BPIQ treatment causes a dramatic dose-responsive increase of late-apoptotic cells in Y79 cells, suggesting the apoptosis-inducing potential of BPIQ on Rb cells.

BPIQ Increases Endogenous ROS Level in Rb Y79

Our results showed that BPIQ inhibits the proliferation of Rb cells. In normal conditions, ROS has been shown to act as a second messenger in cell signaling, and regulates various biological functions (Clerkin et al. 2008; D'Autreaux and Toledano 2007; Ushio-Fukai and Nakamura 2008). However, exposure to higher levels of ROS may lead to aberrance in redox balance that can trigger cell death including cancer cells (Wang and Yi 2008). BPIQ treatment for 24 h increased endogenous ROS levels showed concentrations (0–5 μM) were 3.76 ± 1.08, 18.7 ± 0.68, 20.2 ± 0.20 and 35.2 ± 0.31 % ($n = 3$), respectively. It is well documented that ROS acts in diverse signaling cascades related to different behaviors in cancer cells, such as survival, proliferation, angiogenesis and metastasis (Clerkin et al. 2008; Ushio-Fukai and Nakamura 2008). As shown in Fig. 3, the results showed that BPIQ increases the level of ROS in human Y79 cells in both concentration- and time-dependent manner ($p < 0.05$).

BPIQ-Induced DNA Damage of Rb Cells

γH2AX, a phosphorylation of histone variant H2AX at Ser^{139}, is a biomarker of DNA damage especially the double strand breaks (Valdiglesias et al. 2013). Detection of γH2AX is widely used to monitor DNA damage response. Figure 4a showed the DNA damage induction of BPIQ on Y79 cells using flow cytometry-based detection assay. As shown in Fig. 4b, the folds of γH2AX in cells treated with indicated BPIQ concentrations at 0, 0.5, 1 and 5 μM were 1 ± 0.04, 1.67 ± 0.23, 2.76 ± 0.60 and 4.08 ± 0.66 ($n = 3$) significantly resulted in the activation of γH2AX with a dose-responsive manner, suggesting that the positive role of DNA damage in BPIQ-induced apoptosis of human Y79 cells.

BPIQ Induces Mitochondria-Mediated Apoptosis by Modulating the Pro-Survival Proteins

To investigate the mechanism underlying BPIQ-induced apoptosis of Y79 cells, we examined whether several mitochondrial-related apoptotic markers, such as Bcl-2, Bad, Bax and the endogenous inhibitors of apoptosis (IAP) including survivin and XIAP, involves in regulation of apoptosis in BPIQ-treated Y79 cells. As shown in Fig. 5, the results of Western blot assay showed that BPIQ down-regulates the expression of pro-survival Bcl-2 and two IAP proteins survivin and XIAP, in contrast up-regulates the expression of

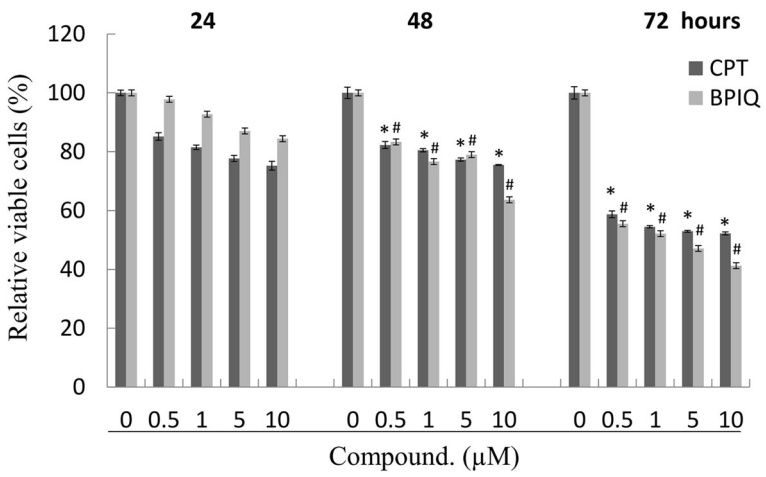

Fig. 1 The effect of BPIQ on cellular proliferation of Rb Y79. Cells were treated with CPT and BPIQ at indicated doses for 24–72 h, respectively. Afterwards, the proliferation rate of cells was determined by XTT assay. The results are presented as a percentage of control by mean ± standard errors (SE) determined from three independent experiments. $^{*}p < 0.005$ compared to CPT control; $^{\#}p < 0.005$ compared to BPIQ control

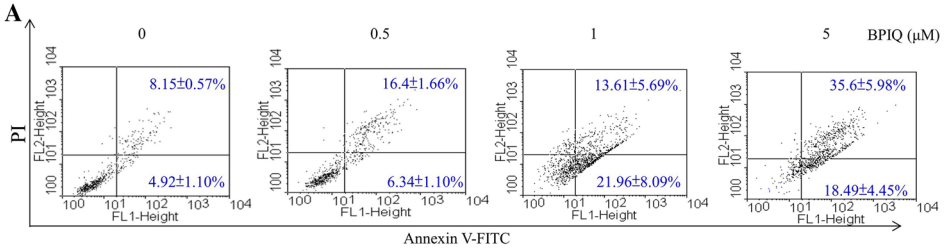

Fig. 2 Flow cytometry-based detection for apoptosis assessment. **a** Cells were treated with indicated concentrations of BPIQ for 36 h, respectively. Afterwards, cells were harvested and detected the apoptosis by Annexin V/PI staining assay. **b** Fluorescence microscopy-based detection using Annexin V/PI double staining after BPIQ treatment. *Scale bars* 100 μm

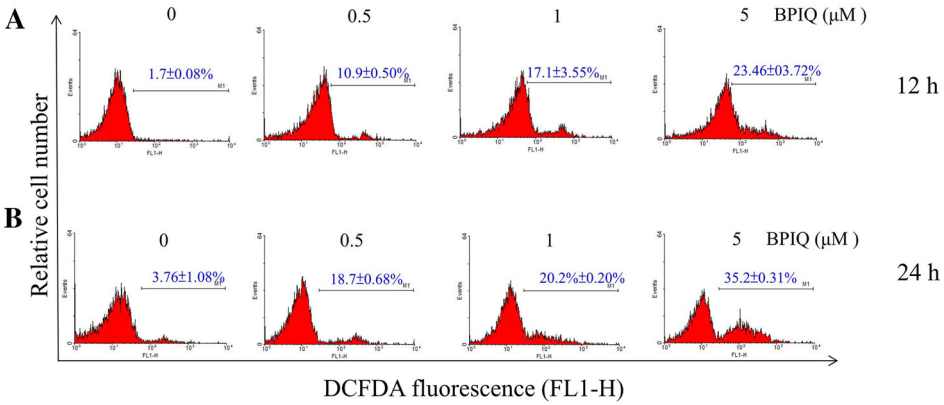

Fig. 3 BPIQ causes the increased endogenous ROS in Y79 cells. Cells were inoculated with indicated concentrations of BPIQ for 12 (**a**) and 24 h (**b**), respectively. Afterwards, the intracellular levels of ROS level were measured by a flow cytometry-based assay described in the section of materials and methods. The results showed that BPIQ induced the increased level of ROS both in time- and dose-dependent manners

pro-apoptotic Bcl-2 proteins including Bad, Bax and Bid (Fig. 5a). Furthermore, the mitochondria-mediated apoptosis pathway, including cytochrome *c* release, the activation of both caspase-9 and caspase-3 were detected following BPIQ treatment (Fig. 5b). Besides, the cleavage of PARP, a substrate of caspase-3 was also observed (Fig. 5c).

ROS Scavenger Rescues BPIQ-Induced Apoptosis of Rb Cell Y79

NAC is commonly used to identify and test ROS inducers, and to inhibit ROS. Based on the above results, we proposed that BPIQ-induced apoptosis of Y79 cells may through the accumulation of intracellular ROS level. To prove the hypothesis, we pretreated Y79 cells with 5 mM NAC, the ROS scavenger, for 1 h; then added BPIQ to a final concentration of 5 μM for further 24 h. As expected, the results of Annexin V/PI showed that pre-treatment of NAC significantly rescues the BPIQ-induced apoptotic cell death of Y79 cells, indicating the role of intracellular ROS in BPIQ-induced apoptosis (Fig. 6).

Discussion

Most tumors of Rb patients are detected at an advanced stage, and there is no expectation that useful vision can be preserved. Therefore, these patients often undergo the enucleation, a surgical technique. To develop ocular salvage approaches, modalities such as chemotherapy and

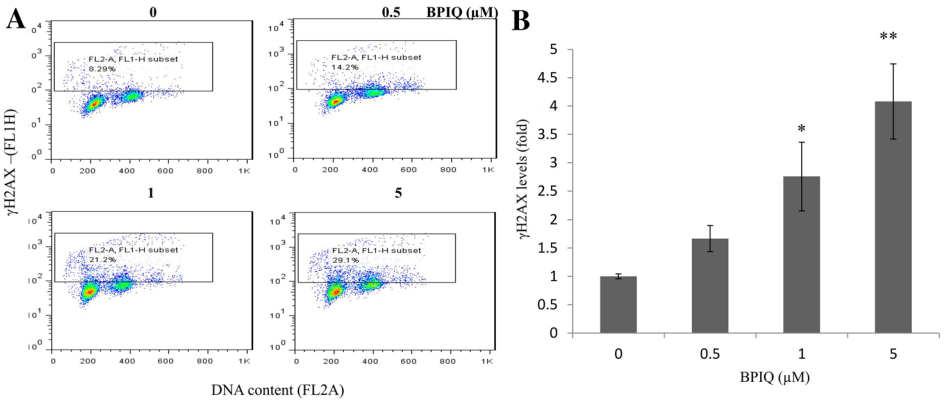

Fig. 4 Assessment of DNA damage induced by BPIQ. **a** Cells were treated with BPIQ indicated doses 0.5, 1 and 5 μM for 24 h, respectively. Data showed that BPIQ increased phosphorylation of γH2AX, a marker of DNA damage in a dose-responsive manner. **b** The phosphorylation of γH2AX in BPIQ-treated Y79 cells and vehicle controls. $*p < 0.005$ compared to control; $**p < 0.001$ compared to control

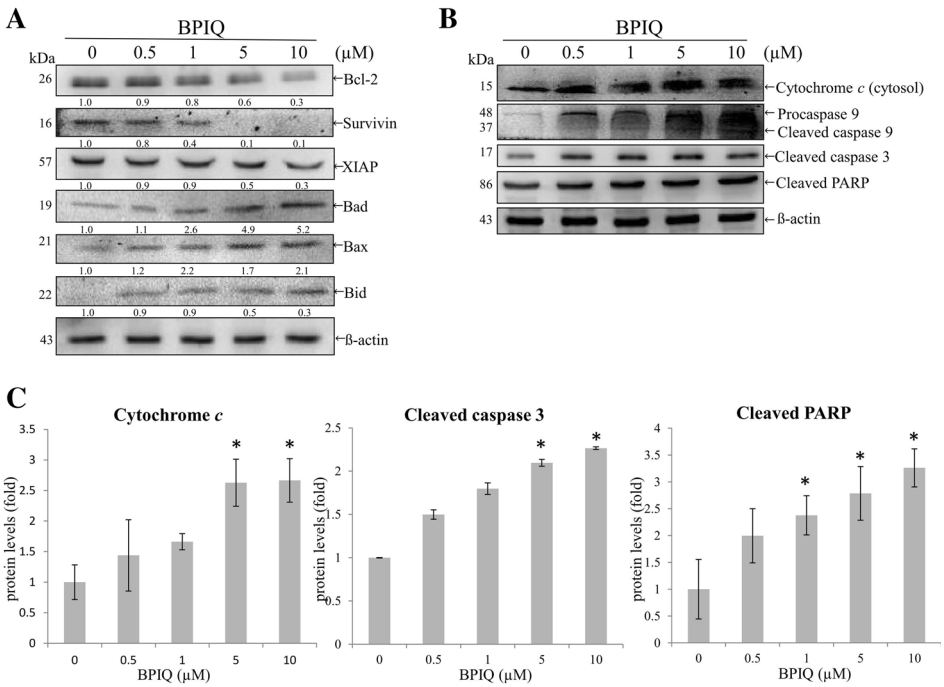

Fig. 5 Analysis of apoptotic markers in BPIQ-treated Y79 cells. Cells were inoculated with indicated concentrations of BPIQ for 24 h. Afterward, Y79 cells were harvested and lysed. The protein lysates were resolved by 10 % SDS-PAGE and analyzed by Western blotting assay. **a** The protein level changes of pro-survival Bcl-2, survivin and XIAP and the pro-apoptotic Bax, Bad and Bid following BPIQ treatments. **b** Detection of the mitochondria-mediated apoptotic cascade, including cytochrome *c*, proteolytic activation of both caspase-9 and the downstream effector caspase-3, as well as the cleavage of poly(ADP-ribose) polymerase (PARP). **c** The quantitative analysis of (**b**) the mitochondria-mediated apoptotic factors, including cytochrome *c*, cleaved caspase-3 and cleaved PARP

local control treatments have been proposed (Shields et al. 2002); however, the results are still controversial. Therefore, to search for compounds that exhibit excellent cancer-killing potential with minimal side effects is the urgent goal of Rb chemotherapy.

2,9-Bis[2-(pyrrolidin-1-yl)ethoxy]-6-{4-[2-(pyrrolidin-1-yl)ethoxy]phenyl}-11H-indeno[1,2-c]quinolin-11-one (BPIQ) is a derivative of 6-arylindeno[1,2-c]quinoline. In previous study, BPIQ was reported to exhibit anti-proliferative effect on non-small cell lung cancer and hepatocellular carcinoma cells (Tseng et al. 2008, 2010). However, there is no study mentioning whether BPIQ has anti-Rb effect. Recently, Han's work showed that CPT induces apoptosis of human Rb cells Y79 (Han and Wei 2011). We

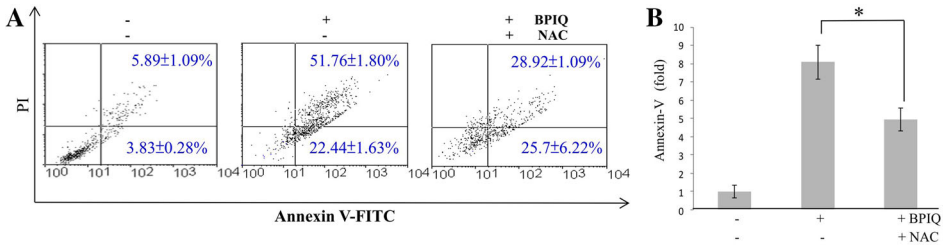

Fig. 6 The effect of ROS scavenger on BPIQ-induced apoptosis of Rb cells. **a** In comparison of BPIQ treatment alone, the pre-treatment of *N*-acetylcysteine (NAC), a well-known ROS scavenger, caused the decrease of BPIQ-induced apoptotic populations of Y79 cells. **b** Results of quantitative analysis. *Data* are presented as mean ± SD ($n = 3$). *$p < 0.05$ vs. BPIQ-treatment alone

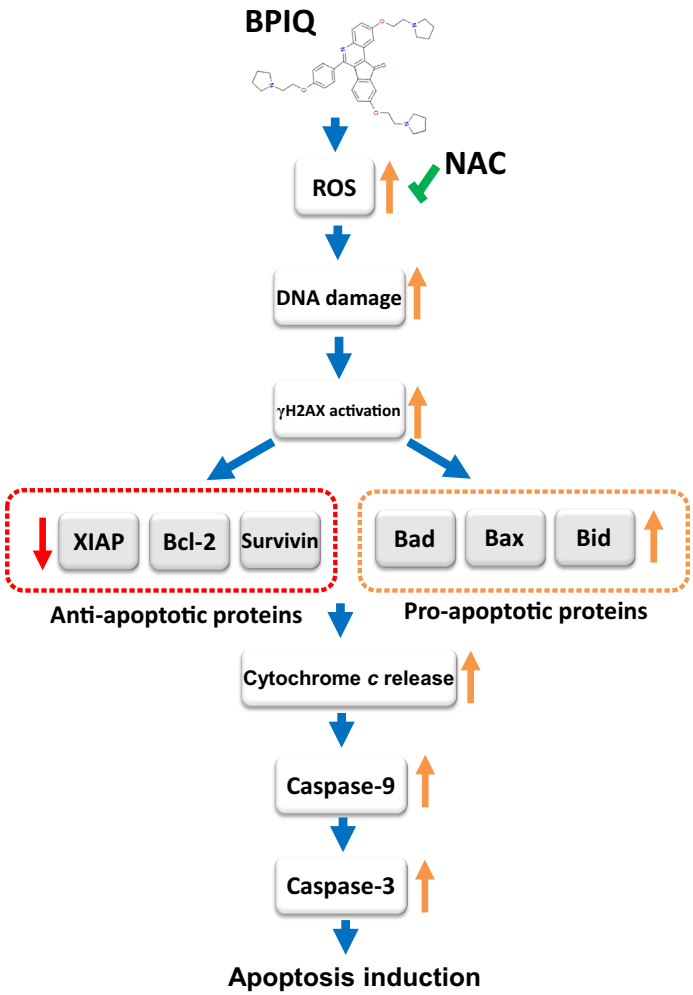

Fig. 7 Proposed mechanism of BPIQ-induced apoptosis of Y79 cells. BPIQ treatment accumulates the levels of endogenous ROS and causes the DNA damage of Rb Y79 cells. γH2AX, an endogenous sensor for detecting DNA damage is activated and results in the disturbance of pro-survival survivin, XIAP and Bcl-2, and pro-apoptotic proteins Bad, Bax and Bid. Sequentially, the imbalance leads to the release of cytochrome *c*, activation of caspase-9 and it downstream effector caspase-3, in turns, resulting in a mitochondria-mediated apoptosis of Rb cells

performed the proliferation assay to make a comparison of CPT and BPIQ, and the results showed that both CPT and BPIQ exert significant anti-Rb activities, suggesting the potential application of BPIQ in Rb treatment (Fig. 1).

Previous studies suggested the mitochondrial-mediated apoptosis may play a critical role in chemotherapy-induced cell death (Kaufmann and Earnshaw 2000). Drugs or another stresses induces the activation of B cell lymphoma 2 (Bcl-2) proteins. Sequentially, the pro-apoptotic Bcl-2 homologues Bad, Bax and Bid, promote the apoptosis cascades. For example, Bax oligomerizes to form a pore in the outer membrane of mitochondria and causes the mitochondrial outer membrane permeabilization (MOMP). The MOMP further causes the release of several factors (Chipuk et al. 2010; Tait and Green 2010), including cytochrome *c*. The release of cytochrome *c* facilitates the

formation of apoptosome, which is composed of cytochrome *c*, caspase-9 and apaf-1. Eventually, the apoptosome activates the downstream effectors caspases such as caspase-3 and caspase-7 (Li et al. 1997), resulting in morphological and biochemical hallmarks of apoptosis (Taylor et al. 2008).

To investigate the role of Bcl-2 proteins in BPIQ-induced apoptosis of human Y79 cells, we examined the levels of both pro-apoptotic proteins and pro-survival Bcl-2 proteins by conducting Western blot assay. Besides, the expression of endogenous inhibitors of apoptosis including XIAP and survivin was examined. After treatment with BPIQ, the level of endogenous ROS increased and this causes the imbalance of the two distinct types of proteins and to facilitate cytochrome *c* release by activating pro-survival proteins Bcl-2, in the meanwhile, inhibiting the expression of pro-apoptotic proteins including Bad, Bax and Bid (Fig. 5a). Furthermore, the results of the Western blot showed the release of cytochrome *c*, the proteolytic activation of both caspase-9 and caspase-3 (Fig. 5b). Additionally, the proteolytically inactivated form of PARP, a hallmark of apoptosis was detected following BPIQ treatment, indicating the mitochondrial-mediated caspase cascade involved in BPIQ-induced apoptosis of Y79 cells.

These results demonstrated that BPIQ inhibits cell growth and induce programmed cell death in Rb cell acting on the balance of pro-survival and pro-apoptotic proteins, suggesting that the anti-Rb effect of BPIQ. The results showed that BPIQ treatment causes a down-regulation of the anti-apoptotic Bcl-2 protein expression and sensitizes human Y79 cells to apoptotic stimuli.

In conclusion, our present study demonstrated the antiproliferative effect of BPIQ in human Y79 cells. The inhibitory effect of BPIQ is, at least partly, mediated by the DNA damage pathway (Fig. 7). BPIQ induces the inhibition of cell growth and apoptosis in Rb cells, indicating that it could be a promising candidate as a chemotherapeutic agent to treat Rb. In summary, our results showed BPIQ may have the potential to serve as an effective chemotherapeutic agent for Rb and the further studies in vitro and in vivo are worthy for investigated.

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