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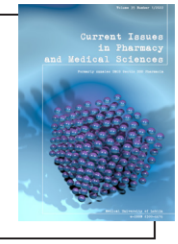

Betulinic acid and its ionic derivatives impaired growth of prostate cancer cells without induction of GRP78 and CHOP

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ABSTRACT

Prostate cancer (PCa) is the most common invasive malignancy for men in the USA. The incidence and mortality rates of PCa are significantly higher among African American men, as compared to those in Caucasian men. Betulinic acid (BA) is a pentacyclic triterpenoid that is often found in the bark of several species of plants. It possesses a variety of biological activities, including anti-cancer activities. We examined the cytotoxic effects and endoplasmic reticulum (ER) stress induced by BA and its ionic derivatives with PCa cells derived from African Americans and Caucasian men. The viability of all PCa cells was reduced by the BA compounds, and the cytotoxicity of these BA compounds was independent of ethnicity and androgen dependency. The BA compounds induced modest effects on ER stress proteins when compared with ER stress inducers, tunicamycin and thapsigargin. The induction of glucose regulated protein 78 (GRP78) was largely correlated with the expression of C/EBP homologous protein (CHOP) and cleaved poly [ADP-ribose] polymerase (PARP)/caspase-3 in the PCa cells. In summary, our data demonstrated that BA compounds impaired the growth of PCa cells regardless of ethnicity – through GRP78- and CHOP-independent pathways.

INTRODUCTION

Prostate cancer (PCa) is one of the most common cancers and second leading cause of cancer-related death among U.S. males. Although many patients are diagnosed with localized PCa, metastatic disease can occur at beginning stages or with development. Most patients with PCa are often initially treated with androgen deprivation therapy, however, eventually, the majority of cells become castration resistant. This clearly demonstrates that PCa progression is highly associated with androgen receptor (AR) signaling, which comprises androgen, its intracellular receptors, transcription factors and a diverse array of genes induced by transcription factors (e.g., steroid receptor coactivator, AR activator, CREB-binding protein) [1]. Several processes, including gene amplifications, gene mutations, changes in co-regulators, and abnormal androgen-generating enzymes have been discovered in the acquisition of AR independence [2].

African American men tend to have early-onset PCa and are generally diagnosed with more advanced stages of PCa at earlier ages in comparison to other races. The fact reflects well on the world's highest incidence and mortality rate of PCa in African American populations [3]. Lines of evidences suggest that environmental, social and genetic factors engender the health disparity of PCa [4]. Indeed, accumulation of biomedical information has revealed some unique phenotypes in PCa cells derived from African American men, for instance, increased cell replication, enhanced metastasis capacity, and altered apoptosis. Furthermore, several researches, particularly, recent genome-wide association studies, have found a substantial link between PCa and certain biochemical markers that are only seen in the African American community (e.g. abnormal protein expression, gene amplification, and unique SNPs) [1,5].

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Betulinic acid (3β-hydroxyl-lup-20(29)-en-28-oic acid, BA) is a natural pentacyclic lupine type triterpene that is developed and extracted from the bark of birch trees [6]. This natural compound has numerous biological properties related to medicine, including anti-inflammatory, anti-cancer and anti-viral activities, that are induced by triggering certain types of molecules in cells [7]. However, the limited solubility of BA in aqueous solutions and common organic solvents such as esters, alcohols, and ethers is a major obstacle in releasing its biological activity [8,9]. When compared to BA, certain BA compounds demonstrate improved water solubility, as well as elevated biological activity [10,11], especially for ionic derivatives of BA [12].

The unfolded protein response (UPR) refers to a set of responses triggered by the accumulation of unfolded proteins in endoplasmic reticulum (ER), and molecules involved in the response primarily relieve ER stress and promote cell survival by regulating the balance between the protein load and folding capacity in the ER. Growing evidence has shown that cellular defense machinery induced by ER stress has a critical role in mediating the viability of cancer cells. Glucose regulating protein 78 (GRP78), an ER chaperone that promotes tumor proliferation, metastasis, apoptosis and resistance to a number of treatments, is among the most essential modulators of the UPR [13]. Although UPR is predominantly a pro-survival activity, sustained ER stress and failure of the adaptive response cause cell death through apoptosis [14]. A previous report demonstrated that BA chemosensitized breast cancer cells by triggering ER stress-mediated apoptosis [15]. In the present study, we assessed ER stress-mediated cytotoxic effects induced by BA compounds in PCa cells.

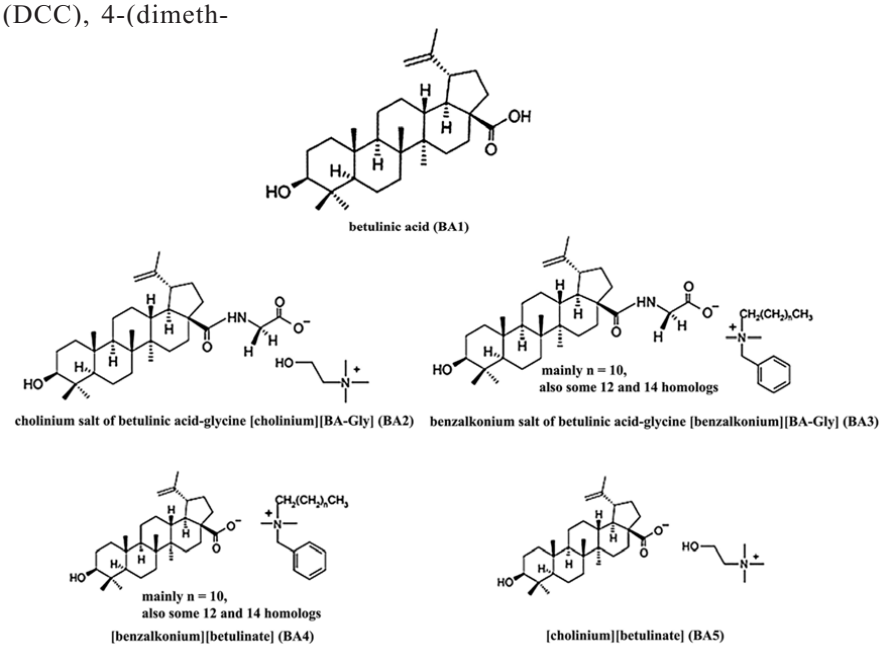
MATERIALS AND METHODS

Preparation of ionic salts of betulinic acid conjugated with glycine

Betulinic acid, glycine methyl ester hydrochloride, N, *N*'-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino) pyridine (DMAP), choline chloride, benzalkonium chloride (BKC) and Amberlyst A26 hydroxide (Sigma-Aldrich, St. Louis, MO) were used to prepare ionic salts of betulinic acid conjugated with glycine. The detailed procedures to prepare ionic derivatives of betulinic acid were described previously [12]. In this study, betulinic acid, betulinic acid-glycine, benzalkonium salt of glycinylated betulinic acid, benzalkonium salt of betulinate and cholinium betulinate are referred as BA1 to BA5 (Fig. 1). All compounds were dissolved in DMSO initially at 10 mg/mL as stock solutions and were further diluted by cell culture media.

Cells and cell culture

LNCap, DU145 and PC-3 cell lines were derived from Caucasian prostate cancer patients. LNCaP was established from a supraclavicular lymph node meta-static lesion o f human prostate adenocarcinoma which exhibited androgen sensitive growth [16]. DU145 was derived from a primary prostate adenocarcinoma metastasized to the central nervous system [17]. DU145 cells express detectable AR mRNA and protein, but are considered as androgen non-responsive [18]. PC-3 was established from a prostatic adenocarcinoma meta-static to bone, which exhibited non-sensitivity against androgen [19]. E006AA-Par, E006AA-hT, and RC77T/E cells were derived from African American men. E006AA-Par was established as spontaneously immortalized cells from a patient with a clinically localized prostate cancer and shows androgen-dependent growth [20], E006AA-hT is a highly tumorigenic subline of E006AA-Par [21]. RC77T/E was established through transformation of the tumor tissue obtained from a radical prostatectomy specimen with HPV-16E6E7 and is responsive to androgen [22]. LNCaP was obtained from American Type Culture Collection (Manassas, VA). PC-3 and DU145 were donated by Dr. Fan (University of California, Irvine). E006AA-Par and E006AA-hT were kind gifts from Dr. Shahriar Koochekpour (Roswell Park Cancer Institute), while RC77T/E was acquired from Dr. Clayton Yates (Tuskegee University) with the kind assistance of Dr. Honghe Wang. LNCaP cells were maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. PC-3, DU-145, E006AA-Par and E006AA-hT cells were cultured and maintained in DMEM medium containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin. RC77T/E cells were maintained in Gibco Keratinocyte Serum-Free Medium (SFM; Thermo Fisher Scientific, USA) supplemented with human recombinant epidermal growth factor and bovine pituitary extract (25 mg) and 1% penicillin/streptomycin. All cells were grown at 37°C with 5% CO_2.



Structures of betulinic acid (BA1) and ionic derivatives (BA2-BA5) used in this study

Figure 1. Betulinic acid and ionic derivatives of betulinic acid

AlamarBlue Cell Viability Assay

Cell viability was assessed using the alamarBlue Cell Viability Assay (Bio-Rad, USA) as described previously [23]. In brief, the PCa cells (5 x 10^3) placed on 96-well plates were incubated overnight, treated with DMSO, 10-50 µM of betulinic acid or its ionic derivatives for 48 hours and then alamarBlue solution was added to each well. Following the 4 to 8-hour incubation at 37 °C with 5% CO_2, the reaction was stopped, and absorbance of 570 nm/600 nm was measured to determine the reduction of resazurin dye. The DMSO-treated cells were set to 100% and then the viability readings from treated wells were calculated as a percentage of the control.

SDS-PAGE and western blots

The PCa cells were treated with 50 µM of BA1 and BA2, 1 µM tunicamycin (TM) and 0.3 µM of thapsigargin (TG) for 48 hours. Cellular proteins were extracted with SDS sample buffer consisting of 62.5 mM Tris (pH 6.8), 10% (v/v) glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.001% bromophenol blue. The samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The blots were incubated for 2 hours at room temperature in blocking buffer consisting of 5% non-fat dry milk in Tris-buffered saline with 0.05% (v/v) Tween 20 (TBST), then overnight at 4°C with primary antibodies diluted in TBST. The blots were incubated with horseradish peroxidase-conjugated mouse, rabbit and rat secondary antibodies (Invitrogen, USA) for 1 hour at room temperature. The images were acquired by the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Gel Doc™ XR+ Gel Documentation System (Bio-Rad, CA, USA). To quantify protein signals on the membranes, the densitometry software known as Image J and AlphaEaseFC (Alpha Innotech, CA, USA) were applied. The following primary antibodies were used to detect poly [ADP-ribose] polymerase (PARP): Cell Signaling Technology #9542 and Invitrogen 14666782, clone HC2R8; cleaved PARP: Cell signaling #5625; caspase-12: Proteintech #55238-1-AP; CHOP: Proteintech #15204-1-AP; caspase-3: Cell Signaling Technology #14220 and Santa Cruz Technology, sc-56053; cleaved caspase-3: Cell Signaling Technology, #9664; and beta Tubulin: Invitrogene MA516308, clone BT7R.

Statistical analysis

Welch's t-test was employed to evaluate the cell viability of betulinic acid and its derivatives on PCa cells.

RESULTS

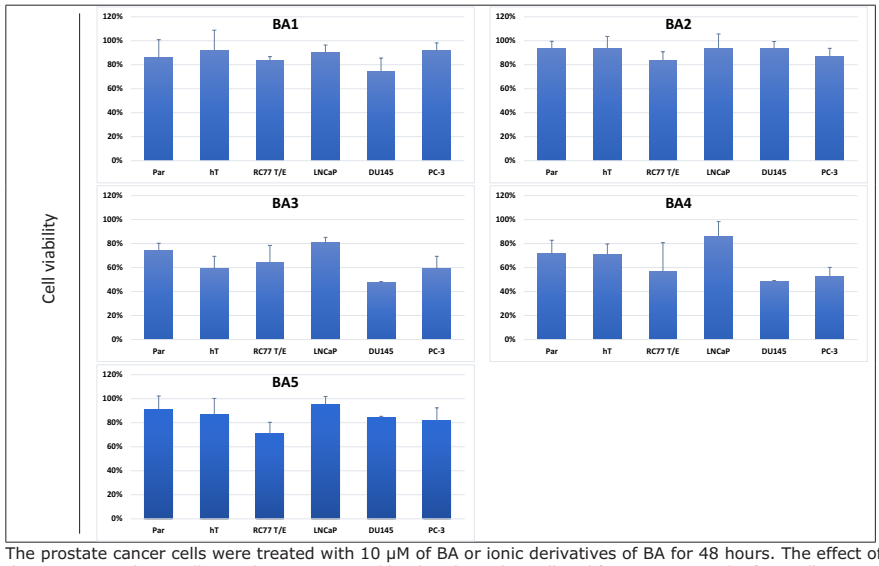
To measure the cytotoxic effect of BA1 and BA derivatives (BA2-BA5) on PCa cells, six PCa cells that were derived from African American and Caucasian men, respectively, were treated with 10 µM of each BA compound for 48 hours. The cell viability was evaluated using an alamarBlue cell proliferation assay that measured the conversion of resazurin to resorufin in viable cells. All compounds reduced the viability of some PCa cells at 10 µM, but the sensitivity against each compound was different among each PCa cell line. BA1, BA2 and BA5 showed moderate cytotoxic effects, and induced 0-30% reduction on cell viability (Fig. 2). BA3 and BA4 exhibited considerably stronger cytotoxicity, and reduced cell viability by 14-52%. Proliferation of RC77 T/E cells was impaired by the treatment of all compounds, BA1-BA5 (Table 1). E006AA-Par and E006AA-hT were resistant except for BA3 and BA4. Proliferation of LNCap and PC-3 was impaired by BA1 and BA3. DU145 showed less growth against BA3, BA4 and BA5. These data suggest that the induced cytotoxic effects of BA and its ionic derivatives could depend on the nature of individual PCa cells rather than the origin of ethnicity or AR dependency.

The cell viability data described above (Tab. 1 and Fig. 2) demonstrate that BA3 and BA4 imposed stronger cytotoxic effects on PCa cells. Both BA3 and BA4 containing the benzalkonium cation and BKC mixtures have been reported to bring about cell death through unknown

Table 1. Comparison of cytotoxicity of betulinic acids and its ionic derivatives toward PCa cell lines

Cell line	Par*	hT*	RC77T/E	LNCaP	DU145	PC-3
Ethnicity**	AA	AA	AA	CA	CA	CA
Androgen***	D	D	D	D	ID	ID
BA1			p<0.01	p<0.05		p<0.05
BA2			p < 0.05			
BA3	p<0.05	p<0.01	p<0.01	p<0.05	p<0.01	p<0.01
BA4	p<0.05	p<0.01	p<0.05		p<0.01	
BA5			p <0.01		p<0.05	

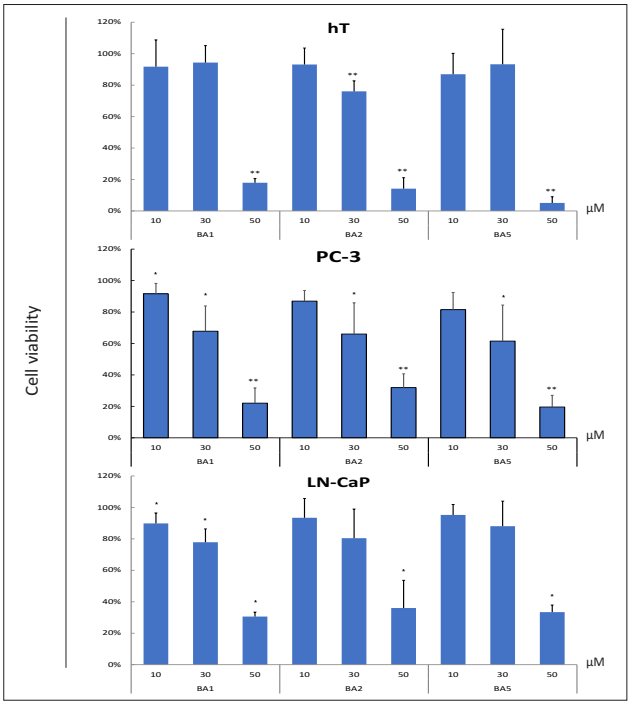
Differences of cell viability to control samples (DMSO) with statistical significance are shown. * Par and hT indicate each E006AA and E006AA-hT. ** Ethnicity shows origins of prostate cancer cells: AA - African American, CA - Caucasian. *** Androgen indicates androgen dependence: D - dependent, ID - independent. Welch's t-test was used to evaluate the cell viability of betulinic acid and its derivatives on PCa cells



The prostate cancer cells were treated with 10 µM of BA or ionic derivatives of BA for 48 hours. The effect of these compounds on cell growth was measured by the AlmarBlue cell proliferation assay. The figure illustrates the relative cell proliferation to the control. The experiments were done in triplicate, and the averages and standard deviations of two or more experiments are shown. Par and hT indicate each E006AA and E006AA-hT

Figure 2. Cell viability of prostate cancer cell lines in the presence of betulinic acid compounds

mechanisms [24,25]. Indeed, both BA2 and BKC displayed cytotoxicity on NIH3T3 cells (a mouse fibroblast cell line) independently [23]. To measure cytotoxic effects of benzalkonium salts-free BA derivatives, the viability of E006AA-hT, LNCaP, and PC-3 cells were assayed with doses of BA1, BA2 and BA5. The cytotoxicity of BA and its ionic derivatives was found to be slightly different among the cells (Fig. 3), and 50 µM of these compounds inhibited growth of all three PCa cells.



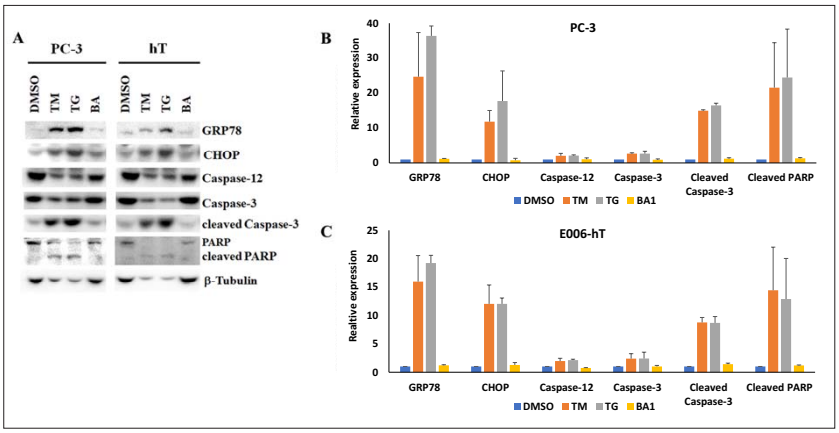
* p < 0.05, ** p < 0.01
Prostate cancer cells were treated with 10, 30, or 50 µM of BA1, BA2 and BA5 for 48 hours. The effect of these compounds on cell growth was measured by the alamarBlue cell proliferation assay. The figure illustrates the relative cell proliferation to the control. The experiments were done in triplicate, and the averages and standard deviations of three or more experiments are shown. Welch's t-test was used to evaluate the cell viability of betulinic acid and its derivatives on PCa cells

Figure 3. Comparison of the viability of PCa cells dosed with betulinic acid compounds

We next tested if the cytotoxicity induced by BA is associated with ER stresses by using E006AA-hT and PC-3 cells. Accordingly, the expression of ER stress and apoptotic signals was assessed by western blots, and the signals on the membranes were quantified by immunedensitometry software. These signals were normalized with the internal control (beta-Tubulin) signals. The effect of BA1 on GRP78 expression was seen to be modest in contrast to tunicamycin (TM) and thapsigargin (TG), which are known as potent ER stress inducers (Fig. 4). We found that the expression of GRP78 was largely correlated with the expression of CHOP, cleaved PARP and cleaved caspase-3. Moreover, the effects of BA1 on ER stress and apoptosis signals were similar between E006AA-hT and PC-3 cells (Fig. 4), while the expression of GRP78, CHOP and caspase-12 in the cells treated with BA1 and BA2 were comparable (data not shown). These data suggest that enhancement of ER stress could induce apoptosis in PCa cells, but ER stress triggered by BA compounds was limited in PCa cells.

DISCUSSION

Among many studies describing anti-cancerous effects of BA1 on the types of cancers [26,27], some papers revealed that BA1 demonstrated cytotoxic effects on PCa cells [28-33]. These reports used PCa cells derived from Caucasian men such as LNCaP, DU145 and PC-3 cells, and showed that BA1 impaired the viability of PCa cells through alteration of cell cycle and pro-survival proteins (e.g., reduction of cyclins, CDKs, vascular endotherial growth factor, survivin, Bcl-2, Akt and nuclear levels of NF-κB/p65). However, the effects of BA compounds on PCa cells derived from African Americans remained unknown because the availability of PCa cell lines established from African American men was highly limited [34]. The current study extended the previous findings by demonstrating that BA1 and derived ionic derivatives of BA (BA2-BA5) reduced the viability of PCa cells derived from both African American and Caucasian men (Fig. 2 and Tab. 1). The cytotoxic effects of BA compounds used in this study varied among PCa cells, and our data suggests that the cytotoxicity of BA compounds depends on how individual PCa cells were established rather than the ethnicity and AR dependence. As a matter of fact, E006AA-hT is a subline of E006AA-Par cells that showed a similar DNA profile with stronger tumorigenicity in intact NUDE and NOG-SCID mice [21], and the cytotoxic effects of BA compounds were similar between the two cell lines (Table 1). Treatment of doses of BA derivatives that did not contain BKC (BA1, BA2 and BA5) indicated that E006AA-hT, PC-3 and DU145 have slightly different sensitivities against BA1, BA2 and BA5 (Fig. 3). These data corroborated the idea that tumor heterogeneity could be a determinant of the cytotoxic effects of BA compounds on PCa cells.



PC-3 and E006-hT (hT) cells were treated with 50 µM of BA1, 300 nM of thapsigargin (TG), and 1 mM of tunicamycin (TM) for 48 hours. The same volume of whole cell lysates was loaded on the acrylamide gels. The proteins on the membranes were detected by the antibodies shown in the panel (**A**). The signals on the membranes were quantified by densitometry software and normalized based on beta-Tubulin in each sample. The relative expression of proteins to control (DMSO treated) cells was shown. The bars represent the average and standard deviation, respectively, calculated from two or more independent measurements in PC-3 (**B**) and E006-hT (**C**) cells

Figure 4. Detection of ER stress and apoptotic signal proteins

ER stress exhibits pro-survival effects on tumor development and progression, but the prolonged activation of ER stress may induce apoptosis [14,35]. It was reported that BA1 elevated GRP78 and triggered CHOP mediated-apoptosis in breast cancer and glioblastoma cells [15,36]. The data prompted us to investigate the effect of BA compounds on some proteins relevant to ER stress in PCa cells. In contrast to our expectations and the previous reports [15,36], the BA compounds showed modest effects on the expression of ER stress and apoptotic signal proteins in both E006AA-hT and PC-3 cells. Interestingly, tunicamycin and thapsigargin induced ER stress and apoptosis in both PCa cells, suggesting that induction of ER stress could trigger apoptosis in PCa cells regardless of ethnicity, although the possibility of independent activation of ER stress and apoptosis by these compounds cannot be excluded [37]. Since the treatment of 50 μM of BA compounds showed 70-80% reduction in cellular metabolic activities measured by alamarBlue cell proliferation assay (Fig. 3), our observations suggest that BA1 and its ionic derivatives could impair metabolic activities through GRP78- and CHOP-independent mechanisms. We suggest that the metabolic activities impaired by BA compounds in PCa cells can be explained by BA1-induced alteration of certain metabolic pathways (e.g., the inhibition of glucose production by modulating cAMP response element-binding protein in hepatoma cells, the impairment of c-Myc-mediated glycolysis in glioblastoma cells) [36,38]. However, further studies are needed to clarify the molecules modulated by BA compounds in PCa cells.

BKC is an organic salt classified as a quaternary ammonium compound that is composed of a mixture of alkylbenzyl dimethylammonium chlorides with several analogues varying in length of their aliphatic alkyl chains. BKC shows a variety of biological effects, including antimicrobial activities, the enhancement of phagocytosis, and the induction of cell death [24,25,39]. The main purpose of our current study was to assess the cytotoxic effects of BA2 and BA5 on different types of PCa cells, but the data using BA3 and BA4 suggests that BKC expresses synergistic effects on the viability of PCa cells. We noted that LNCaP and PC-3 cells were sensitive against BA3, but not against BA2 and BA4 (Table 1). Since BA3 and BA4 contain benzalkonium salt along with BA2 and BA5, our data implies that benzalkonium salt could enhance the cytotoxic effects of BA compounds.

Our work demonstrated that the viability of E006AA-Par, E006AA-hT and PC-3 cells was impaired by BA3, but not BA2. The data also suggest that BKC could enhance the cytotoxic effects of BA compounds, although cytotoxic effects induced by benzalkonium salts alone cannot be excluded in these PCa cells – as observed in experiments using NIH3T3 cells [23]. A previous report using human cancer cell lines, A375 (melanoma), SH-SY5Y (neuroblastoma), MCF7 (breast adenocarcinoma), and A431 (epidermoid carcinoma) demonstrated that IC50 values for BA3 were lower than those for BA2 [12]. In contrast, IC_{50} values on the cytotoxicity of BA4 were comparable or higher than those for BA5 in these cells. Previous work also reported that quaternary ammonium compounds, including cetrimonium bromide, benzethonium chloride and BKC, selectively increased the cytotoxicity induced by doxorubicin, but not camptothecin in RKO cells expressing E6 [40]. Thus, the cytotoxic effect enhanced by benzalkonium salts could depend on both target cells and the combination of drug molecules.

CONCLUSION

Our data demonstrated that BA and its ionic derivatives inhibited the growth of PCa cells regardless of ethnicity and AR dependency and suggested that apoptosis could be inducible through ER stress in PCa cells. These observations indicate that BA compounds and other chemicals inducing ER stress could be potentially useful in PCa therapeutics.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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