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A novel benzotriazole derivative inhibits proliferation of human hepatocarcinoma cells by increasing oxidative stress concomitant mitochondrial damage

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Abstract

Benzotriazole derivatives have been shown to be able to induce growth inhibition in cancer cells. In the present study, we synthesized bioactive compound, 3-(1H-benzo [d] [1,2,3] triazol-1-yl)-1-(4-methoxyphenyl)-1-oxopropan-2-yl benzoate (BmOB), which is a novel benzotriazole derivative. BmOB displayed anti-proliferative effects on several human tumor cell lines. Human hepatocarcinoma BEL-7402 cell line was selected as a model to illustrate BmOB's inhibition effect and its potential mechanism, since it was the highest susceptible cell line to BmOB. It was shown that treatment with BmOB resulted in generation of reactive oxygen species, disruption of mitochondrial membrane potential ($\Delta\Psi m$), and cell death in BEL-7402 cells. BmOB induced cytotoxicity could be prevented by antioxidant vitamin C and mitochondrial permeability transition inhibitor cyclosporine A. cyclosporine A could also protect the BmOB induced collapse of $\Delta\Psi m$ in BEL7402 cells, while vitamin C did not show similar effects. The results suggest that BmOB could inhibit BEL-7402 cell proliferation, and the cell death may occur through the modulation of mitochondrial functions regulated by reactive oxygen species. It appears that collapse of $\Delta\Psi m$ prior to intracellular reactive oxygen species arose during the cytotoxic process in our experimental system.

Keywords: Benzotriazole; Apoptosis; Mitochondrial membrane potential; Reactive oxygen species

1. Introduction

Benzotriazole derivatives are of wide interest because of their diverse biological activity and potential clinical applications (Katarzyna et al., 2004; Kamal et al., 2006). The 1H-benzotriazol compounds regulate important pharmacological activities such as anti-inflammatory, antiviral, antifungal, antineoplastic and anti-depressant activities [Katarzyna et al., 2004; Al-Soud et al., 2003; Wu et al., 2006]. 1- and 2-[3-(1-piper-azinyl) propyl]-benzo-triazoles showed in vitro remarkable antiserotonergic, antiadre-nergic and antihistaminic activity, as well as in vivo analgesic action (Al-Soud et al., 2003). A class of stable benzotriazole esters was also reported as mechanism-based inactivators of SARS-3CL^{pro}, which has been shown to be essential for replication of SARS virus (Wu et al., 2006). It was noted that 4,5,6,7-tetrabromobenzotriazole (TBB),is a good inhibitor of hepatitis C virus helicase. The mode of action of the inhibitors in the case of the helicases is not by interaction with the catalytic ATP-binding site, but rather by occupation of an allosteric nucleoside/nucleotide binding site (Borowski et al., 2003).

It has been proposed but not yet demonstrated that the benzotriazole derivatives have an effect on cancer development. The cell growth studies showed that steroidal C-17 benzoazoles inhibit the growth of prostate cancer cells (Handratta et al., 2005). It is reported that 4, 5, 6, 7-tetrabromo-1H-benzotriazole (TBB) is a potent inhibitor of protein kinase CK2 (Sarno et al., 2001; Ruzzene et al., 2002). Evidence is accumulating concerning the involvement of CK2 in apoptotic events, such as its interaction with Fas-associated factor-1 (Guerra et al., 2001), and its possible role in regulating p53 function (Landesman-Bollag et al., 1998; Sayed et al., 2001). By blocking CK2, TBB displays a remarkable

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pro-apoptotic effect on a number of tumor derived cell lines (Szyszka et al., 1995; Sarno et al., 2001). The complex [2-(4,5-dihydro-1H-imidazol-2-yl)-1H-benzotriazole]-dichlorocopper (II) showed very potent SOD activity and inhibited the growth of seven human tumor cell lines (Saczewski et al., 2007). It is interesting to note that a series of [4-(2H-1,2,3-benzotriazol-2-yl) phenoxy]alkanoic acids has been synthesized and tested as agonists of peroxisome proliferator-activated receptor (PPAR) α, γ and δ (Sparatore et al., 2006). PPARγ is a member of the nuclear receptor superfamily of ligand activated transcription factors (Rosen and Spiegelman, 2001; Willson et al., 2001), which is highly expressed in many tumor samples and cancer cell lines derived from hematopoietic and nonhematopoietic tumors (Ikezoe et al., 2001). Several studies show that PPARγ agonists inhibit growth and/or induce apoptosis in multiple cancer cell lines and in *in vivo* tumor models (Place et al., 2003; Qin et al., 2003; Chang and Szabo, 2000; Elstner et al., 1998). The newly synthesized 3-(1H-benzo[d][1, 2, 3]triazol-1-yl)-1-(4- methoxyphenyl)-1-oxopropan-2-yl benzoate in our lab builds upon and relates to these studies [Handratta et al., 2005; Sarno et al., 2001; Ruzzene et al., 2002; Guerra et al., 2001; Szyszka et al., 1995; Saczewski et al., 2007; Sparatore et al., 2006].

It is generally recognized that tumor growth occurs when cells lose the normal balance between cell proliferation and apoptosis (Rust and Gores, 2000; Ge et al., 2000; Kim et al., 2005). Recent evidence implicates apoptosis as a contributing mechanism of chemotherapy-induced tumor cytotoxicity (Eastman, 1990). Accumulating evidences have indicated that a reduction in mitochondrial transmembrane potential ($\Delta\Psi m$) accompanies early apoptosis in many situations and oxidative stress also appears to regulate apoptosis(Green and Reed, 1998; Danial and Korsmeyer, 2004). Although the possible role of 4, 5, 6, 7-tetrabromo-1H-benzotriazole (TBB) on cancer cell apoptosis have been documented, the regulating pathway of other benzotriazole derivatives on cell growth has not been assessed. Building on our research on the synthesis of benzotriazole derivatives (Wan et al., 2006), nine benzotriazoles compounds were studied using cytotoxicity assay. The results of this assay lead us to select 3-(1H-benzo[d][1, 2, 3] triazol-1-yl)-1-(4- methoxyphenyl)-1-oxopropan-2-yl benzoate (BmOB) for the present investigation because of its obvious growth inhibition in hepatocarcinoma BEL-7402 cells. Our research investigated the anti-proliferation effects of this compound as well as the potential mechanisms involved in this action.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium, fetal bovine serum (FBS), Dexamethasone, 2, 7-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Gibco (BRL, Gaithersburg, MD, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), Trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Streptomycin sulfate, Penicillin G sodium salt, 5-Fluorouracil, Trypan Blue, RnaseA, were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Synthesis of BmOB

BmOB was synthesized according to the protocol we published (Wan et al., 2006). The structure of the compound was established on the basis of elemental analysis and spectral data (Scheme 1).

2.3. Cell culture

The cell lines used in the present study are listed in Table 1. Human hepatocarcinoma BEL-7402 cells and human breast MCF-7 cells are a generous gift from Dr. XiuKun Lin, Institute of Oceanology, Chinese Academy of Sciences. Human prostatic stromal cells are propagated as described [Janssen et al., 2000]. All other cell lines were acquired from the American Type Culture Collection and were cultured under conditions recommended by the vendor. Hepatocarcinoma BEL-7402 cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml, final concentration, the same below), streptomycin (100 μg/ml).All cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. BmOB was dissolved in dimethyl sulfoxide (DMSO), and equal amounts of this solvent were included in control reaction mixtures.

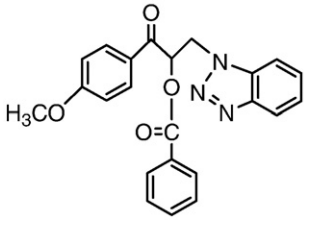
2.4. Cell survival assays

The conversion of the dye MTT to formazan crystals by cellular dehydrogenases was used as an index of cell viability. After incubating the cells with BmOB under different experimental conditions, MTT was added to cultures at a final concentration of 0.5 mg/ml and incubated for 2 h. Then an equal volume of 20% sodium dodecyl sulphate (SDS) in 50% dimethylformamide was added to each well and the reaction product was resuspended overnight. Results were read as the absorbance at 570 nm (Berridge and Tan, 1993).

ID_{50} were determined in duplicate in every set of experiments, and each experiment was repeated three times under identical conditions. The cells were incubated for 48 h at 37 °C. ID_{50} were defined as drug concentrations that induced 50% cellular death in comparison with untreated controls and calculated by nonlinear regression analysis (Jiang et al., 1998).

2.5. DNA fragmentation and flow cytometry assay

After treatment with BmOB, the cells were collected and washed three times with PBS. The cells were then disrupted, and



Scheme 1. 3-(1H-benzo[d][1,2,3]triazol-1-yl)-1-(4-methoxyphenyl)-1-oxopropan-2-yl benzoate (BmOB).

Table 1
The inhibitory activity of BmOB against various human tumor cell lines

Cell type	Cell line	IC_{50} (mM)
Normal hepatocytes (human)		3.360±0.027
Hepatocarcinoma (human)	BEL 7402	0.082±0.008[a]
Human prostatic stromal cells		1.235±0.031
Prostate cancer	PC3	0.150±0.016[b]
Human kidney epithelium	HK-2	0.983±0.025
Kidney cancer	ACHN	0.256±0.034[c]
Normal human mammary epithelial cells	184-A1	0.783±0.038
Breast cancer	MCF-7	0.154±0.018[d]
Normal human lung cells	NL20	2.330±0.083
Human NSCLC	H 460	1.338±0.024[e]

Data are presented as means±S.D., [a]$P<0.05$ *versus* normal hepatocytes; [b]$P<0.05$ *versus* human prostatic stromal cells; [c]$P<0.05$ *versus* human kidney epithelium; [d]$P<0.05$ *versus* normal human mammary epithelial cells; [e]$P<0.05$ *versus* normal human lung cells.

the nucleoplasm was separated from the high-molecular-weight chromatin. DNA fragments were purified and analyzed by conventional electrophoresis on a 1.2% agarose gel containing 0.375 mg/l ethidium bromide as described previously (Spencer et al., 2000; Galeano et al., 2005; Canelles et al., 1997). The Annexin V Apoptosis kit (Gene Research Lab, Taiwan) was used to assay apoptosis according to the manufacturer's instructions, and the cells were analyzed by flow cytometry (CyFlow®SL, Partec, Germany).

2.6. Preparation of mitochondria

Liver mitochondria from 1-month-old Swiss Mice were prepared by differential centrifugation as described (Qu et al., 1999). The respiratory control ratio (state 3/state 4) with succinate as substrate was measured to ensure that the mitochondria obtained were intact.

2.7. Measurement of reactive oxygen species and mitochondrial membrane potential

Production of intracellular reactive oxygen species was monitored by the fluorescence emission of DCFH-DA within the cell (Carmody and Cotter, 2001). 10^6 cells at different experimental conditions were incubated with 2 μM DCFH-DA for 30 min at 37 °C. The cells were then washed twice with PBS and the fluorescence emission was read at excitation of 503 nm and emission of 529 nm in a F-4500, Hitachi (Japan) fluorescence spectrophotometer. Intracellular levels of reactive oxygen species were also determined by fluorescence microscope image analysis. Mitochondrial membrane potential ($\Delta\Psi m$) was measured by incubating the cells or isolated mitochondria with JC-1 at 37 °C for 20 min as described previously (Reers et al., 1991).

2.8. Statistics

All data are reported as means±S.D., except where indicated. Comparisons among multiple groups were subjected to a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference post-hoc test. The sample *t*-test was used in two group comparisons. In case the data were not normally distributed, a Wilcoxon signed ranks test was used. The 95% confidence limit ($P<0.05$) was considered statistically significant. All the data presented in the figures were obtained from at least three independent experiments.

2.9. Statement

All the experimental procedures were carried out following the guidelines of the Qingdao University of Science and Technology.

3. Results

3.1. BmOB's inhibitory effect on tumor cell lines

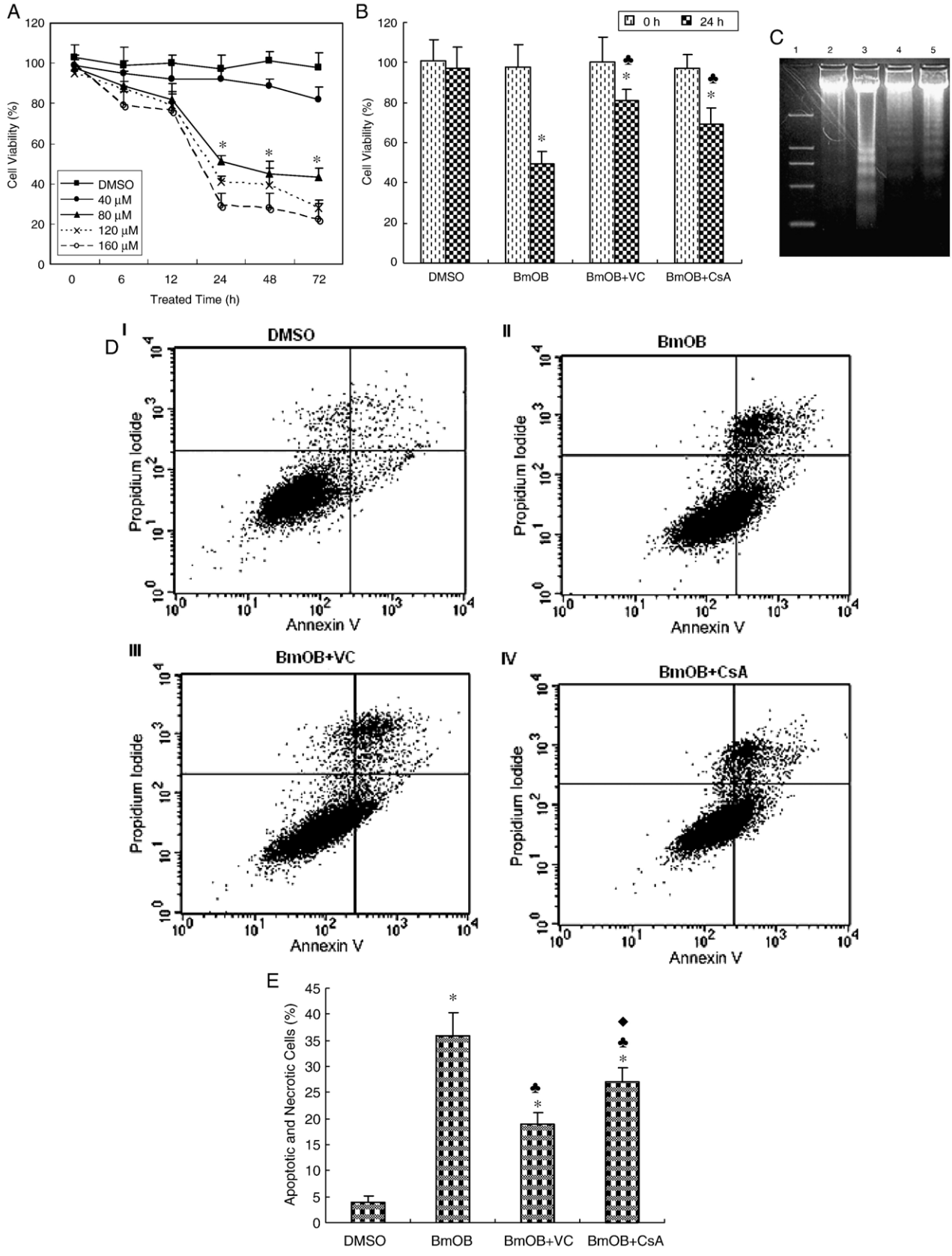
The inhibitory effect of cell growth of BmOB was demonstrated in various human tumor cell lines (Table 1). Human hepatocarcinoma BEL-7402 cells displayed the highest susceptibility to BmOB, as indicated by the IC_{50} value, which was about 80 μM. Tumor cell lines of prostate, breast, lung, and kidney cancer were less susceptible, having IC_{50} values of 150 to 1338 μM. The normal human hepatocytes had the lowest susceptibility to BmOB, which is about 40 times less sensitive to cytotoxic effect of BmOB than hepatocarcinoma. Other normal cell lines are about 2–8 times less sensitive to BmOB induced cytotoxic than the related tumor cell lines. The hepatocarcinoma BEL 7402 cell line was selected for this study since it showed the highest susceptibility to the BmOB.

3.2. Cell viability and cytotoxicity of BmOB

The inhibitory effect of BmOB on BEL-7402 cells was tested on adherent cells by MTT assay (Berridge and Tan, 1993). The cells were incubated at the concentrations indicated for different times as shown in Fig. 1 A. Proliferation of the cells was inhibited significantly upon treatment with BmOB in a concentration- and time-dependent manner. Since the dose required for half-maximal

Fig. 1. Effect of BmOB on BEL-7402 cell viability and cytotoxicity. (A) BEL-7402 cells were treated with increasing concentrations of BmOB and harvested at the times indicated. Data are expressed as means±S.D. of five or more separate MTT experiments. *$P<0.05$ *versus* DMSO at the indicated times. (B) The cells were treated with 80 μM BmOB, or 80 μM BmOB+1 mM Vitamin C, or 80 μM BmOB+10 μM cyclosporine A (CsA) for 24 h. Data are expressed as means±S.D. of five or more separate experiments. *$P<0.05$ *versus* DMSO; ♣$P<0.05$ *versus* BmOB. (C) Internucleosomal DNA fragmentation. BEL-7402 cells were treated for 24 h. lane 1 marker, lane 2 DMSO, lane 3 BmOB 80 μM, lane 4 BmOB+VC, lane 5 BmOB+CsA. (D).Contour diagram of FITC-annexin V/PI flow cytometry of BEL-7402 cells treated with 80 μM BmOB, or 80 μM BmOB+1 mM Vitamin C, or 80 μM BmOB+10 μM CsA for 24 h. The lower left quadrant in each panel represents viable cells, which excluded PI and are negative for FITC-annexin V binding. The upper right quadrants contain non-viable, necrotic cells, which are positive for FITC-annexin V binding and for PI uptake. The lower right quadrants contain the apoptotic cells, FITC-annexin V-positive and PI negative, indicating cytoplasmic membrane integrity and apoptosis. One representative experiment of the three flow cytometric determinations is shown. (E) The percentage of apoptotic and necrotic cells of flow cytometric assay shown in (D). Data are expressed as means±S.D. *$P<0.05$ *versus* DMSO; ♣$P<0.05$ *versus* BmOB. ♦$P<0.05$ *versus* BmOB+VC.

inhibition of cell viability was about 80 μM, this single concentration was chosen for subsequent studies. The viability was not decreased significantly until the cells were treated with BmOB for 24 h. Antioxidant vitamin C could significantly prevent the loss of viability after the cells were treated with 80 μM BmOB for 24 h (Fig. 1 B). It is interesting to note that cyclosporine A, an



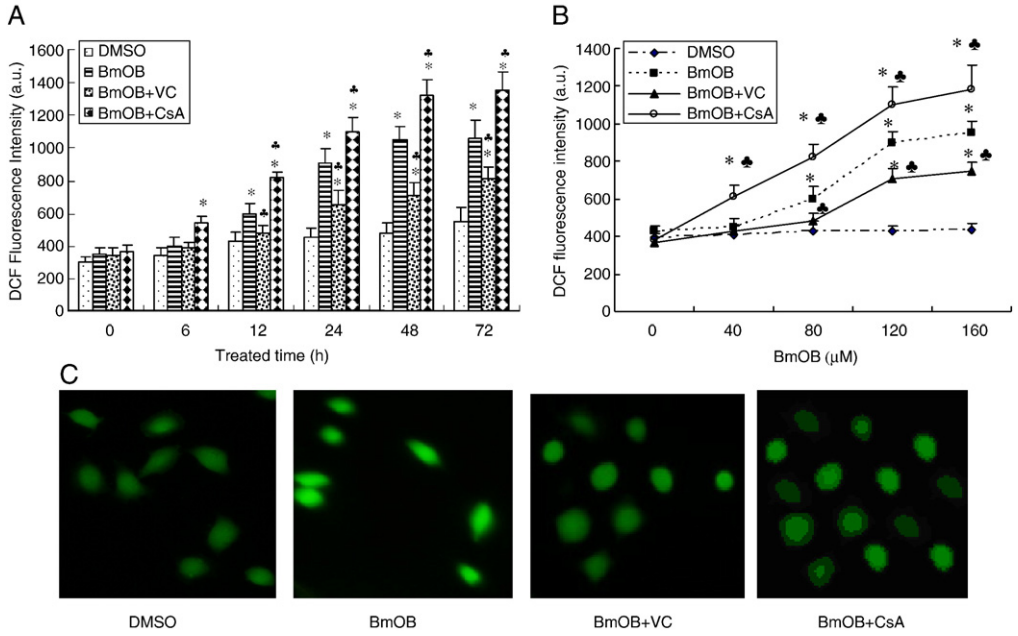


Fig. 2. BmOB-induced production of reactive oxygen species in cells. (A) Intracellular reactive oxygen species production after the BEL-7402 cells treated with 80 μM BmOB, or 80 μM BmOB + 1 mM Vitamin C, or 80 μM BmOB + 10 μM CsA at different times. Data represent means ± S.D. for six independent samples. *$P<0.05$ *versus* DMSO; ♣$P<0.05$ *versus* BmOB. (B) Intracellular reactive oxygen species production after the BEL-7402 cells treated with different concentrations of BmOB, or different concentrations of BmOB plus 1 mM Vitamin C, or plus 10 μM CsA for 12 h. Data represent means ± S.D. for six independent samples. *$P<0.05$ *versus* DMSO; ♣$P<0.05$ *versus* BmOB. (C) Intracellular reactive oxygen species levels after cells were treated with 80 μM BmOB and BmOB plus 1 mM Vitamin C, or plus 10 μM CsA for 24 h, observed by fluorescence microscope. Pictures are representative of three independent experiments. At least five fields were viewed in each of the experiment.

inhibitor of mitochondria permeability transition, could also enhance cell viability (Fig. 1 B). It seems that vitamin C has a better protective effect than cyclosporine A in preventing the loss of cell viability although there is no statistically significant difference (Fig. 1 B).

To initially determine BmOB induced cell death, BEL-7402 cells were exposed to increasing concentrations of BmOB and the fragmented DNA was analyzed by agarose gel electrophores (Canelles et al., 1997; Galeano et al., 2005). As shown in Fig. 1 C, BmOB -mediated formation of DNA ladders appeared after the cells were exposed to 80 μM BmOB. In contrast, untreated control cells showed no DNA laddering. Vitamin C and cyclosporine A could partly prevent the formation of DNA laddering in BmOB treated cells (Fig. 1 C). BmOB induced cytotoxicity was confirmed by flow cytometric assay. Compared with control, BmOB treatment increased the apoptotic and necrotic fractions significantly (Fig. 1 D, E). Vitamin C and cyclosporine A could decrease the formation of apoptotic and necrotic fractions; while vitamin C has a better protective effect than cyclosporine A to BmOB induced cytotoxicity (Fig. 1 D, E).

3.3. Effect of BmOB on intracellular reactive oxygen species levels

To understand the mechanisms underlying BmOB-induced growth inhibition and cytotoxicity, oxidative stress was examined through DCFH-DA analysis. Excessive production of reactive oxygen species is one potential explanation for cell death (Carmody and Cotter, 2001). An increase in fluorescence intensity was used to quantify the generation of net intracellular reactive oxygen species (Yi et al., 2002). As shown in Fig. 2 A, an increase in reactive oxygen species levels was observed after cells were incubated with 80 μM BmOB for 12 h, as evidenced by the shift in DCF fluorescence. Treatment with ≥120 μM BmOB resulted in more significant generation of reactive oxygen species in experimental cells compared to the control cells (Fig. 2 B).Vitamin C could effectively prevent the oxidative stress in BmOB treated cells. In contrast, cyclosporine A could not protect the cells from the oxidative stress. It even increased the DCF fluorescence in cells (Fig. 2 A,B). BmOB-induced intracellular oxidative stress was confirmed by fluorescence images of DCF staining (Fig. 2 C).

3.4. Disruption of mitochondrial membrane potential

Mitochondria play an important role in the regulation of apoptosis (Hengartner, 2000; Raha and Robinson, 2000; Preston et al., 2001). In order to determine whether the cell death induced by BmOB is related to a reduction of $\Delta\Psi m$, mitochondrial membrane potential was investigated with the fluorescent probe JC-1 (Reers et al., 1991; Smiley et al., 1991). JC-1 is a ratiometric, dual-emission fluorescent dye that is internalized and concentrated by respiring mitochondria and can reflect changes in $\Delta\Psi m$ in living cells (Fig. 3 A, B). Fig. 3 C shows that the mitochondrial membrane potential was depolarized by BmOB in both a concentration- and time-dependent manner in BEL-7402 cells. It was shown that BmOB at concentrations greater than 80 μM, significantly reduced the $\Delta\Psi m$, while BmOB at concentrations less than 40 μM had little effect. This is consistent with the result of Fig. 2, which suggest that 80 μM is the key concentration for BmOB to take effect. Mitochondrial

membrane potential is also decreased significantly after the cells cultured with 80 μM BmOB for 24 h (data not shown).

Cyclosporine A, an inhibitor of the mitochondrial permeability transition pore (Zamzami et al., 1995), could protect the BmOB-induced collapse of ΔΨm in BEL-7402 cells (Fig. 3 D). In contrast to the effect of cyclosporine A, Fig. 3 D shows that antioxidants vitamin C could not significantly prevent the collapse of ΔΨm in BmOB treated cells.

Since the time needed for BmOB to affect cell mitochondrial membrane potential is quite short, we surmised that this chemical has a direct effect on mitochondria. Therefore, fresh isolated mouse liver mitochondria which parallel with BEL-7402 cells were examined (Whiteman et al., 2005). Fig. 4 shows

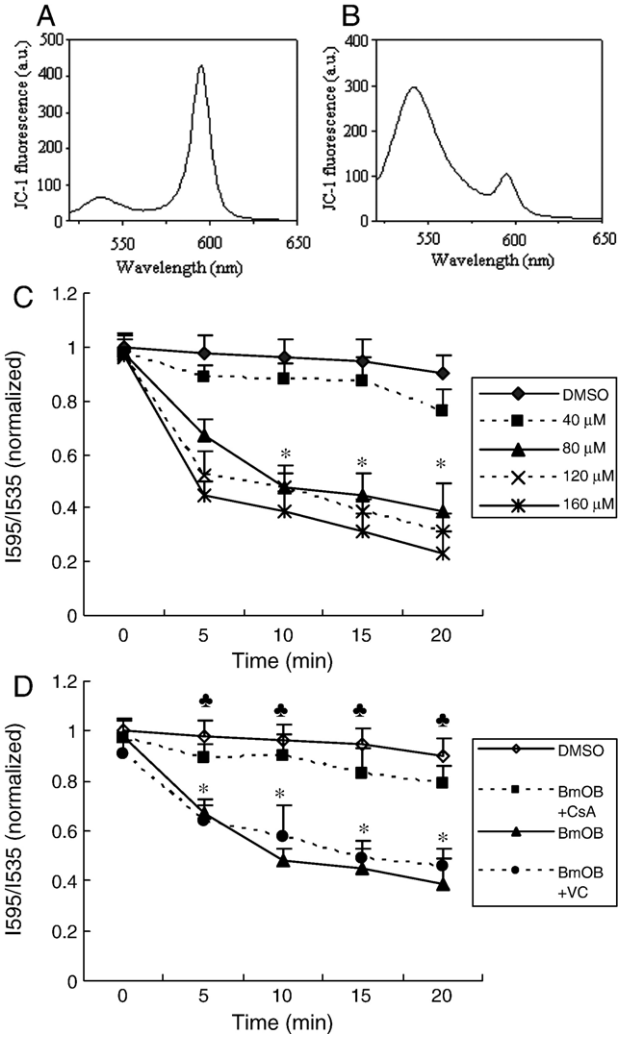


Fig. 3. Effect of BmOB on mitochondrial membrane potential on BEL 7402 cells. (A) JC-1 emission spectrum after cells was excited 495 nm. (B) JC-1 emission spectrum after cells were treated with 80 μM BmOB for 20 min. (C) The ratio of fluorescence intensities at 595 nm and 535 nm (I_{595}/I_{535}) which show concentration- and time-dependent effects of BmOB on the mitochondrial membrane potential. Data represent means±S.D. from five cell preparations. *$P<0.05$ *versus* DMSO.(D) The ratio of fluorescence intensities after 10 μM CsA and 1 mM vitamin C added to cells treated with 80 μM BmOB. Data represent means±S.D. from five cell preparations. *$P<0.05$ *versus* DMSO; ♣$P<0.05$ *versus* BmOB.

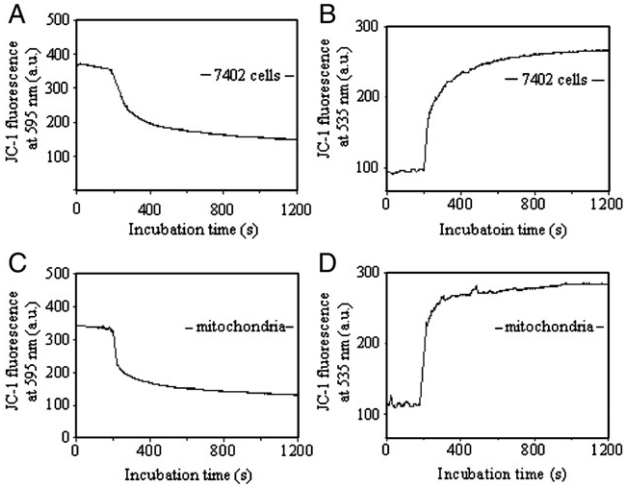


Fig. 4. Loss of ΔΨm induced by BmOB in BEL-7402 cells and isolated mitochondria. The BEL-7402 cells (A, B) or fresh isolated intact mitochondria (C,D) were incubated with JC-1 for 20 min at 37 °C, then 80 μM BmOB was added. The fluorescence emissions at 595 nm and 535 nm (with excitation at 495 nm) correspond to the emission peak of J-aggregate and that of JC-1 monomer, respectively.

that the addition of 80 μM BmOB to intact mitochondria and cells both decreased the fluorescence intensity of JC-1 aggregates (Fig. 4 A, C). In contrast, the fluorescence intensity of JC-1 monomer at 535 nm was enhanced in intact mitochondria and cells respectively (Fig. 4 B, D).

4. Discussion

It is well known that the growth inhibition is the important determinants of the response of cancers to chemotherapeutic agent (Carnero, 2002). Accumulating evidence has indicated that chemotherapeutic agents induce tumor regression through inhibition of proliferation (Kowaltowski et al., 2001). Therefore, compounds that induce cell-cycle arrest and cell death may provide potent anti-cancer effect for cancer treatment. A novel benzotriazole derivative, 3-(1H-benzo [d] [1, 2, 3] triazol-1-yl)-1-(4-meth-ox-yphenyl)-1-oxopropan-2-yl benzoate (BmOB), was synthesized in our lab recently. Although the molecular uptake of benzotriazole derivatives interacting with biomolecules is less well-known (Poznański et al., 2007), BmOB exhibits significant cytotoxic activity to several tumor cell lines, but have low toxicity to normal cells. Our data are consistent with the findings of Pinna group who had examined a panel of 33 protein kinases with benzotriazole derivative 4,5,6,7-tetrabromobenzotriazole (TBB), and observed 60 μM TBB could promote Jurkat cell death and apoptosis (Sarno et al. 2001; Ruzzene et al., 2002). Recently, Saczewski et al. have also synthesized a series of copper(II) complexes with chelating bidentate 2-substituted benzotriazole ligands which exhibited both pronounced SOD-mimicking and cytotoxic activities (Saczewski et al., 2007, 2006). Cytotoxicity studies with seven human tumor cell lines *in vitro* showed that the complexes inhibited the growth of cancer cells significantly, but none of the these compounds displayed any particular selectivity for any cell line (Saczewski et al., 2007). In our

system, hepatocarcinoma BEL-7402 is the most sensitive cancer cell line to the new synthesized benzotriazole derivative.

Oxidative stress, originating from reactive oxygen species and free radicals, provides a constant challenge to eukaryotic cell survival. Since many agents that produce oxidative stress and reactive oxygen species induce apoptosis and necrosis, such stress may be a common initiator and/or mediator of the process (Adams and Cory, 1998; Behrend et al., 2005). In the present study, we used human hepatocarcinoma BEL-7402 cells to analyze the production of reactive oxygen species after exposure to low concentrations of newly synthesized BmOB for different times. BmOB $\geq$ 80 μM could significantly increase the production of cellular reactive oxygen species in cultured cells for 12 h or more (Fig. 2). The protective effects of antioxidant against the loss of cell viability suggest the reactive oxygen species maybe a mediator of cytotoxicity.

It is generally assumed that after the loss of the outer mitochondrial membrane integrity and the release of cytochrome c from the mitochondria to the cytosol, the cells are committed to apoptosis (Martindale and Holbrook, 2002). We have shown that within 20 min of exposure, BmOB clearly induced the loss of $\Delta\Psi$m in BEL-7402 cells (medium and serum free) and that cyclosporine A could prevent the collapse of mitochondria membrane potential. Similar to the potential decrease observed with intact cells, the ability of BmOB to collapse the membrane potential was confirmed in isolated mitochondria (Fig. 4). These results suggest that the $\Delta\Psi$m in BEL-7402 cells might be altered by BmOB instantly and the depolarizing effect of BmOB on mitochondria might be direct and persistent. This is also confirmed by the observation that antioxidants could effectively block the formation of reactive oxygen species but could not prevent the BmOB-induced loss of $\Delta\Psi$m (Figs. 1 and 3).

It is interesting to note that BmOB displayed significant growth inhibitory activity on cancer cell lines by increasing oxidative stress, and causing a drop in mitochondrial membrane potential not only in hepatocarcinoma cells, but also in mitochondria isolated from mouse liver (Fig. 4), but it is low cytotoxic on normal human hepatocytes and other normal cells (Table 1). It seems that the loss of mitochondria integrity is necessary but not sufficient to induce cell death. This may result from the introduction of substituents on the benzotriazole nucleus, which produced a good activity of PPARγ agonist (Sparatore et al., 2006). And the oxy bridge that characterizes BmOB seems to favor activity and potency on PPARγ (Sparatore et al., 2006). It is well documented that PPARγ is a nuclear receptor (Dreyer et al., 1992; Kliewer et al., 1994) that is expressed in an adipose-selective way (Chawla et al., 1994; Tontonoz et al., 1994), as well as in a number of tissues that are important in human cancer, including the colon, prostate, bladder, and breast (Elstner et al., 1998; Kubota et al., 1998; Mueller et al., 1998; Sarraf et al., 1998; Chang and Szabo, 2000; Huin et al., 2002). It was, however, reported that breast cancer cells express a higher level of PPARγ protein than normal breast epithelial cells (Elstner et al., 1998). Sarraf et al also reported that PPARγ maintains at very high levels in high-grade malignant colon tumors and very poorly differentiated cell lines than normal human colon cells (Sarraf et al., 1998;). Koga et al. first identified a significant expression of both PPARγ mRNA and PPARγ protein in human hepatoma cell lines (Koga et al., 2001). Thus, treatment of colon, prostate, bladder, liver, and breast cancer cells with PPARγ agonists results in a more differentiated, less malignant state, a reduction in growth rate and an enhancement of cytotoxicity (Koga et al., 2001; Elstner et al., 1998; Kubota et al., 1998; Mueller et al., 1998; Sarraf et al., 1998; Chang and Szabo, 2000; Huin et al., 2002). Our results agree with these observations, indicating that BmOB shows an effect similar to a PPARγ agonist. This helps explain why BmOB could inhibit growth and induce prominent cytotoxicity of cancer cells tested but low cytotoxicity to normal cells. On the other hand, Han et al. suggests that the predominant G2/M arrest by PPARγ ligands in human cholangiocarcinoma cells is likely mediated by coordinated induction of p53-dependent GADD45 (Han et al., 2003). Bonofiglio et al. found that PPARγ ligand exposure up-regulates both p53 mRNA and protein levels with a concomitant increase of p21 expression in MCF7 breast cancer cells (Bonofiglio et al., 2006).

In conclusion, our results demonstrate that the compound BmOB, which had been synthesized in our lab, could induce cell death by triggering the collapse of mitochondria membrane potential, producing reactive oxygen species and DNA fragmentation. Disruption of $\Delta\Psi$m seems to be an earlier event than reactive oxygen species function in the process of cell death. Other mechanism may be also involved and future investigation may isolate those factors, but in this study the primary factor would appear to be BmOB.

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