Benzyl Isothiocyanate (BITC) and Phenethyl Isothiocyanate (PEITC)-Mediated Generation of Reactive Oxygen Species Causes Cell Cycle Arrest and Induces Apoptosis via Activation of Caspase-3, Mitochondria Dysfunction and Nitric Oxide (NO) in Human Osteogenic Sarcoma U-2 OS Cells

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ABSTRACT: Benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), a member of the isothiocyanate family, have been shown to exhibit antineoplastic ability against many human cancer cells. In this study, we found that exposure of human osteogenic sarcoma U-2 OS cells to BITC and PEITC led to induce morphological changes and to decrease the percentage of viable cells in a time- and dose-dependent manner. BITC and PEITC induced cell cycle arrest at G2/M phase at 48 h treatment and inhibited the levels of cell cycle regulatory proteins such as cyclin A and B1 in U-2 OS cells but promoted the level of Chk1 and p53 that led to G2/M arrest. BITC and PEITC induced a marked increase in apoptosis (DNA fragmentation) and poly(ADP-ribose)polymerase (PARP) cleavage, which was associated with mitochondrial dysfunction and the activation of caspase-9 and -3. BITC and PEITC also promoted the ROS production in U-2 OS cells and the *N*-acetylcysteine (NAC, an antoxidant agent) was pretreated and then treated with both compounds which led to decrease the levels of ROS and increase the cell viability. Interestingly, BITC and PEITC promoted the levels of NO production and increased the iNOS enzyme. Confocal laser microscope also demonstrated that BITC and PEITC promoted the release of cytochrome *c* and AIF, suggesting that both compounds induced apoptosis through ROS, caspase-3 and mitochondrial, and NO signaling pathways. Taken together, these molecular alterations and signaling pathways offer an insight into BITC and PEITC-caused growth inhibition, G2/M arrest, and apoptotic death of U-2 OS cells. J Orthop Res 29:1199–1209, 2011

Keywords: BITC; PEITC; apoptosis; NO; human osteogenic sarcoma U-2 OS cells

Bone cancer is one of the major causes of death in the human population worldwide. In Taiwan, bone cancer causes about 0.4 persons per 100,000 to die annually based on the 2008 report from the Department of Health, ROC (TAIWAN). It also represents about 0.2% of all malignant tumors with an incidence of 3 cases/million - population/year.[1] Osteosarcoma, a highly malignant bone tumor, is a primary malignant bone tumor that usually develops in children and young adults during periods.[2,3] Currently, surgery, radiation, chemotherapy, or a combination of radiotherapy and chemotherapy were used in clinical patients for the treatment of bone cancer but it is still unsatisfying. Numerous studies have focused on gene and protein levels to investigate the pathogenesis and development of osteosarcoma, and the results found many osteosarcoma related genes and proteins related to familial genetics, cell cycle biology, DNA damage pathways, and the use of chemotherapy.[4–7] Many compounds that have been used for cancer preventative agents or even as cancer therapy drugs have been found in natural products.[8] Epidemiological studies suggest that a dietary intake of cruciferous vegetables may protect against different malignancies.[9–11]

Benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) are present in cruciferous plants and both are a member of the isothiocyanate family, which have been demonstrated to be protective against carcinogenesis.[12–14] Much evidence has shown that BITC induced G2/M cell cycle arrest via decreasing Cdk1, cyclin B1, and Cdc25B protein levels.[15–17] It was also reported that BITC produces the formation of reactive oxygen species (ROS) that induces cell death through apoptosis.[14,18] Other investigators also reported that BITC treatment effectively inhibits growth of human breast cancer cells by inducing apoptotic cell death.[19–21] PEITC has been shown to decrease the percentage of viability of cancer cells in culture through the induction of apoptosis and autophagy.[19,22,23]

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Furthermore, PEITC has been demonstrated to exert growth inhibition, the induction of apoptosis and cell cycle arrest in lung,[24] prostate,[25] and ovarian cancer.[26]

Induction of apoptosis from cancer cells is one of the best strategies in chemotherapy and radiotherapy.[27] Apoptosis pathway can be divided into the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway.[28] The extrinsic pathway (caspases 8/10 are the initiator caspases)[29] begins with external death receptors on the cell surface, after ligands bind to their specific receptor resulting in intercellular signaling to form the cleavage and activation of caspase-8[30,31] before caspase-8 cleave affects caspase-3 to induce apoptosis directly.[32] In some cases, the activated caspase-8 can also trigger the activation of the intrinsic pathway involving key mitochondrial events including the anti-apoptotic protein Bcl-2, Bcl-XL and the pro-apoptotic protein Bax, Bak, Bik, Bad, and Bid.[33,34] This affects mitochondrial membrane integrity and triggers cytochrome *c* release[35] to cause the activation of caspase-9 and subsequently leads to the activation of effector caspase-3[36] for causing apoptosis. In some cases, the mitochondrial dysfunction of cells will release apoptosis-inducing factor (AIF)[37] and endonuclease G (Endo G)[38] to induce apoptosis directly.

Although many studies have shown that BITC and PEITC induced cell cycle arrest and apoptosis in many human cancer cell lines, there is no available information to address the effect of BITC and PEITC on human bone cancer cells. Thus, the present study aims to investigate the effect of BITC and PEITC on molecular signaling pathway to cause the cell cycle arrest and induction of apoptosis in human osteogenic sarcoma U-2 OS cells.

MATERIALS AND METHODS

Reagents

BITC, PEITC, dimethyl sulfoxide (DMSO), propidium iodide (PI), and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). McCoy's 5A medium, L-glutamine, fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were obtained from Gibco BRL/Invitrogen (Carlsbad, CA). Primary antibodies (cyclins A and B1, chk1, p53, catalase, Mn–SOD, iNOS, cytochrome *c*, caspase-9 and -3, PARP, and β-actin) and second antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and diluted in PBS Tween-20 before use.

Cell Culture and Treatments

Human osteogenic sarcoma U-2 OS cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). U-2 OS cells were plated onto 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 atmosphere and grown with 90% McCoy's 5A medium with 2 mM L-glutamine adjusted to contain 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.[39] The cells were maintained in 5% CO_2 at 37°C until reaching approximately 50–70% confluence, and then treated with different amounts of BITC and PEITC as indicated.

Morphological Changes and Viability

A U-2 OS cell at 2×10^5 cells/well was maintained on 12-well plates for 24 h before different concentrations of BITC and PEITC were added to each well at final concentrations of 0, 5, 7.5, and 10 μM or 0, 5, 10, and 15 μM, respectively. They were then incubated for 0, 6, 12, 24, and 48 h. All cells in the well were directly examined and photographed under phase contrast microscope for morphological change examinations. All cells from each treatment were trypsinized and harvested by centrifugation at 1,500 rpm for 5 min, washed twice with PBS before 5 μg/ml PI in PBS was added to the cells and viable cells were determined by using FACSCalibur utilizing CellQuest software (Becton-Dickinson, San Jose, CA) for determination of viable cells as previously described.[40,41]

Cell Cycle Analysis

The U-2 OS cells were maintained on 12-well plates and cultured under the conditions described above. After treatment with BITC and PEITC for 48 h, cells were isolated, washed with ice-cold PBS, and then fixed in 70% ethanol overnight. Then cells were re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in dark room for 30 min at room temperature.[42] Cell cycle analyses and sug-G1 (apoptosis) were analyzed with a flow cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm wavelength. The analyses were performed in triplicate for statistical evaluation.

Morphology and Mitotic Phase Assays

After U-2 OS cells were treated with 10 μM BITC and PEITC for 24, 48, and 72 h, cells from each treatment were harvested and smeared on slides. The slides were air dried, fixed in methanol, and stained with Giemsa at room temperature for 15 min. Alterations of nuclei, membrane, and morphological features were observed by light microscopy. Cells in mitotic phase were recognized by the appearance of chromosomes dispersed in the cytoplasm and by the disappearance of nuclear membranes.[43]

Comet Assay for DNA Damage

In order to prevent further DNA damage, all steps were performed in the dark. The alkaline comet assay was carried out according to the method described previously.[41,42] U-2 OS cells (5×10^4 cells/ml) were treated with 7.5 μM of BITC or 10 μM of PEITC for 24 and 48 h. Cells were harvested by centrifugation, isolated and examined for DNA damage by using the Comet assay. We quantified the DNA damage of nuclei with tail randomly selected under microscope at 100× magnification after staining with 2 μg/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min by using a fluorescent microscope. The tail moment (TM) was used to evaluate the degree of DNA damage in all samples.

DAPI Staining

U-2 OS cells (5×10^4 cells/ml) were plated onto 6-well plates and were treated with 7.5 μM of BITC or 10 μM of PEITC for 24 and 48 h. Cells were harvested and U-2 OS cells (5×10^4 cells/ml) were treated with 7.5 μM of BITC or 10 μM of PEITC for 24 and 48 h. Cells were harvested by centrifugation and stained by 10 μg/mL DAPI for apoptotic cells as previously described.[40] After staining, the cells were examined and photographed using a fluorescence microscope.

Western Blotting

The U-2 OS cells at density of 1×10^6 cells/well on 12-well plate were treated with 10 μM BITC or PEITC for incubation of 24 h and then the cells were harvested by trypsinized and

were lysed in lysate buffer composed of 50 mM tris (pH 8.0), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 0.5% NP-40 with protease inhibitor solution (Roche, Mannheim, Germany). The protein concentration from each treatment was determined using a protein assay (Bio-Rad®, Hercules, CA). Equal amount of proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide electrophoretic gel (SDS–PAGE) and transferred to nitrocellulose membranes and then were blocked with 5% dry milk in tris buffered saline–Tween-20 and probed with the appropriate primary antibodies and secondary antibodies. Membranes were then developed using enhanced chemiluminescence methods.[40,42]

Flow Cytometer Assay for the Production of Reactive Oxygen Species and the Levels of Mitochondrial Membrane Potential ($\Delta\Psi m$)

The U-2 OS cells were plated onto 12-well plates and were treated with 7.5 and 10 μM, respectively, of BITC and PEITC for 0, 12, 18, and 24 h before being harvested, washed twice, and counted from each treatment. 1×10^5 cells were re-suspended in the 500 μl of ROS indicator 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA), or 500 μl of the $\Delta\Psi m$ indicator 3,3′-dihexyloxacarbocyanine iodide (DiOC6). Then all samples were incubated at 37°C for 30 min and the levels of ROS and $\Delta\Psi m$ were measured by using flow cytometric assay as previously described.[42,44] Cells were pretreated with or without cyclosporine A (CsA, an inhibitor of mitochondrial permeability transition pore)[45] at 0, 2.5, 5, and 10 μM and then were treated with 7.5 and 10 μM, respectively, of BITC and PEITC for 24 h before being harvested to measure the levels of $\Delta\Psi m$ as described elsewhere.[41]

Confocal Laser Scanning Microscope

The location of cytochrome *c* and AIF was determined by confocal laser scanning microscopy. The U-2 OS cells at a density (5×10^4 cells/well) were plated on 4-well chamber slides and were treated individually with 7.5 and 10 μM, respectively, BITC or PEITC for 24 h. Then cells from each treatment were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton X-100 in PBS for 1 h with blocking of nonspecific binding sites using 2% BSA as described previously.[46,47] At the end of fixation, the fixed cells were stained with primary antibodies to cytochrome *c* and AIF (1:100 dilution) (green fluorescence) for overnight. Then cells were washed and stained by secondary antibody (FITC-conjugated goat antimouse IgG at 1:100 dilution), followed by the staining with PI (red fluorescence) before being washed twice. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope as described previously.[41]

Statistical Analysis

Student's *t*-test was used to analyze the differences between BITC or PEITC-treated and control groups. $p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

BITC and PEITC Induced Morphological Changes Decreased the Percentage of Viable U-2 OS Cells

The morphological changes of U-2OS cells were observed at 24 h after treatment with 5, 10, and 15 μM of BITC and PEITC as shown in Figure 1A and these effects are dose-dependent manners. Figure 1A showed that some cells after being exposed to BITC or PEITC, became smaller, round, and blunt in size and these observations in BITC treatment were more obvious than that of PEITC treatment. After being examined and photographed, the cells in each well were harvested by centrifugation to determine the percentage of viable cells by flow cytometric assay and the results are shown in Figure 1B. The data indicated that BITC and PEITC decreased the percentage of viable U-2 OS cells and the influences are dose- and time-dependent manners. Percentage of viability decreased by more than 50% in U-2 OS cells exposed to 10 μM PEITC or 7.5 μM BITC after 48 h treatment. Thus the concentration of 10 or 7.5 μM, respectively, for both test agents was used in all further experiments.

BITC and PEITC Induced Cell Cycle Arrest in U-2 OS Cells

Based on the growth inhibition results, further studies were conducted to investigate the possible mechanisms by which PEITC and BITC exhibits the inhibitory effects in U-2 OS cells in vitro. The results from flow cytometric assay, as shown in Figure 2A, revealed that BITC induced dramatic accumulation of U-2 OS cells in G2/M phase at 5 μM but induced accumulation of cells G0/G1 phase at 10 μM BITC. However, PEITC induced dramatic accumulation of U-2 OS cells in G2/M phase at 5 and 7.5 μM but induced dramatic accumulation of U-2 OS cells in S phase at 10 μM. All observations are obtained from BITC and PEITC treatment for 48 h. The results also showed that BITC or PEITC caused cell cycle arrest in different doses.

BITC and PEITC Affected Cell in Mitotic Phase and G2/M Arrest Associated Protein Levels in U-2 OS Cells

To examine whether the growth inhibition effect of BITC and PEITC on U-2 OS cells were mediated through specific inhibition of mitosis, we investigated the mitosis stain by Gimsa staining and the results are shown in Figure 2B. The results indicated that both BITC and PEITC compounds induced the inhibition of mitotic phase in examined U-2 OS cells. We examined whether the G2/M arrest in U-2 OS cells by BITC and PEITC were mediated through the effects on associated protein levels. The results in Figure 2C show that similar effects were observed with BITC and PEITC treatment in U-2 OS cells for 48 h, and both compounds decreased the levels of cyclin A and cyclin B1 but increased the levels of p53 and Chk1.

BITC and PEITC Induced Apoptosis and DNA Damage in U-2 OS Cells

To examine the decrease of the percentage of viable U-2 OS cells from treatment of BITC and PEITC through DNA damage and apoptosis, cells were examined by DAPI staining and Comet assay. The results shown in Figure 3 indicated that both examined compounds induced condensation of nuclei (apoptosis) (Fig. 3A). The Comet assay showed that both examined compounds induced DNA damage in U-2 OS cells (Fig. 3B). After the

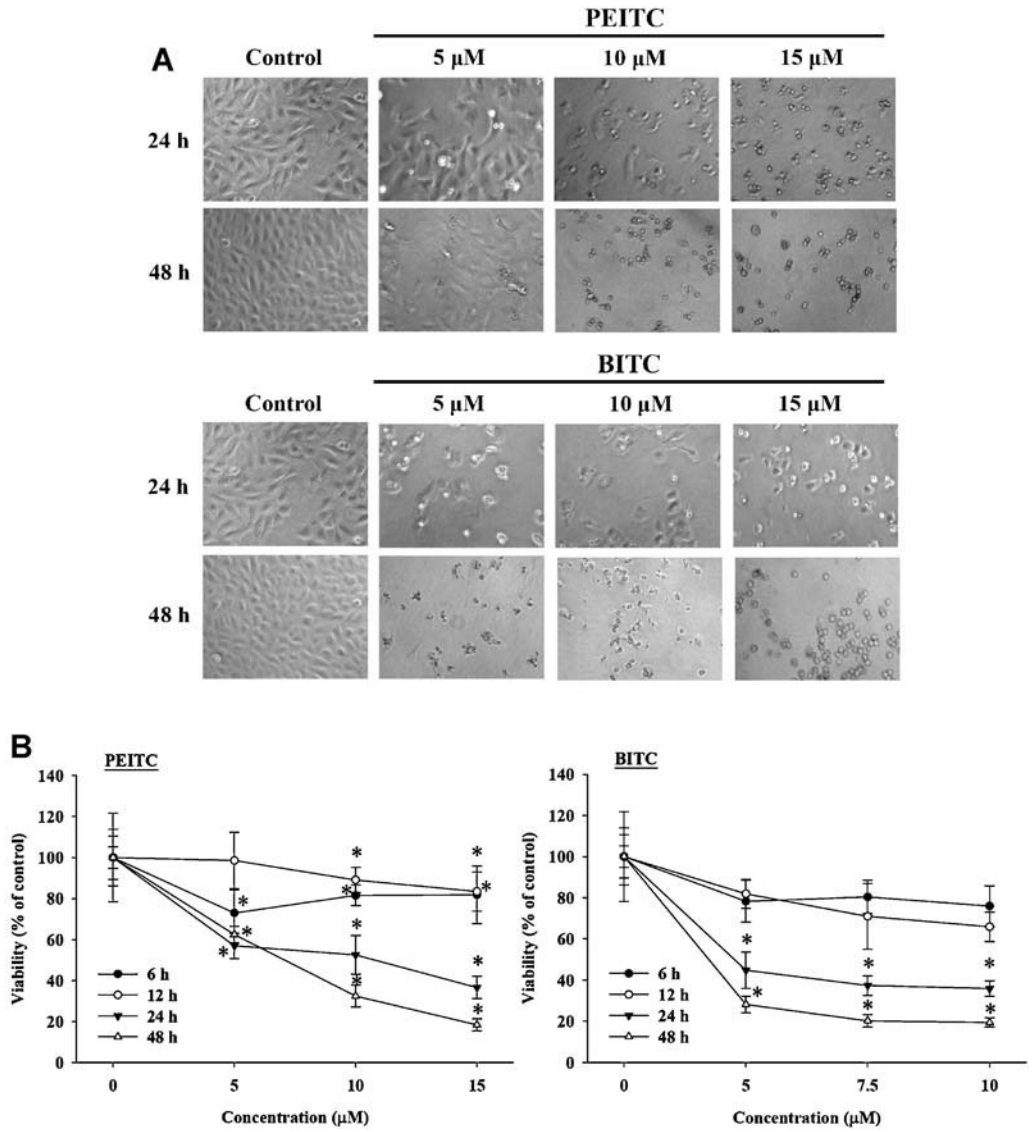

Figure 1. PEITC and BITC induced morphological changes and decreased the percentage of viable U-2 OS cells. Cells were treated with different concentrations (5–15 μM) of BITC and PEITC for 0, 6, 12, 24, and 48 h and cells morphological changes were examined under phase contrast microscope at 200× (A) and cells were harvested to measure the percentage of viable cells by flow cytometric assay (B). Data are given as relative inhibitory rates compared with untreated control group. The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{*}p < 0.05$. Significantly different from vehicle control treated cells.

calculation, both compounds induced apoptosis in U-2 OS cells and these effects are time-dependent (Fig. 3C).

NAC Affected the Effects of BITC and PEITC on the ROS and NO Production in U-2 OS Cells

To examine whether the induction of apoptosis in U-2 OS cells came from the treatment of BITC and PEITC through ROS production, cells also pretreated with or without *N*-acetylcysteine (NAC) were then harvested for measuring ROS production by flow cytometric assay. The results indicated that both examined compounds promoted ROS production in U-2 OS cells in a dose-dependent manner (Fig. 4A). Both compounds promoted NO production and the levels of catalase (Fig. 4C), but BITC decreased the levels of Mn–SOD and PITC yet did not significantly affect the levels of Mn–SOD (Fig. 4C). Both compounds also promoted NO production and the levels of iNOS (Fig. 4B and D) in a time-dependent manner. After U-2 OS cells were pretreated with NAC then treated with BITC or PEITC, cell viability was determined by flow cytometric assay and the results are shown in Figure 4E. They indicate that NAC can increase the percentage of viable cells from both compounds of treated groups and the percentage over the control group. These observations indicated that BITC and PEITC induced growth inhibition through the ROS and NO production in U-2 OS cells.

CsA Did Not Alter the Effects of BITC and PEITC on the Level of Mitochondrial Membrane Potential ($\Delta\Psi m$) and NAO in U-2 OS Cells

To examine whether the effects of $\Delta\Psi m$ in U-2 OS cells came from the treatment of BITC and PEITC, cells also pretreated with or without cyclosporine then were

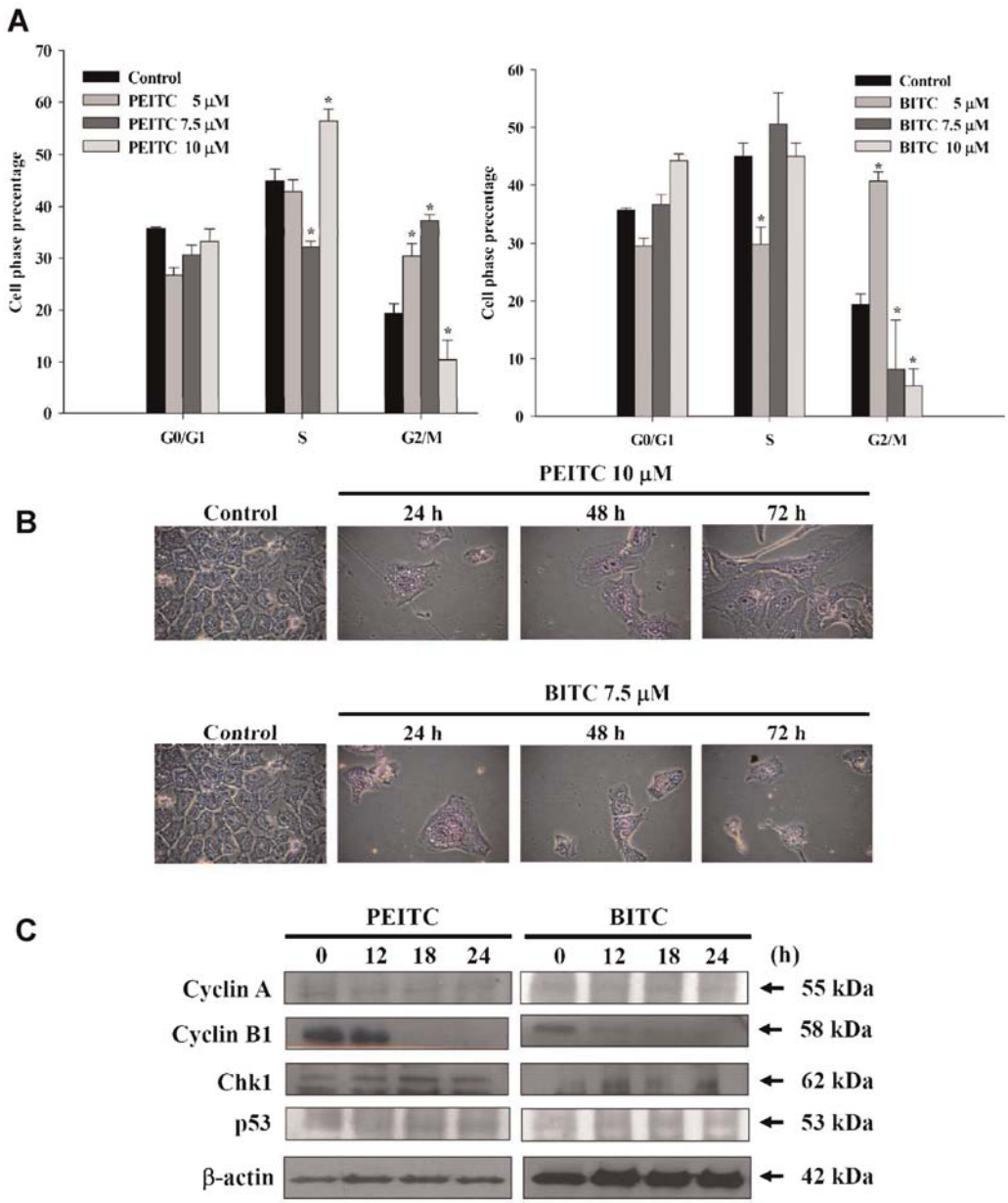

Figure 2. BITC and PEITC induced cell cycle arrest, affected cell in mitotic index, and G2/M arrest associated protein levels in U-2 OS cells. Cells were treated with different concentrations (5–10 μM) of BITC and PEITC for 24 and 48 h and then were harvested for measuring the cell cycle distribution as described in the Materials and Methods Section. (A) Percentage of cells in G0/G1, S, and G2/M phase of cell cycle. The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{*}p < 0.05$. Significantly different from vehicle control treated cells. Cells were treated with 10 μM PEITC and 7.5 μM BITC for different time periods and then were harvested for measuring the mitotic index by Gimsa staining (B) and also for measuring the changes of G2/M arrest associated protein levels by Western blotting (C). Results were obtained from three independent experiments.

harvested for measuring the levels of $\Delta\Psi m$ and the production of NAO by flow cytometric assay. The results are shown in Figure 5, indicating that BITC and PEITC decreased the levels of $\Delta\Psi m$ and these effects are of a time-dependent manner (Fig. 5A). Both compounds increased the NAO production after 12 h treatment (Fig. 5B). However, cells were pretreated with CsA and did not change the effects of BITC and PEITC decreasing the levels of $\Delta\Psi m$ in U-2 OS cells (Fig. 5C).

PEITC and BITC Affected Apoptosis Associated Protein Levels and Translocation in U-2 OS Cells

Based on the results from apoptotic cell death, further studies were conducted to investigate the possible mechanisms by which BITC and PEITC induced apoptosis in U-2 OS cells in vitro. The results from Western blotting revealed that PEITC and BITC increased the protein levels of AIF, cytochrome *c*, caspase-9, caspase-3, and PARP (Fig. 6A) in U-2 OS cells and these effects are time-dependent manner. The

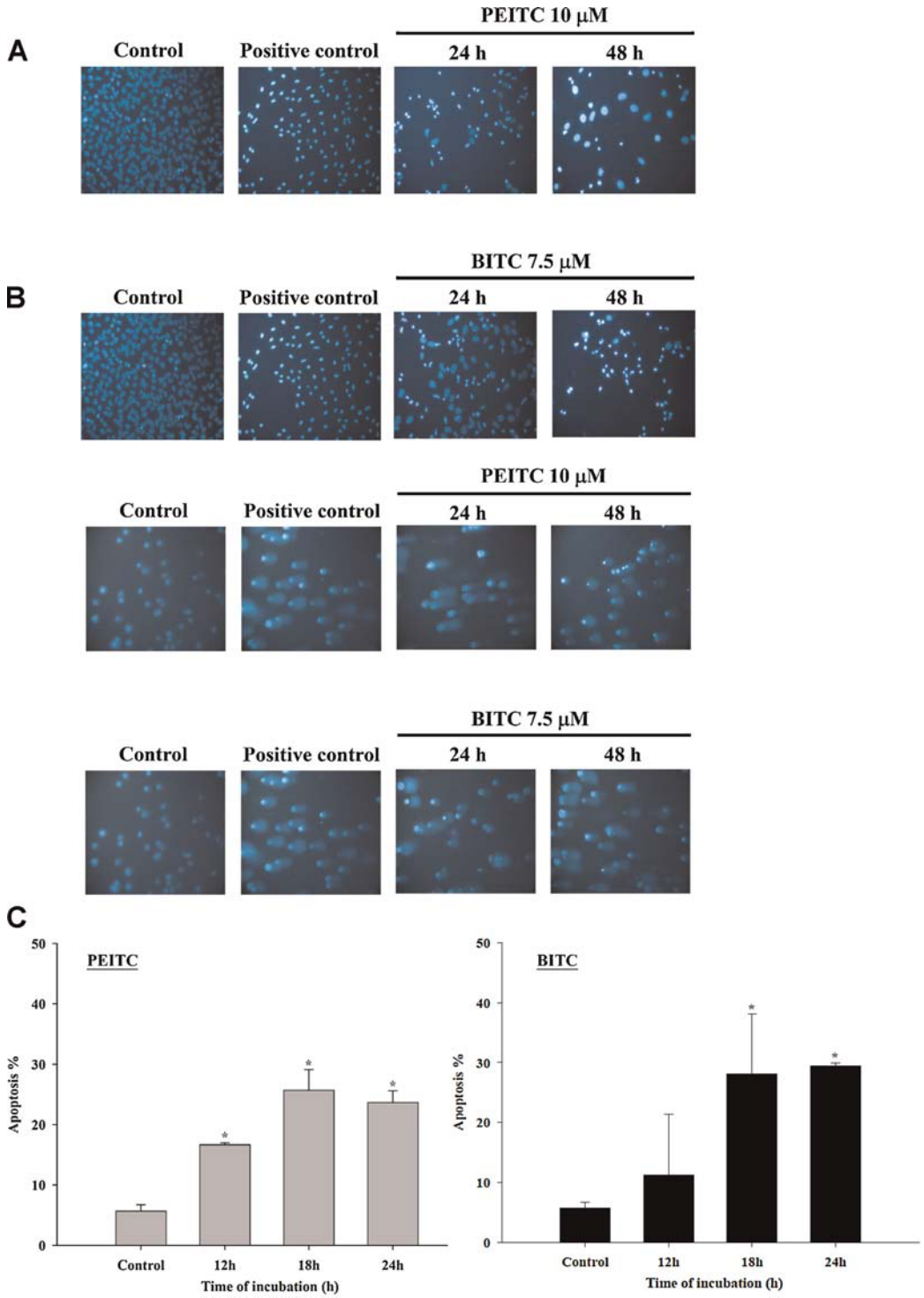

Figure 3. BITC and PEITC induced apoptosis and DNA damage in U-2 OS cells. Cells were treated with different concentrations of BITC and PEITC for different time periods and then were harvested for measuring the apoptosis by DAPI staining (A) and for DNA damage examination by Comet assay (B) then to calculate the percentage of apoptosis based on sub-G1 from flow cytometric assay (C). The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{*}p < 0.05$. Significantly different from vehicle control treated cells.

confocal laser microscope examination indicated that PEITC and BITC promoted the release of cytochrome *c* and AIF, but only AIF moved into the nuclei (Fig. 6B,C).

DISCUSSION

It is well known that some anticancer and DNA damage agents work are via the cell cycle arrest at different phases and then induce apoptosis in cancer cells.[48–50]

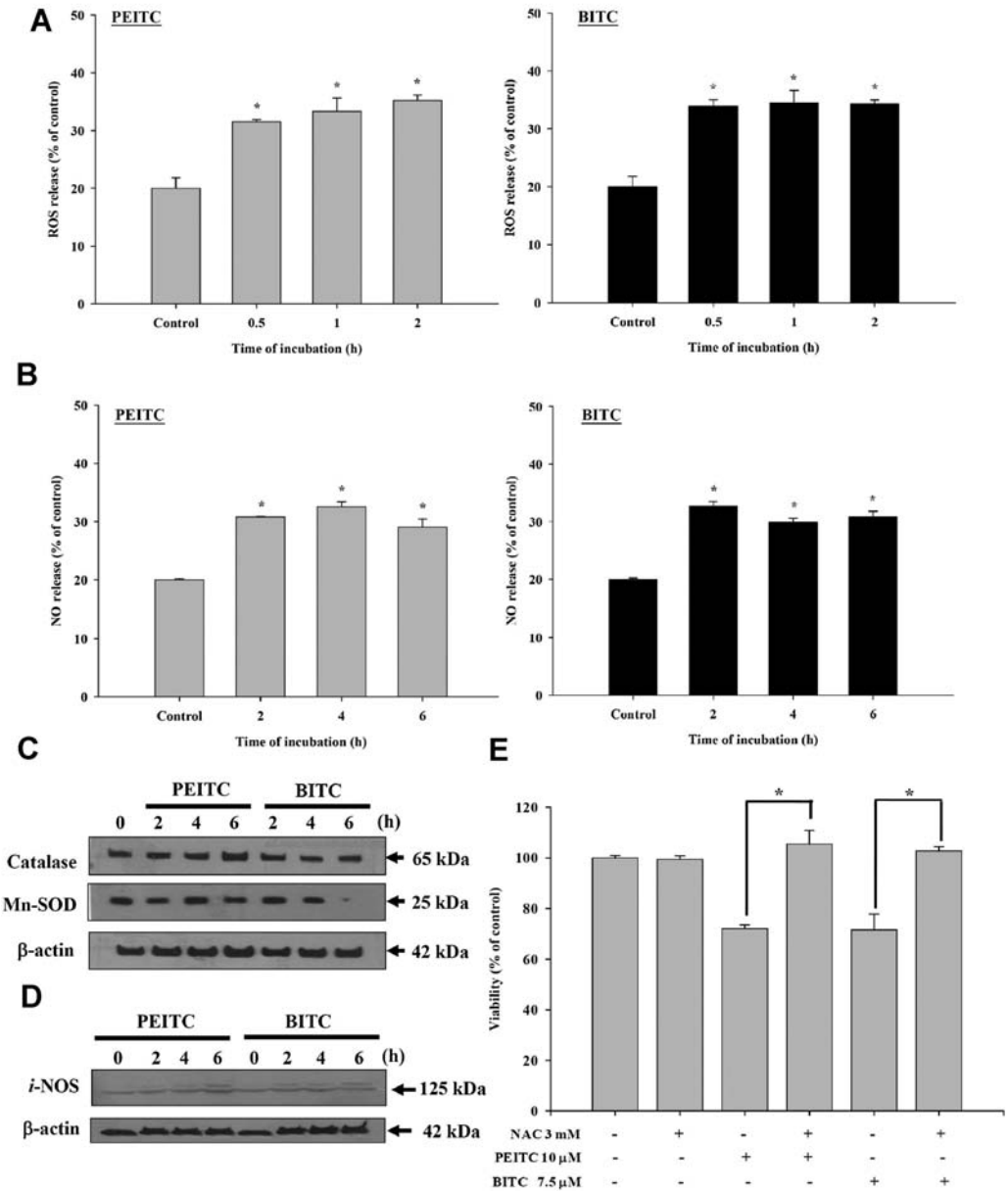

Figure 4. NAC affected the effects of BITC and PEITC on the ROS and NO production in U-2 OS cells. Cells were pre-treated with or without NAC and then were treated with 10 μM PEITC and 7.5 μM BITC for different time periods before being harvested for measuring the ROS production by H_2DCF-DA (A) and NO production by DAF/FM (B) were analyzed by flow cytometric assay. For determination of ROS (C) and NO (D) production associated proteins by Western blotting and then to calculate the percentage of ROS production and whether or not it was affected by NAC from flow cytometric assay (E). The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^*p < 0.05$. Significantly different from vehicle control treated cells.

It was also well documented that cell cycle have checkpoints for ensuring cells have time to repair the damaged DNA, whereas apoptotic cell death can eliminate irreparable or unrepaired damaged cells. Although numerous studies have reported that BITC and PEITC can induce cell cycle arrest and apoptosis in many human cancer cell lines, the molecular mechanism is still unclear.

Herein, the aim of the present study was to elucidate the molecular mechanism of action by which BITC and PEITC induced cytotoxic effects on human osteogenic sarcoma U-2 OS cells in vitro. The results can be summarized as (1) BITC and PEITC induce morphological changes and decreased the percentage of viable cells; (2) BITC and PEITC induced dramatic accumulation of U-2 OS cells in G2/M phase at 5 μM, however, PEITC induced dramatic accumulation of U-2 OS cells in S phase at 10 μM; (3) BITC and PEITC both induced apoptosis in time-dependent manners; (4) BITC and PEITC both promoted the production of ROS and Ca^{2+} but decreased the levels of MMP; (5) BITC and PEITC both promoted the production of NO but decreased the levels of NAO.

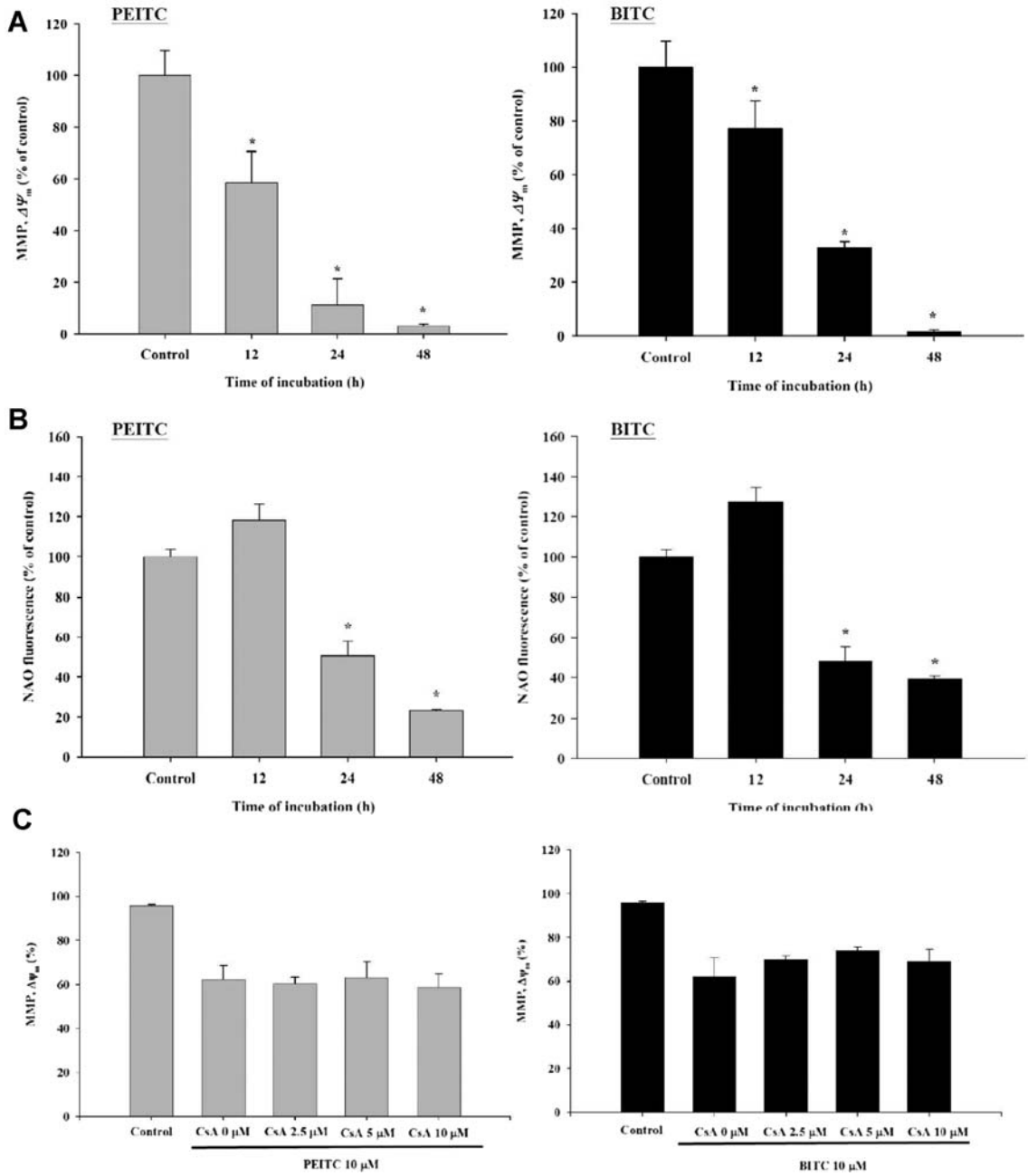

Figure 5. CsA affected the effects of PEITC and BITC on the level of mitochondrial membrane potential ($\Delta\Psi m$) and NAO production in U-2 OS cells. Cells were pre-treated with or without CsA and then were treated with 10 μM PEITC and 7.5 μM BITC for different time periods and then were harvested for measuring the level of $\Delta\Psi m$ (A) and for determination of NAO production (B) then finally to calculate the level of $\Delta\Psi m$ and whether or not they were affected by CsA from flow cytometric assay (C). The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{*}p < 0.05$. Significantly different from vehicle control treated cells.

Figure 2 indicates that BITC and PEITC induced an accumulation of U-2 OS cells in the G2/M phase of the cell cycle. It was reported that the microtubule-stabilizing agents[51] induced G2/M phase arrest in cancer cells. Our results showed that the novel finding of BITC and PEITC induced G2/M phase arrest in U-2 OS cells. In mammalian cells, a number of Cdks have shown to regulate the cell cycle event,[52,53] and Cdk1 and Cdk2 kinases are activated primarily in association with cyclin A and B1 in the G2/M phase progression. Results also showed that BITC and PEITC both decreased the protein levels of cyclin A and B1 (Fig. 2C). It was reported that the cyclin B1/Cdk1 complex is the primary regulator of transition from G2 to M phase.[54] BITC and PEITC both decreased the percentage of viable cells via the apoptotic cell death with cell cycle arrest.

Cells failing to progress to mitosis may be destined to apoptosis by BITC and PEITC because our results from Gimsa staining also showed that both compounds inhibited the mitotosis (Fig. 2B). We also saw that BITC and

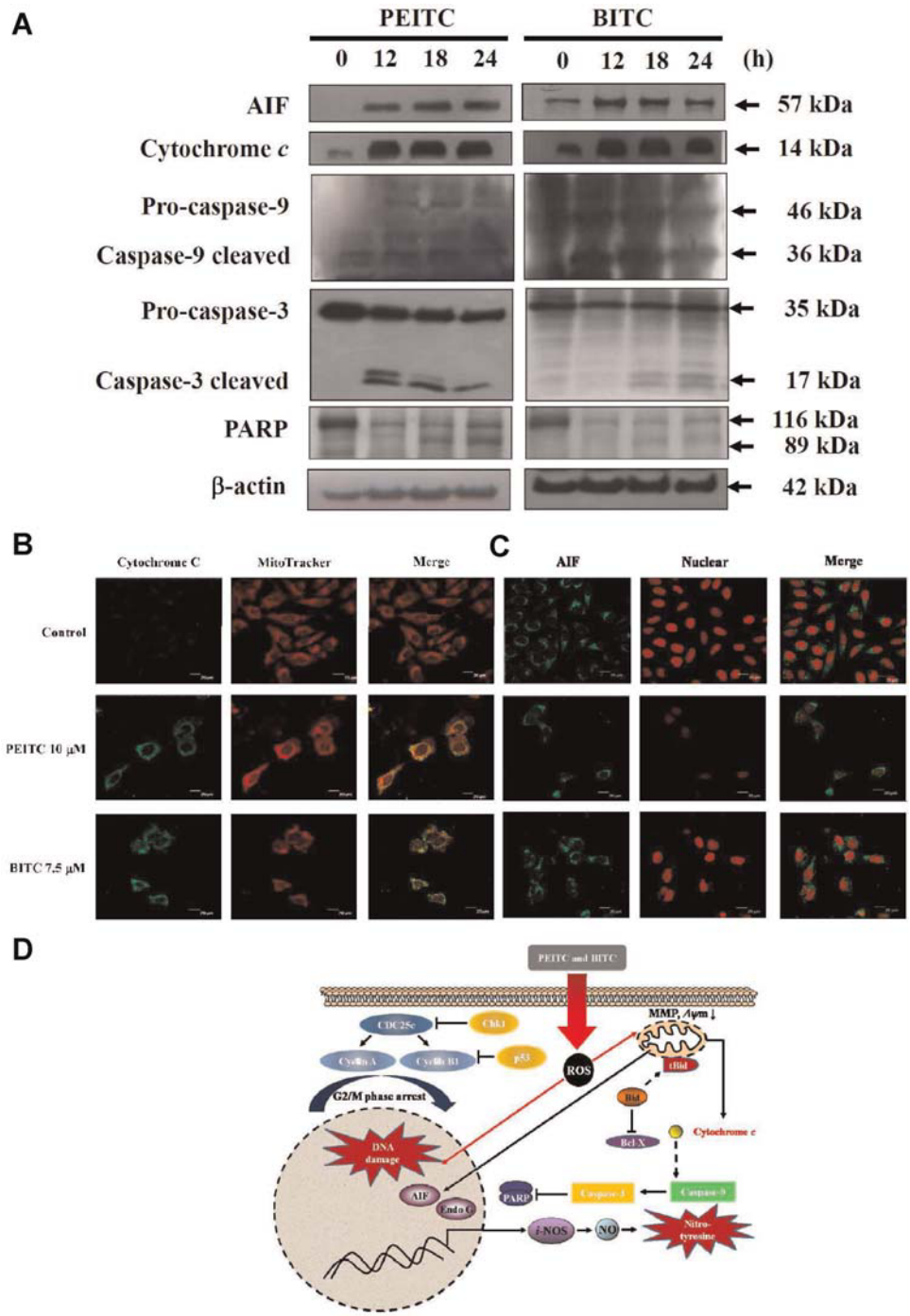

Figure 6. PEITC and BITC affected apoptosis associated protein levels and translocation in U-2 OS cells. Cells were treated with 10 μM PEITC and 7.5 μM BITC for 0, 12, 18, and 24 h and then were harvested for measuring the apoptotic associated proteins by Western blotting (A). Cells were also measured for the translocation of cytochrome *c* and AIF by confocal laser microscope at 200× (B). Results were obtained from three independent experiments. The proposed signaling pathway for BITC and PEITC affecting the induction of cell cycle arrest and apoptosis in U-2 OS cells (C).

PEITC induced apoptosis in U-2 O cells (Fig. 3C) and this is also confirmed through the morphological changes, DNA fragmentation, sub-G1 increase, and PARP cleavage (Fig. 6A). It is well documented that some of the anticancer agents induced apoptosis via caspases.[55] In particular, the caspase-3 is an executioner caspase, which can be activated directly from caspase-8 or -9[56,57] before leading to apoptosis. Based on the results (Fig. 6A) from Western blotting, it indicated that BITC and PEITC increased the active form of caspase-9 and -3.

Our results also showed that BITC and PEITC decreased the levels of $\Delta\Psi m$ which may be through the release of caspase-9 and then activated the caspase-3 for apoptosis to occur. However, the cells were

pretreated with CsA, and then cells were exposed to BITC and PEITC for examining the levels of $\Delta\Psi m$. Results indicated that there was no significant difference in BITC or PEITC exposure (Fig. 5C). Therefore, BITC and PEITC induced apoptosis in U-2 OS cells may be through other different signaling pathways. We also used confocal laser microscope to examine the translocation of AIF which indicated that BITC and PEITC both promoted the release of AIF from mitochondria to cytoplasm, and this observation indicated that both compounds may promote the AIF release to induce apoptosis (Fig. 6C). Our results also showed that BITC and PEITC both promoted the productions of ROS and Ca^{2+} in U-2 OS cells. However, cells were pretreated with NAC, then exposed to BITC and PEITC then led to decrease the production of ROS and Ca^{2+} but increased the percentage of viable cells. This observation also showed that BITC and PEITC induced cytotoxic effects was through the induction of ROS (Fig. 4). However, the NAC pretreatment (Fig. 4D) did not completely rule out the dead cells which means that BITC and PEITC compounds induced cell death through other signal pathways. Thus, we investigated the levels of NO and the results indicated that both compounds promoted the NO production which then also led to apoptosis in U-2 OS cells. These results suggested involvement of ROS, NO, and mitochondrial pathways in BITC and PEITC-induced apoptosis.

In conclusion, BITC and PEITC arrested G2/M phase in the cell cycle distribution and induced apoptosis of U-2 OS cells and the possible signal pathways are summarized in Figure 6D. BITC and PEITC-induced G2/M phase arrest was associated with reduction of cyclins A and B1. BITC and PEITC-induced may have gone through the ROS production, dysfunction of mitochondria, caspase-3 activation, AIF release from mitochondrial, and promotion of NO for causing apoptosis in U-2 OS cells. Taken together, these findings provide more information regarding the possible molecular mechanisms and possible signal pathways of the anticancer activity of BITC and PEITC.

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