3-Bromopyruvate sensitizes human breast cancer cells to TRAIL-induced apoptosis via the phosphorylated AMPK-mediated upregulation of DR5

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Abstract. Previous studies have indicated that the sensitivity of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is associated with the expression of death receptors on the cell membrane. However, drug resistance limits the use of TRAIL in cancer therapy. Numerous studies have indicated that death receptors, which induce apoptosis, are upregulated by the endoplasmic reticulum (ER) stress response. 3-Bromopyruvate (3-BP), an anticancer agent, inhibits cell growth and induces apoptosis through interfering with glycolysis. In the present study, it was demonstrated that 3-BP synergistically sensitized breast cancer cells to TRAIL-induced apoptosis via the upregulation of death receptor 5 (DR5). Furthermore, we found that the protein levels of glucose-related protein 78 (GRP78) and CCAAT-enhancer-binding protein homologous protein (CHOP) increased following treatment with 3-BP. The expression of Bax (in MCF-7 cells) and caspase-3 (in MDA-MB-231 cells) increased following co-treatment with 3-BP and TRAIL, whereas the expression of the anti-apoptotic protein Bcl-2 decreased. In order to investigate the molecular mechanism regulating this effect, the expression of adenosine monophosphate-activated protein kinase (AMPK), activated by 3-BP, was determined. It was demonstrated that phosphorylated-AMPK was upregulated following treatment with 3-BP. Notably, Compound C, an AMPK inhibitor, reversed the effects of 3-BP. Finally, a synergistic antitumor effect of 3-BP and TRAIL was observed in MCF-7 cell xenografts in nude mice. In conclusion, these results indicated that 3-BP sensitized breast cancer cells to TRAIL via the AMPK-mediated upregulation of DR5.

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Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR5, death receptor 5; 3-BP, 3-bomopyruvate; CHOP, CCAAT/enhancer-binding protein-homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; DMSO, dimethyl sulfoxide; AMPK, AMP activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; PI, propidium iodide; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinyl difluoride; H&E, hematoxylin and eosin; ATP, adenosine tri-phosphate

Key words: 3-bromopyruvate, TRAIL, apoptosis, ER stress

Introduction

Breast cancer is the leading cause of cancer-related deaths among females and is the most frequently diagnosed type of cancer in women. The incidence of breast cancer in China is increasing by 2.2% per year (1). The currently available treatments for patients with breast cancer includes chemotherapy, surgical resection and radiotherapy. Considering that drug resistance is often associated with targeted therapies, there is a requirement for novel therapeutic strategies for the treatment of cancer (2).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has a crucial role in cancer therapy; by binding to death receptor (DR)4 and DR5, it selectively induces apoptosis in cancer cells. TRAIL has reduced toxicicity compared with other members of the tumor necrosis factor (TNF) protein family, such as TNFα and Fas ligand (CD95L) (3). Pre-clinical trials have determined that TRAIL effectively inhibits tumor growth without inducing toxicity in both mice and non-human primates (4,5). However, cancer cells may aquire resistence to TRAIL-induced apoptosis via mutation or deficiency of DR4 or DR5. A number of studies have investigated whether the use of recombinant TRAIL may reverse this effect, by enabling the interaction of TRAIL with mutated receptors, or whether the combination of treatment with TRAIL and

Chinese herbology (6) may increase the therapeutic efficacy and decrease the toxicity of TRAIL (7).

The expression of TRAIL receptor 2 (TRAIL-R2 or DR5) is crucial in TRAIL-induced apoptosis in cancer cells (8), and is upregulated following the inhibition of ATP synthesis in cancer cells (9). Cancer cells and healthy cells may be characterized according to their different energy metabolisms (10). In 1956, Warburg demonstrated that cancer cells produce energy predominantly via fermentation rather than through oxidative respiration as exhibited by healthy cells, even when sufficient oxygen is present (11). However, fermentation is associated with the higher consumption of glucose relative to oxidative respiration, as well as the greater production of lactate.

3-Bromopyruvate (3-BP), a hexokinase II inhibitor, can induce apoptosis in hepatocellular carcinoma cells by inducing endoplasmic reticulum (ER) stress (12). ER stress occurs upon accumulation of misfolded proteins in the ER, and results in the activation of the ER stress chaperone protein glucose-related protein 78 (GRP78) and the pro-apoptotic transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP). Previous studies have indicated that DR5 is upregulated during the ER stress response (13-15). Adenosine monophosphate-activated protein kinase (AMPK) is a nutrient and energy marker in the cells, and can also induce apoptosis (16-18). Notably, numerous studies have suggested that c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), is potentially a regulator of AMPK-induced apoptosis. JNK regulates apoptosis via the phosphorylation of apoptosis related-proteins, such as B-cell f lymphoma 2 (Bcl-2), or via the activation of activator protein-1 (AP-1) (19). In addition, 3-BP suppresses the levels of cellular adenosine tri-phosphate (ATP) and activates AMPK, as well as the AMPK-mediated upregulation of Bcl-2-associated X protein (Bax), in which subsequently induces cell death via the mitochondrial pathway (20).

Our previous study indicated that the hexokinase inhibitor 3-BP induced cell death in colon cancer cells via a number of different mechanisms (21). Therefore, we aimed to investigate whether 3-BP upregulated DR5, thus enhancing the anticancer effect of TRAIL. The results of the present study demonstrated that AMPK was induced by 3-BP to act as a regulator of apoptosis. Thus, we hypothesized that AMPK played a role in the ER stress response, and subsequent upregulation of CHOP and DR5. Furthermore, the results of the present study revealed that co-treatment with 3-BP and TRAIL induced apoptosis in breast cancer cells in a Bax- and caspase-dependent manner.

Materials and methods

Reagents and antibodies. Recombinant human TRAIL was purchased from Genentech Inc. (San Francisco, CA, USA). 3-BP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The ATP assay kit was purchased from Merck KGaA. The AMPK inhibitor Compound C was purchased from Selleck Chemicals (Houston, TX, USA). A rabbit polyclonal antibody against DR5 (1:1,000 dilution; cat. no. ab199357) and a rabbit monoclonal antibody against CHOP (1:2,000 dilution; cat. no. ab179823) were purchased from Abcam (Cambridge, MA, USA). The rabbit polyclonal antibody against caspase-3 (1:1,000 dilution; cat. no. LBP72217) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The GRP78 (1:1,000 dilution; cat. no. SC-3177) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The rabbit polyclonal antibodies against AMPK-α1 (1:500 dilution; cat. no. 10929-2-AP), Bax (1:5,000 dilution; cat. no. 50599-2-Ig) and Bcl-2 (1:1,000 dilution; cat. no. 12789-1-AP) were supplied by ProteinTech Group, Inc. (Chicago, IL, USA). The rabbit polyclonal antibody against phosphorylated (p)-AMPKα (Thr172) (1:1,000 dilution; cat. no. 50081) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All reagents were prepared to the recommended dilutions according to the manufacturer's instructions.

Cell culture. The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 U/ml). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cells were seeded in 96-well plates ($6x10^3$ cells/well) and treated with different concentrations of 3-BP (0, 40, 80, 160 and 320 μmol/l) or TRAIL (25, 50, 100, 200 and 400 ng/ml) for 24 h. Phosphate-buffered saline (PBS) containing 5 mg/ml MTT (15 μl) was added to each well, and the cells were incubated for a further 4 h. Subsequently, the medium was then replaced with dimethyl sulfoxide (DMSO; Biosharp, Inc., Hefei, China; 150 μl/well) in order to solubilize the formazan crystals. Finally, absorbance was determined at 490 nm using a plate reader (Synergy HT, Inc. Winooski, VT, USA).

PI staining. Cells were seeded in 12-well plates ($1.5x10^5$ cells/well) and incubated for 24 h, until the cells reached exponential phase. Subsequently, cells were treated for 24 h with various concentrations of 3-BP, TRAIL or both. Cells were subsequently stained using propidium iodide (PI; 600 μl/well) for 2 h and then analyzed using flow cytometry (Accuri™ C6 system; BD Biosciences, Franklin Lakes, NJ, USA).

ATP quantification. CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) was used to investigate ATP levels, according to the manufacturer's instructions. Cells ($1.5x10^5$/well) were seeded in 12-well plates for 24 h and then treated with different concentrations of 3-BP for 4 h at 37˚C. Following this, cells were collected and then lysed using radioimmunoprecipitation assay (RIPA) lysis buffer for 10 min on ice. Cell lysates were subsequently centrifuged at 13,225 x g for 5 min at 4˚C. A nucleotide-releasing buffer (100 μl/well), ATP-monitoring enzyme (10 μl/well), and cell lysate (30 μl/well) were added to 96-well plates. Developed signals were then detected using a Luminoskan luminometer and a Varioskan™ Flash spectral scanning multimode reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell surface staining. Cells ($2.5x10^5$/ml) were seeded in 6-well plates, treated with PBS or 3-BP (80 μmol/l), and then incubated for 24 h. Subsequently, non-specific antibody binding sites were blocked using PBS containing 10% FBS for 20 min. Cells were then washed with PBS, re-suspended in 200 μl PBS, aliquoted into two tubes and incubated with 20 μl of an antibody-containing solution (rabbit anti-DR5 in PBS with 1% FBS; dilution 1:1,000) for 30 min on ice. Subsequently, the cells were washed twice with PBS, pelleted and incubated with 100 μl FITC-conjugated goat anti-rabbit IgG (1:100 dilution; cat. no. BL033A; Biosharp) for 30 min on ice. Cell surface staining was investigated via flow cytometry using the Accuri™ C6 flow cytometer (BD Biosciences).

Western blot analysis. Harvested cells were washed with PBS and then lysed using RIPA buffer for 30 min on ice. Subsequently, cell lysates were centrifuged at 13,225 x g for 30 min at 4˚C. The proteins were determined by BCA assay. Subsequently, proteins (50 μg) were loaded per lane, and then separated on a 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were then blocked using 5% skimmed milk in PBS for 4 h at 20-25˚C and incubated overnight at 4˚C with the primary antibodies. After washing with Tris-buffered saline containing 1% Tween®-20 (Beyotime Institute of Biotechnology, Haimen, China), membranes were incubated with the corresponding secondary antibodies. β-actin was used as the loading control.

In vivo experiments. In order to investigate the antitumor effect of 3-BP and TRAIL, female nude mice (BALB/c; 4-5-weeks old and 18-20 g) were used. Mice were purchased from the Animal Experimental Center of Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained under specific pathogen-free conditions (26-28˚C, air pressure difference was 10-20 kPa, 10-h light/14-h dark cycle, food and water were taken *ad libitum*). Mice were injected subcutaneously with MCF-7 cells ($4x10^6$ cells/mouse) to induce tumor formation. A total of 20 mice with tumors of >100 mm^3 were randomly divided into four groups (five per group), and injected intraperitoneally with either PBS (0.2 ml), 3-BP (8 mg/kg), TRAIL (0.1 mg/kg) or both 3-BP (8 mg/kg) and TRAIL (8 mg/kg) every 4 days. Body weight was monitored prior to each injection. Tumor volume was calculated using the following formula: Length x $width^2/2$. Mice were sacrificed by cervical dislocation after 28 days of treatment. Following treatment for 28 days, the solid tumors were resected from mice, stored in 4% formalin solution, cut into sections and subsequently subjected to with hematoxylin and eosin (H&E) or TUNEL staining. All procedures performed in this study involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Bengbu Medical College.

Statistical analysis. Data are expressed as the mean ± the standard error of the mean (SEM). Statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The difference among groups was calculated by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

3-BP enhances TRAIL-induced apoptosis in breast cancer cells. MCF-7 and MDA-MB-231 cells, were selected as they are estrogen/progesterone receptor positive and negative, respectively, which frequently exhibit different drug sensitivities. To investigate the effects of 3-BP and TRAIL on cell viability, MCF-7 and MDA-MB-231 cells were treated with various concentrations of 3-BP and TRAIL for 24 h. The results of the MTT assay and PI staining analyses demonstrated that 3-BP (80 and 160 μmol/l) and TRAIL (400 ng/ml) significantly inhibited cell viability (Fig. 1A). Notably, concomitant treatment with 3-BP (80 μmol/l) and TRAIL (200 ng/ml) inhibited cell viability to a greater extent compared with treatment with 3-BP or TRAIL alone ($P<0.05$, Fig. 1B and C). Therefore, the results indicated that co-treatment with 3-BP and TRAIL synergistically induced apoptosis in breast cancer cells.

3-BP inhibits ATP generation and upregulates the expression of DR5. Taking the above-mentioned results into consideration, we investigated whether 3-BP affected the activity and/or expression of one or more mediators in the enhancement of the anticancer activity of TRAIL in breast cancer cells. To investigate this, we determined whether 3-BP affected ATP levels and/or the expression of DR5. It was demonstrated that treatment with 3-BP induced ATP depletion in breast cancer cells (Fig. 2A). Similarly, DR5 staining revealed that the expression of DR5 was enhanced in breast cancer cells treated with 3-BP compared with control cells (Fig. 2B).

3-BP upregulates CHOP, GRP78 and the phosphorylation of AMPK and augments TRAIL-induced Bax and caspase-3 levels. Previous studies have demonstrated that 3-BP induced apoptosis in hepatocellular carcinoma cells via ER stress (12). Therefore, the present study aimed to investigate whether 3-BP induced ER stress in breast cancer cells by determining the expression of ER stress-associated proteins in cells treated with 3-BP. The results of western blot analysis revealed that CHOP and GRP78 levels were enhanced in breast cancer cells following treatment with 3-BP (Fig. 3A). In addition, previous studies have demonstrated that 3-BP induced apoptosis via the disruption of energy metabolism (20). Therefore, the present study aimed to investigate the level of p-AMPK in cells treated with 3-BP. The results of western blot analysis revealed that treatment with 3-BP increased levels of p-AMPK in a dose-dependent manner, as well as the AMPK-α levels overall (Fig. 3A). Furthermore, the expression levels of Bax in MCF-7 cells and caspase-3 in MDA-MB-231 cells were increased in breast cancer cells following co-treatment with 3-BP and TRAIL. However, co-treatment with 3-BP and TRAIL decreased the expression of Bcl-2 in breast cancer cells (Fig. 3B).

The AMPK inhibitor Compound C reduces the TRAIL-synergizing effect of 3-BP on cell growth inhibition and apoptosis. Subsequently, we investigated whether 3-BP sensitized breast cancer cells to growth inhibition and

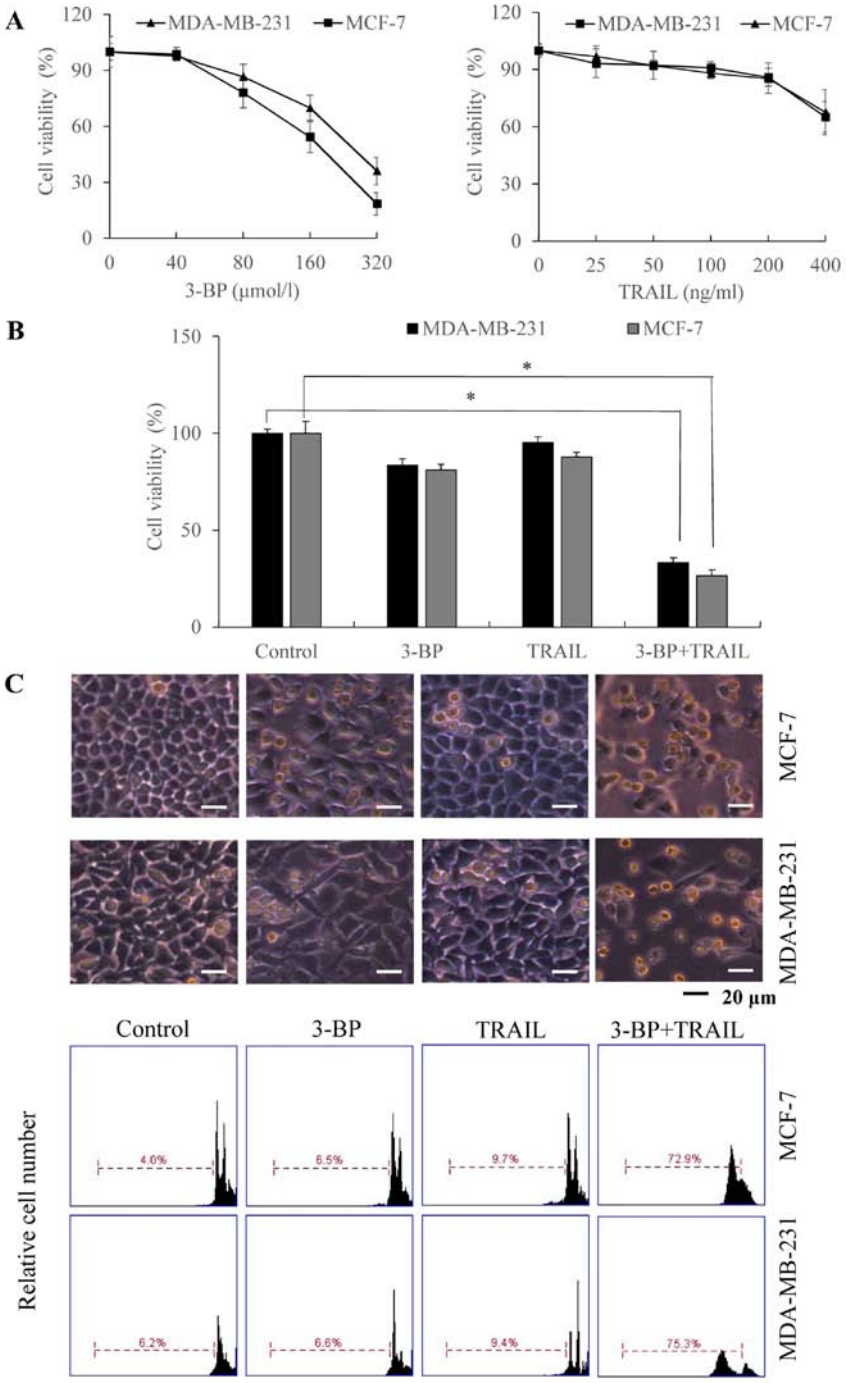

Figure 1. Inhibitory and apoptotic effect of the combinatory 3-BP and TRAIL treatment on breast cancer cells. (A) MCF-7 and MDA-MB-231 cells were treated with medium (Control) or 3-BP (40, 80, 160 or 320 μmol/l) and TRAIL (25, 50, 100, 200 or 400 ng/ml). Cell viability was determined using an MTT assay. (B and C) MCF-7 and MDA-MB-231 cells were treated with either medium (Control), 80 μmol/l 3-BP, 200 ng/ml TRAIL or 3-BP and TRAIL for 24 h. (B) Cell viability was determined using an MTT assay. (C) Cell morphology was investigated using light microscopy, and the rate of apoptosis was determined using PI staining and flow cytometry. Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 vs. the control group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; 3-BP, 3-bomopyruvate.

apoptosis by TRAIL via the AMPK activation. MCF-7 and MDA-MB-231 cells were treated with Compound C (an AMPK inhibitor), and 3-BP, TRAIL or both 3-BP and TRAIL. The results of the MTT assays demonstrated that the viability of cells treated with either 3-BP, TRAIL or 3-BP and TRAIL and Compound C did not exhibit a significant difference compared with the control group (Fig. 4A). Similar results were obtained using PI staining, which indicated the number of apoptotic cells upon each treatment (Fig. 4B). Therefore, the results suggested that Compound C attenuated the TRAIL-synergistic effect of 3-BP.

Compound C inhibits 3-BP-induced upregulation of DR5, GRP78, CHOP and p-AMPK apoptosis-associated proteins. To investigate the regulatory role of AMPK in mediating the effects of 3-BP associated with the upregulation of ER stress- and apoptosis-associated proteins, MCF-7 and MDA-MB-231 cells were treated with Compound C. Membrane receptor

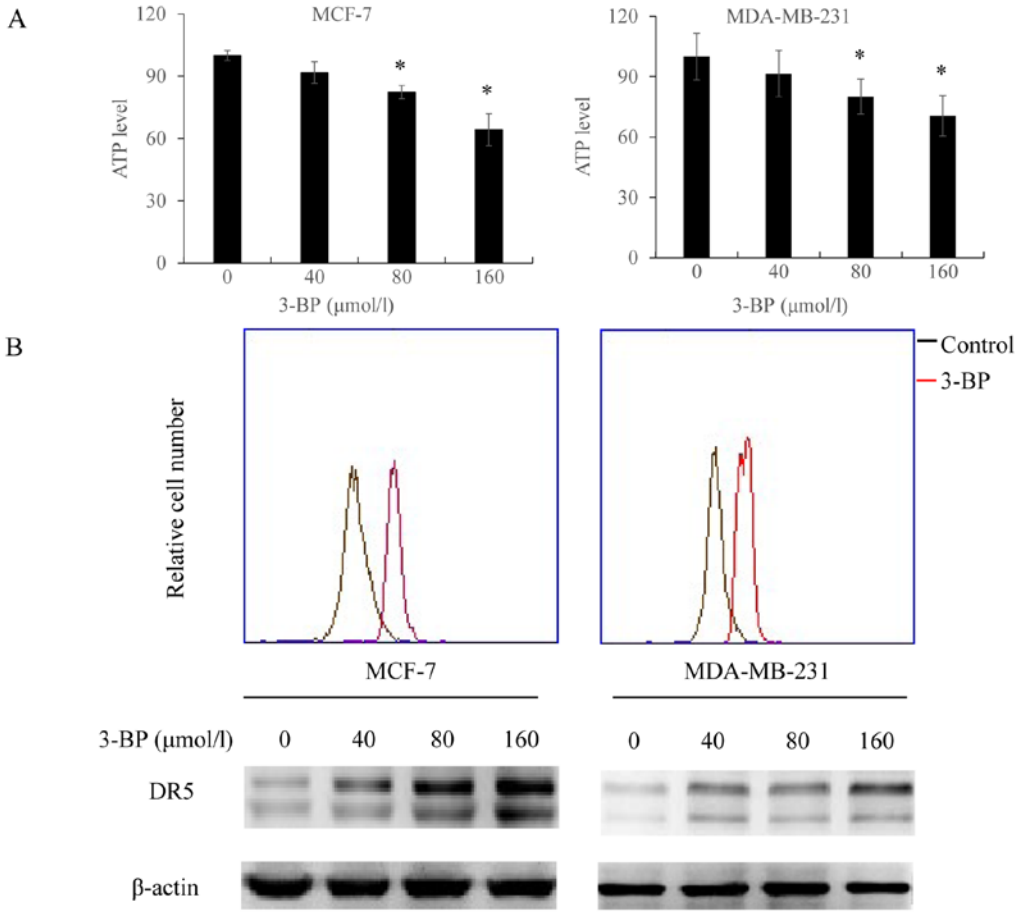

Figure 2. 3-BP inhibits ATP generation and upregulates DR5 expression. (A) MCF-7 and MDA-MB-231 cells were treated with various concentrations of 3-BP (0, 40, 80 and 160 μmol/l) for 4 h, and ATP levels were determined using an ATP assay kit. *P<0.05 vs. the control group. (B) Upper panels: MCF-7 and MDA-MB-231 cells were treated with medium (Control) or 80 μmol/l 3-BP for 24 h, and investigated using flow cytometry. Lower panels: MCF-7 and MDA-MB-231 cells were treated with various concentrations of 3-BP (0, 40, 80 and 160 μmol/l) for 24 h. Western blot assays were performed to determine the expression level of DR5. DR5, death receptor 5; 3-BP, 3-bomopyruvate; ATP, adenosine tri-phosphates.

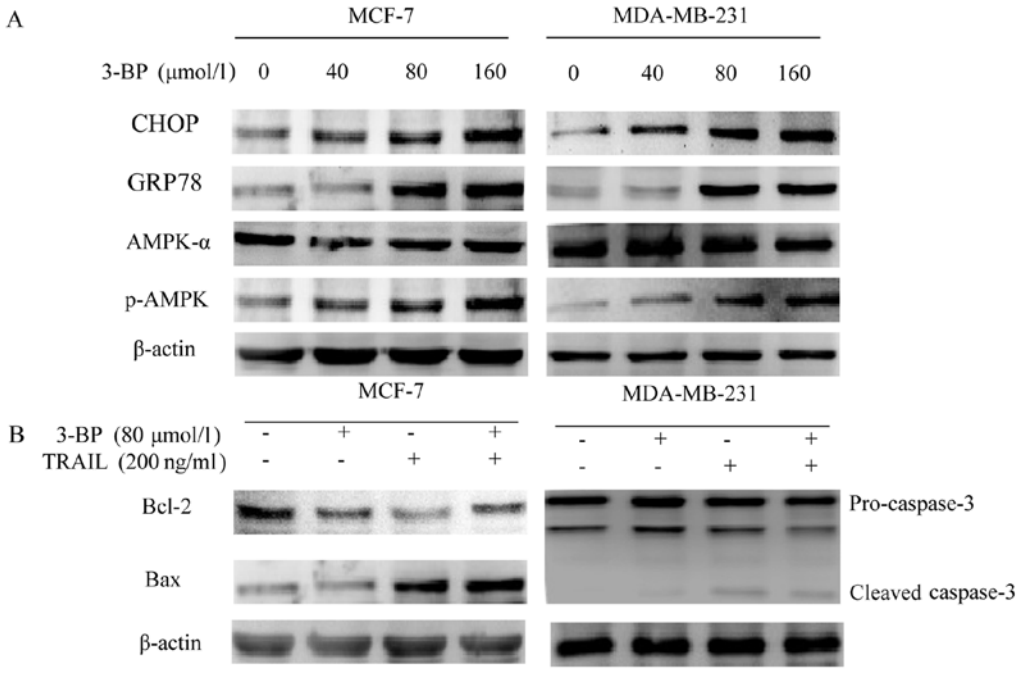

Figure 3. 3-BP induces AMPK phosphorylation and induces cell death. (A) The levels of CHOP, GRP78, AMPK-α and p-AMPK were detected in breast cancer MCF-7 and MDA-MB-231 cells treated with 3-BP (0, 40, 80 and 160 μmol/l) for 24 h via western blot analysis. (B) The expression levels of Bax in MCF-7 cells and caspase-3 protein in MDA-MB-231 cells were detected via western blotting in cells treated with 80 μmol/l 3-BP and 200 ng/ml TRAIL. 3-BP, 3-bomopyruvate.

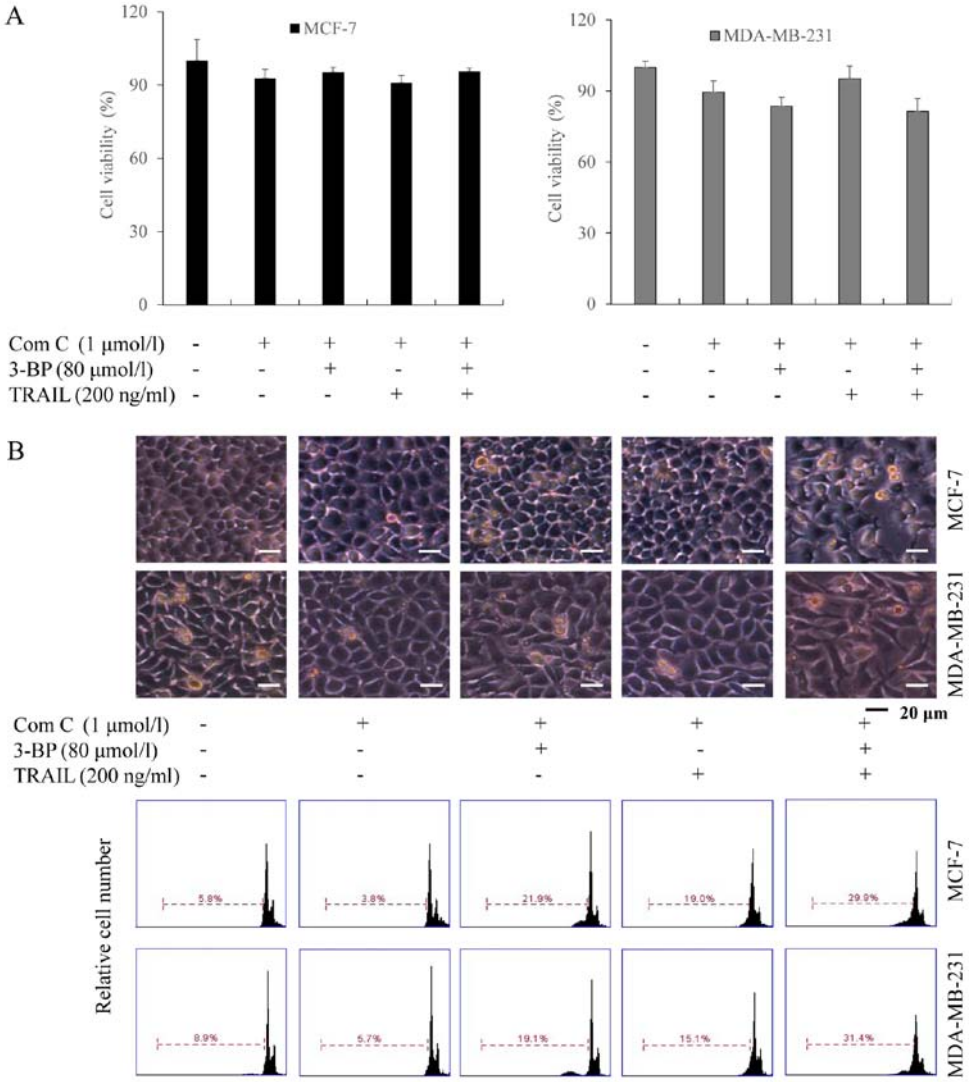

Figure 4. The AMPK inhibitor Compound C attenuates the effects of 3-BP on breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with 1 μmol/l Compound C (Com C), 80 μmol/l 3-BP, and 200 ng/ml TRAIL, or both 3-BP and TRAIL, as indicated. (A) Cell viability was determined using an MTT assay. (B) Cell morphology was examined via light microscopy and apoptosis rate was determined using the PI staining method and flow cytometry. Data are expressed as the mean ± standard error of the mean (n=3). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; 3-BP, 3-bomopyruvate.

staining revealed that the fluorescence intensity of DR5 did not increase in breast cancer cells following treatment with Compound C (Fig. 5A). In addition, the results demonstrated that DR5, CHOP, GRP78 and p-AMPK levels did not significantly increase in cells treated with various concentrations of 3-BP and Compound C compared with the control cells (Fig. 5B). Similarly, the expression of Bax and Bcl-2 in MCF-7 cells and caspase-3 levels in MDA-MB-231 cells treated with Compound C, 3-BP, TRAIL, or 3-BP and TRAIL did not demonstrate a significant increase compared to with the control cells (Fig. 5C). These results suggested that AMPK activation may be involved in the ER stress response induced by 3-BP in breast cancer cells.

Antitumor efficacy of 3-BP and TRAIL in tumor xenografts. MCF-7 cells were inoculated hypodermically into the forelimb of nude mice to induce the formation of xenograft tumors. Once the tumors reached a mean volume of ~100 mm^3, mice matched for tumor volumes were divided into 4 groups and treated with either PBS, 3-BP, TRAIL or 3-BP and TRAIL. Following 28 days of treatment, the tumor volumes of mice treated with either PBS, 3-BP, TRAIL, or 3-BP and TRAIL were ~1,200±100, ~850±71, ~700±77 and ~232±40 mm^3, respectively (Fig. 6A). To investigate the hepatotoxicity and nephrotoxicity of the aforementioned treatments, the levels of serological markers including aspartate aminotransferase (AST), alanine-aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (Cr) were determined. The results revealed that mice did not exhibit marked levels of toxicity following treatment with either PBS, 3-BP, TRAIL or 3-BP and TRAIL, as the expression of these markers was unchanged (Fig. 6B). The results of H&E staining demonstrated that necrosis occurred in the tumors of groups treated with 3-BP, TRAIL or both 3-BP and TRAIL, and the necrotic area in the group treated with both 3-BP and TRAIL was larger compared with the other treatment groups (Fig. 6C). H&E staining of the liver and kidney revealed that no evident damage was induced following the treatments (Fig. 6C). Finally, TUNEL staining demonstrated that the number of apoptotic cells in the group treated with 3-BP and TRAIL

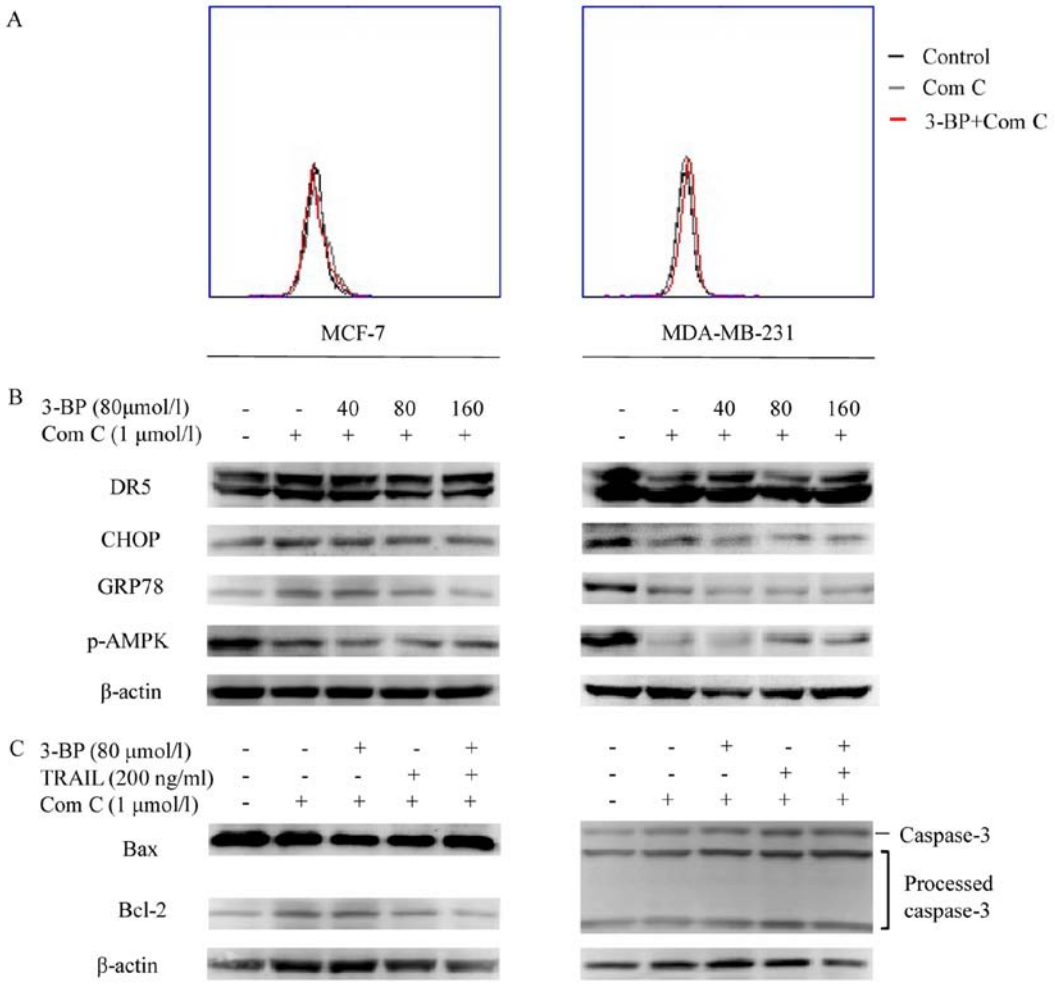

Figure 5. AMPK induces ER stress and sensitizes breast cancer cells to TRAIL in response to treatment with 3-BP. (A) Cells treated with medium (Control), 1 μmol/l Compound C (Com C) or Compound C combined with 80 μmol/l 3-BP for 24 h were investigated via flow cytometry. (B) MCF-7 and MDA-MB-231 cells pre-treated with 1 μmol/l Compound C for 1 h were subsequently treated with 0, 40, 80 or 160 μmol/l 3-BP for 24 h. The expression levels of AMPK, GRP78, CHOP and DR5 were investigated with western blotting. (C) Cells pre-treated with or without 1 μmol/l Compound C for 1 h, were treated with medium, Compound C, 80 μmol/l 3-BP, 200 ng/ml TRAIL or both 3-BP and TRAIL, as indicated, for 24 h. The expression levels of Bax and Bcl-2 were determined in MCF-7 cells and caspase-3 was investigated in the MDA-MB-231 cells by western blotting. β-actin served as loading control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; 3-BP, 3-bomopyruvate; DR5, death receptor 5.

was increased compared with the other groups (Fig. 6D). Therefore, the results suggested that the antitumorigenic effect of 3-BP and TRAIL was associated with low hepatotoxicity and nephrotoxicity *in vivo*.

Discussion

Breast cancer is an aggressive malignancy. It is the most frequently diagnosed cancer and its incidence has been increasing in both economically developed and developing countries (22). Numerous therapeutic strategies are available for the treatment of patients with breast cancer, including chemotherapy, surgery and radiotherapy (23). However, the mortality rate for patients with breast cancer remains high. Therefore, novel and effective therapies for the treatment of patients with breast cancer are required. It has been demonstrated that TRAIL is an effective anticancer agent that inhibits the proliferation of cancer cells and tumor growth in xenograft models (24-26). However, loss or mutation of the death receptors targeted by TRAIL may lead to drug resistance (27). Therefore, it is important to determine the molecular mechanisms underlying TRAIL-induced apoptosis in order to develop novel therapeutic agents that circumvent resistance (28,29).

The ER stress response pathway regulates cancer cell fate, and may induce autophagy, senescence, or apoptosis (30). Protein sensors such as PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activator transcription factor 6 (ATF6) initiate the ER stress response and regulate the unfolded protein response (UPR) under both pathological and physiological conditions (31). Specifically, the PERK/eukaryotic initiation factor 2 (eIF2α)/activator transcription factor 4 (ATF4) axis upregulates CHOP, which subsequently induces apoptosis (32,33). The expression of DR5 is regulated by CHOP, the predominant regulator of the ER stress response. Several previous studies point in this direction: Lu *et al* revealed that ER stressors regulated the transcription of DR5 via the UPR mediator, CHOP (15). In addition, Guo *et al* demonstrated that tunicamycin sensitized human colon cancer cells to TRAIL-induced apoptosis via the JNK-CHOP-mediated upregulation of DR5 expression (13).

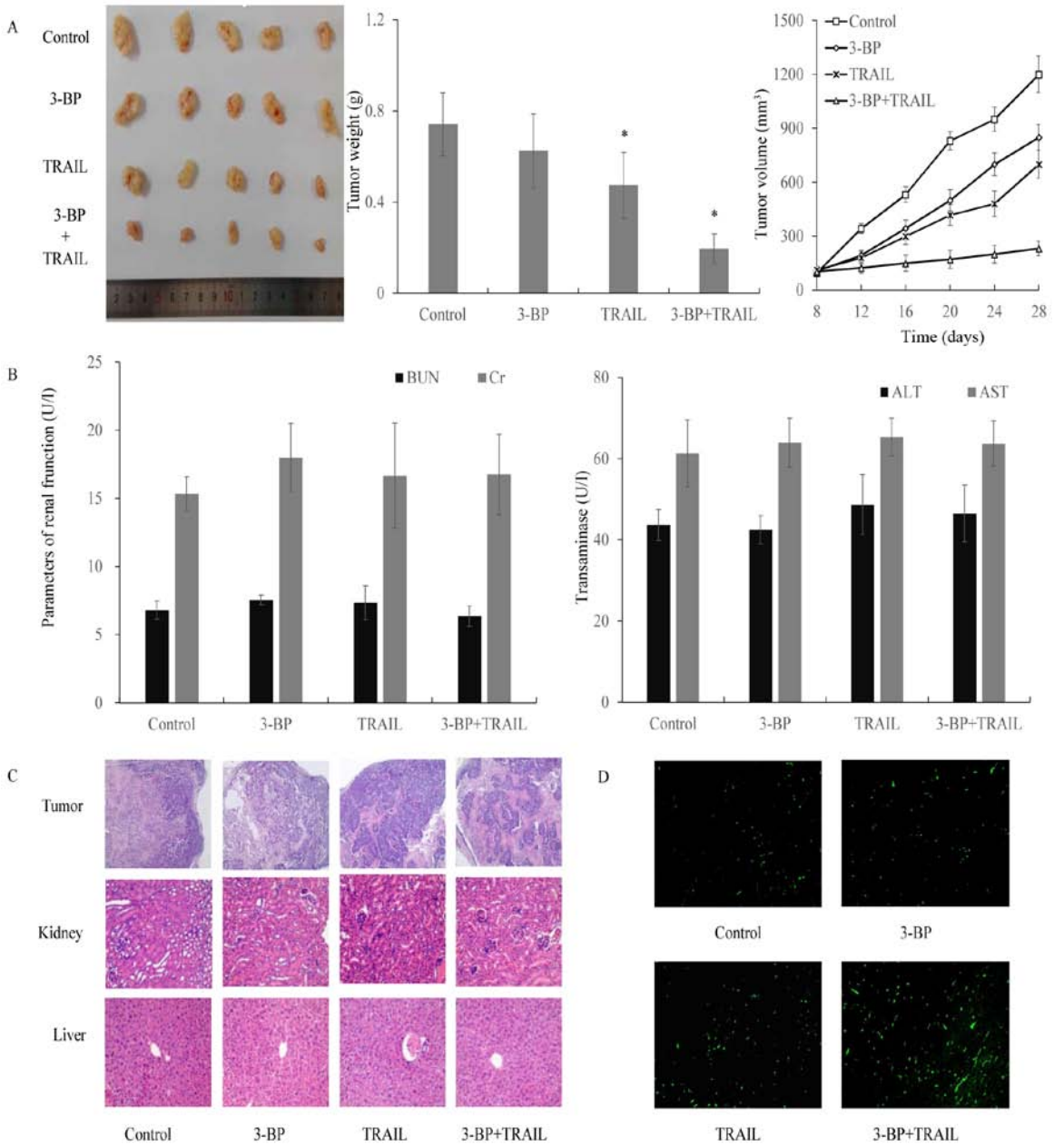

Figure 6. Efficacy of treatment with 3-BP and TRAIL in tumor xenografts. MCF-7 cells were injected subcutaneously into mice ($4x10^6$ cells/mouse) to induce tumor formation. Mice were randomly assigned into four groups, and injected intraperitoneally with either vehicle (200 μl), 3-BP (8 mg/kg BW), TRAIL (0.1 mg/kg BW) or a combination of 3-BP and TRAIL, every 4 days. Mice were sacrificed after 28 days of treatment. (A) Representative tumors isolated from each group. Data are expressed as the mean ± standard error of the mean (n=5). (B) Investigation of hepatotoxic and nephrotoxic levels in nude mice treated with 3-BP, TRAIL or 3-BP and TRAIL *in vivo*. AST, ALT, BUN and Cr levels were determined using an assay kit, and the activities of the indicated markers are presented as unit/l (U/l). Tumor tissues were subjected to (C) H&E and (D) TUNEL staining. BW, body weight; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; 3-BP, 3-bomopyruvate; AST, aspartate aminotransferase; ALT, alanine-aminotransferase; BUN, blood urea nitrogen; Cr, creatinine.

Chen *et al* determined that the expression of DR5 in human esophageal cancer cells is regulated by the ATF4-CHOP-DR5 axis (14). In addition, treatment with caffeic acid phenethyl ester upregulated the protein level of DR5 and promoted apoptosis in hepatocarcinoma Hep3B cells through CHOP (34). While ER stress sensors and mediators have been revealed to be involved in the regulation of death receptors, the molecular mechanisms underlying 3-BP and TRAIL-induced apoptosis in human breast cancer cells have not yet been clarified. In the present study, it was demonstrated that treatment with 3-BP and TRAIL in MCF-7 and MDA-MB-231 breast cancer cells is associated with the suppression of cell viability in a dose-dependent manner. Furthermore, the results revealed that co-treatment with 3-BP and TRAIL significantly increased apoptosis compared with 3-BP or TRAIL alone, thus suggesting that 3-BP and TRAIL exhibit a synergistic anticancer effect both *in vitro* and in a tumor xenograft model. Furthermore, 3-BP was demonstrated to induce the ER stress response and subsequently to upregulate GRP78 and CHOP levels in MCF-7 and MDA-MB-231 cells. Simultaneously, 3-BP was revealed to increase the protein expression level of DR5, which subsequently enhanced the sensitivity of cells to TRAIL. These data suggested that the anticancer efficacy of 3-BP in human breast cancer cells is regulated via the activa-

tion of ER stress and the upregulation of DR5, which, in turn, sensitizes cells to TRAIL treatment.

There are three types of cell death, namely apoptosis, autophagic cell death and necrosis, largely defined by the morphology of the dying cells (35). The death receptor and mitochondrial pathways play major roles in apoptotic cell death despite the existence of additional regulatory pathways associated with apoptosis. DR5 is upregulated in ER stress-induced apoptosis and is an important factor in this mechanism (35). DR5 binds to its ligands and then induces apoptosis via recruitment of the caspase-activation platform. UPR sensitizes cancer cells to TRAIL-induced apoptosis by enhancing the enzymatic activity of caspase-8 and caspase-3/7. Conversely, mitochondrial outer membrane permeabilization (MMOP) has an important role in the mitochondrial pathway of apoptosis (36). MMOP is regulated by members of the Bcl-2 family. Bcl-2 proteins are classified into three groups: The pro-apoptotic effector proteins (e.g., Bax and Bak), anti-apoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-xL and Mcl-1), and BH3-only proteins (e.g., Bad, Bim, Bid and Noxa) (37). The results of the present study suggested that co-treatment with 3-BP and TRAIL decreased the expression of Bcl-2 in MCF-7 cells and upregulated the expression of Bax in MCF-7 cells and caspase-3 in MDA-MB-231 cells. Bax expression was investigated in MCF-7 cells, as these cells do not express caspase-3 (38). The results of the present study indicated that the co-treatment with 3-BP and TRAIL may be involved in caspase-3- and Bcl-2 family-mediated apoptosis in breast cancer cells.

AMPK is a sensor of cellular energy status that regulates metabolism in cellular processes. AMPK is activated by changes in the adenosine mono-phosphate (AMP)/ATP or adenosine di-phosphate (ADP)/ATP ratio (16,17). Numerous studies indicated that activated AMPK induced apoptosis in pancreatic cancer cells, renal carcinoma Caki cells, neuroblastoma and colorectal cancer cells (39-42). Furthermore, previous studies demonstrated that the inhibition of glycolysis, or caloric restriction may promote AMPK-induced apoptosis in cancer cells (43,44), which indicated that the activation and phosphorylation of AMPK could be used as a therapeutic strategy for the treatment of cancer. We hypothesized that 3-BP, an inhibitor of glycolysis, activated AMPK through an effect on cellular energy status. Western blot assays demonstrated that levels of p-AMPK were increased following treatment with 3-BP in a concentration-dependent manner. However, the expression of AMPK-α did not show a significant difference post-treatment. To investigate the association between p-AMPK and ER stress response, MCF-7 and MDA-MB-231 cells were treated with Compound C, an inhibitor of AMPK. Western blot analysis revealed that the increased expression levels of ER stress response associated proteins following treatment with 3-BP and TRAIL was attenuated following treatment with Compound C. In addition, the viability of cells treated with Compound C and 3-BP and/or TRAIL significantly decreased, while the expression of Bcl-2 family proteins (Bcl-2 and Bax) and caspase-3 did not significantly differ compared with the control group. These results indicated that the 3-BP induced ER stress response may be associated with the activation and phosphorylation of AMPK.

In conclusion, the results of the present study demonstrated that 3-BP, a hexokinase II inhibitor that interferes with glycolysis, upregulated GRP78, CHOP and DR5 expression levels and enhanced TRAIL-induced apoptosis in MCF-7 and MDA-MB-231 breast cancer cells. These results revealed that the ER stress response had a crucial role in sensitizing human breast cancer cells to TRAIL-induced apoptosis, potentially via the activation and phosphorylation of AMPK. Further studies are required to determine whether the simultaneous treatment with 3-BP and TRAIL may represent a novel therapeutic strategy for patients with cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HL and SZ conceived and designed the experiments. YC and LW performed the experiments. XZ, XL, YC, SZ, LZ, SZ and HL analyzed the data. Image processing was conducted by QL and QP. YC wrote and proofread the paper. SZ and HL revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in this study involving animals were in accordance with the ethical standards of the Bengbu Medical College Experimental Animal Ethics Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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