3-Bromopyruvic acid regulates glucose metabolism by targeting the c-Myc/TXNIP axis and induces mitochondria-mediated apoptosis in TNBC cells

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Abstract. Aerobic glycolysis is commonly observed in tumor cells, including triple-negative breast cancer (TNBC) cells, and the rate of aerobic glycolysis is higher in TNBC cells than in non-TNBC cells. Hexokinase 2 (HK2) is a key enzyme in the glycolytic pathway and a target of the transcription factor c-Myc, which is highly expressed in TNBC and promotes aerobic glycolysis by enhancing HK2 expression. As an inhibitor of HK2, 3-bromopyruvic acid (3-BrPA) exhibits good therapeutic efficacy in intrahepatic and extrahepatic tumors and inhibits the proliferation of human tumor cells with high expression levels of c-Myc *in vivo* and *in vitro*. In addition, 3-BrPA combines with photodynamic therapy to inhibit TNBC cell migration. Thioredoxin-interacting protein (TXNIP) competes with c-Myc to reduce glucose consumption in tumor cells to restrain cell proliferation. A comparative analysis was performed in the present study in TNBC (HCC1143) and non-TNBC (MCF-7) cell lines to explore the effect of 3-BrPA on energy metabolism in TNBC cells and to investigate the possible mechanism of action. Cell viability and apoptosis were detected through Cell Counting Kit-8 and flow cytometry assays, respectively. Expression levels of HK2, glucose transporter 1, TXNIP, c-Myc and mitochondria-regulated apoptosis pathway proteins were measured through western blotting. 3-BrPA inhibited cell proliferation, downregulated c-Myc and HK2 expression, and upregulated TXNIP expression in TNBC cells, but it doesn't have the same effect on non-TNBC cells. Furthermore, 3-BrPA induced the typical manifestations of mitochondrial-mediated apoptosis such as decreasing Bcl-2 expression and increasing Bax, Cyt-C and Caspase-3 expression. The present results suggested that 3-BrPA promoted TXNIP protein expression and reduced HK2 expression in TNBC cells by downregulating c-Myc expression, inhibiting glycolysis including suppressing lactate generation, intracellular ATP generation and HK activity, inducing mitochondrial-mediated apoptosis and eventually suppressing TNBC cell proliferation. These findings may reveal a novel therapeutic target for the clinical treatment of TNBC.

Introduction

Breast cancer is one of the most frequent malignant tumors that occurs in females worldwide, and its incidence is increasing annually (1). Furthermore, breast cancer is a disease with multiple subtypes (2) and 12-17% of cases are characterized as triple-negative breast cancer (TNBC) with the following features: Lack of expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (3). The highly invasive nature of TNBC is manifested in its poor clinical prognosis and rapid relapse (3). Although the diagnosis and treatment of breast cancer have improved, TNBC cannot be cured using endocrine therapy or current conventional chemotherapies (4). Therefore, it is essential to understand the biological properties of TNBC and discover innovative, effective and reliable drugs for TNBC treatment to further improve patient prognosis. Normally differentiated cells mainly rely on oxidative phosphorylation of mitochondria to power cells, whereas most tumor cells rely on aerobic glycolysis through the phenomenon known as the 'Warburg effect' (5). In this process, most pyruvate is reduced to lactic acid by lactate dehydrogenase rather than entering mitochondria in cancer cells (5). TNBC cells also exhibit abnormal glucose metabolism with a higher rate of glucose absorption and glycolysis (6).

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Hexokinase 2 (HK2) serves an important role in the first step of glycolysis as a key enzyme (7). As an inhibitor of HK2, 3-bromopyruvic acid (3-BrPA) has a good therapeutic effect on intrahepatic and extrahepatic tumors, and no obvious toxic reaction is observed (8). In addition, 2-deoxyglucose, which is a glycolytic inhibitor, and 3-BrPA cooperate with photodynamic therapy to inhibit the migration of TNBC cells, demonstrating that 3-BrPA could inhibit cell proliferation by affecting aerobic glycolysis (9). A previous study also demonstrated that 3-BrPA inhibits human tumor cells with high Myc expression *in vivo* and *in vitro* but has a minimal effect on cells with low Myc expression (10). HK2 has also been demonstrated to be a target of c-Myc (11). As transmembrane transporters, glucose transporters (GLUTs) represent another important factor in glycolysis for the entry of glucose into cells (12). TNBC displays higher levels of GLUT1, one of the most common subtypes of GLUT, than other subtypes of breast cancer (13). The inhibition of GLUT1 expression has been detected in lymphoma cells after treatment with 3-BrPA (14).

Thioredoxin-interacting protein (TXNIP), a binding partner with negative regulatory effect of thioredoxin, could inhibit glucose uptake and aerobic glycolysis (15). TXNIP also has pro-apoptosis and anti-proliferative activities in cancer cells (16). A negative regulatory effect of TXNIP on tumor cells has been reported in several cancer types, including hepatocellular carcinoma and bladder cancer (17,18). Particularly in breast cancer, the tumor-suppressive effect of TXNIP is well established, with one study demonstrating that higher TXNIP expression portended an improved patient prognosis, suggesting that TXNIP is likely to serve an important role in the suppression of breast cancer (19,20). Recently, another study demonstrated that TNBC cells have unique molecular characteristics, including high levels of c-Myc and low levels of TXNIP (21). Furthermore, c-Myc promotes glucose consumption in TNBC to maintain proliferation, directly competing with TXNIP (21). Another study identified the EGFR-MYC-TXNIP axis as an important regulator of the TNBC metabolism and demonstrated the association between the $EGFR_{high}$-MYC_{high}-$TXNIP_{low}$ gene signature with aggressive glycolytic metabolism and a poor survival rate in TNBC (22). Based on these findings, it can be hypothesized that 3-BrPA may inhibit c-Myc, downregulate HK2 and GLUT1 expression, upregulate TXNIP expression, and suppress glycolysis, subsequently inhibiting proliferation and inducing apoptosis in TNBC cells.

To confirm this scientific hypothesis, the present study analyzed the effects of 3-BrPA on aerobic glycolysis in TNBC using human TNBC (HCC1143) and non-TNBC (MCF-7) cell lines. After treatment with 3-BrPA at different concentrations and for different durations, the effects of 3-BrPA were evaluated in terms of cell viability using a Cell Counting Kit-8 (CCK-8) assay and apoptosis using flow cytometry. HK activity, lactate generation, ATP generation and the expression levels of TXNIP, GLUT1, HK2 and c-Myc, as well as mitochondria-mediated apoptosis pathway proteins were examined by western blotting to further clarify the internal mechanism by which 3-BrPA may inhibit TNBC cell viability and induce apoptosis.

Materials and methods

Cells and cell culture. TNBC (HCC1143) and non-TNBC (MCF-7) cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in complete growth medium consisting of DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.). Cells were incubated in an incubator (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2 and passaged every 3-4 days. Morphological changes were observed under an inverted fluorescence microscope (IX73; Olympus).

CCK-8 assay. CCK-8 (Dojindo Laboratories, Inc.) was used to detect the viability of cells. Briefly, ~$1x10^4$ cells/well were added to 96-well plates and cultured in an incubator with 5% CO_2 at 37°C. Subsequently, cells were treated with 3-BrPA (Sigma-Aldrich; Merck KGaA) at 37°C at different concentrations (0, 20, 30, 40, 50 and 60 μM) and for different durations (24 and 48 h). CCK-8 reagent (10 μl) was added to each well, mixed and then incubated for an additional 2 h. The absorbance values of the cells were immediately detected at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The inhibitory rate was calculated as $(1-A_{sample}/A_{control})x100\%$, where A_{sample} is the absorbance value of the sample treated with 3-BrPA at different concentrations and time points and $A_{control}$ is the absorbance value of the control group (untreated cells) recorded at the same time point. Each experiment was performed in triplicate. The viability curves and IC_{50} of 3-BrPA for each cell line were calculated by GraphPad Prism 8.0.1 (GraphPad Software, Inc.).

Apoptosis analysis. Cells were treated with 0, 20, 40 and 60 μM 3-BrPA for 24 h. Approximately $1x10^5$ cells were collected after centrifugation at 300 x g with pre-cooled phosphate buffer saline for 10 min at 4°C. Then cells were stained with 5 μl Annexin V-allophycocyanin (Annexin V-APC) and 10 μl 7-amino-actinomycin D (7-AAD) according to the manufacturer's instructions for the Annexin V-APC/7-AAD cell apoptosis kit (Hangzhou Lianke Biotechnology Co., Ltd.). After gentle vortexing, cells were incubated at room temperature for 5 min in the dark. Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences) with CellQuest Pro 5.2.1 (BD Biosciences). Each experiment was performed in triplicate.

Measurement of HK activity. Cells were seeded into 6-well plates at a density of $2x10^6$ cells/well and were then incubated with 3-BrPA (0, 20 and 40 μM) for both 24 and 48 h at 37°C in 5% CO_2. HK activity detection was performed the Hexokinase Activity Detection kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The absorbance values at 340 nm at 20 sec (A1) and 320 sec (A2) after sample addition were measured using a spectrophotometer (Thermo Fisher Scientific, Inc.). Then, A1 and A2 were plugged into the formula HK(U/10^4 cell)=$[(\Delta A)(V_{total}/(\epsilon xd)x10^9]/(500xVs_{sample}/V_{sample\_total})/t=2.226x\Delta A(\Delta A=A2-A1)$, where V_{total} refers to the total volume of the reaction system

$(1.038 \times 10^{-3}$ l), ε is the NADPH molar extinction coefficient $(6.22 \times 10^3$ l/mol/cm), d represents the diameter of the cuvette (1 cm), V_{sample} is the sample volume (0.03 ml), V_{sample_total} is the extract volume (1 ml), t is the reaction time (5 min), and 500 is the total number of cells (500×10^4). These factors were used to calculate HK activity. An enzyme activity unit (1 unit) is defined as 1 nmol of NADPH produced per min per 10,000 cells.

Measurement of lactate generation. The two cell lines were seeded into 96-well plates at a density of 1×10^4 cells/well. After attachment, the cells were treated with different concentrations (0, 20 and 40 μM) of 3-BrPA for 24 or 48 h. The culture medium was collected to measure the level of lactate generation using the lactate assay kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. The optical density at 530 nm was measured using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.).

ATP generation assay. ATP generation was measured using the ATP Assay Kit (Beijing Solarbio Science & Technology Co., Ltd.). The two cell lines were seeded into 96-well plates at a density of 1×10^5 cells/well and cultured overnight at 37˚C. Subsequently, the cells were incubated with 3-BrPA (0, 20 and 40 μM) for 4 or 8 h (based on our preliminary experiments, the ATP generation after 24/48 h incubation with 3-BrPA is already very low and cannot be measured accurately, therefore 4/8 h was selected). Then, the cells were treated according to the manufacturer's protocol. The absorbance values at 340 nm at 10 sec (A1) after sample addition and 3 min after a 25˚C water bath (A2) were measured using a spectrophotometer (Thermo Fisher Scientific, Inc.). Both A1 and A2 were plugged into the formula ATP $(\mu mol/10^6$ cell)$=(\Delta A_{sample})/(\Delta A_{standard}/C_{standard})V/n(\Delta A=A2-A1)$, where $C_{standard}$ refers to the concentration of the standard solution (0.625 μmol/ml), V denotes the added volume of extraction solution (1 ml), and n indicates the number of cells $(x10^6)$. These factors were used to calculate ATP production.

Western blotting. The two cell lines were treated with 0, 20 and 40 μM 3-BrPA for 24 or 48 h. Total protein was extracted using a Total Protein Extraction kit (Beijing Solarbio Science & Technology Co., Ltd.) for western blot analysis. The protein concentration was determined using a BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). The proteins (30 μg per lane) were separated on a denaturing 12% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane. Then the membrane was cultured in QuickBlock Blocking Buffer for Western Blot (Beyotime Institute of Biotechnology) at room temperature for 1 h. The membrane was incubated with antibodies against GLUT1 (1:1,000; cat. no. TA312796; OriGene Technologies, Inc.), c-Myc (1:1,000; cat. no. TA150121; OriGene Technologies, Inc.), TXNIP (1:1,000; cat. no. TA349090; OriGene Technologies, Inc.), HK2 (1:1,000; cat. no. TA500856; OriGene Technologies, Inc.), Bcl-2 (1:2,000; cat. no. TA806591; OriGene Technologies, Inc.), Bax (1:2,000; cat. no. TA810334; OriGene Technologies, Inc.), cytochrome *c* (Cyt-C; 1:5,000; cat. no. ab13575; Abcam), Caspase-3 (1:1,000; cat. no. TA301776; OriGene Technologies, Inc.) and β-actin (1:1,000; cat. no. TA811000;

OriGene Technologies, Inc.) at room temperature overnight. Then, the membrane was incubated with the IRDye® 800CW goat anti-rabbit IgG secondary antibody (1:10,000; cat. no. 926-32211; LI-COR Biosciences) and the IRDye® 800CW goat anti-mouse IgG secondary antibody (1:10,000; cat. no. 926-32210; LI-COR Biosciences) at room temperature for another 2 h. Protein bands were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences), and the grayscale values of the bands were determined using Odyssey Application software 3.0 (LI-COR Biosciences). Each experiment was performed in triplicate.

Statistical analysis. All results were presented as the means ± standard deviations and images were analyzed using SPSS 20.0 statistical software (IBM Corp.) and GraphPad Prism 8.0.1 software (GraphPad Software, Inc.). Statistical analysis was performed using one-way ANOVA and Bonferroni's correction. $P<0.05$ was considered to indicate a statistically significant difference. The experiments were performed in triplicate.

Results

3-BrPA inhibits TNBC cell viability in vitro. The effects of 3-BrPA on cell viability in TNBC (HCC1143) and non-TNBC (MCF-7) cell lines were examined using a CCK-8 assay. Morphological changes were observed under an inverted fluorescence microscope, and numerous morphological changes occurred in cells after treatment with 3-BrPA. Untreated cells attached closely to one another, and were polygonal in shape (Fig. 1A and B). However, cells treated with 3-BrPA became round or inflated with few cellular contacts and a reduction in the number of viable cells. Cell shrinkage, condensation of cytoplasm and chromosomes inside the cell were observed. Compared with the untreated control group, 3-BrPA treatment markedly reduced the cell viability of breast cancer cells. The IC_{50} values of HCC1143 and MCF-7 were 44.87 μM (95% CI, 40.72-48.69 μM) and 111.3 μM (95% CI, 104.4-117.7 μM) after 24 h and 41.26 μM (95% CI, 36.41-45.71 μM) and 75.87 μM (95% CI, 69.83-82.08 μM) after 48 h of exposure to 3-BrPA, respectively. The inhibitory effect was dose- and time-dependent. Notably, the inhibitory effect of 3-BrPA on the TNBC cell line was stronger than that on the non-TNBC cell line. The full range of results used to calculate the IC_{50} of 3-BrPA in MCF-7 cells is shown in Fig. S1.

3-BrPA promotes TNBC cell apoptosis in vitro. Flow cytometry was performed to evaluate cell apoptosis. The results demonstrated that the early apoptosis rates of the TNBC cell line (HCC1143) were significantly increased in a dose-dependent manner compared with those of the corresponding untreated control cells (Fig. 2A and B). However, 3-BrPA did not exert the same effect on the non-TNBC cell line (MCF-7). The late apoptosis rates were only significantly increased at highest 40 μM concentration in both TNBC cell line and non-TNBC cell line (Fig. 2A and B).

3-BrPA suppresses lactate generation in TNBC cells. Lactate is a vital final product in glycolysis that reflects glycolytic metabolism in cells (23). To study if 3-BrPA regulates
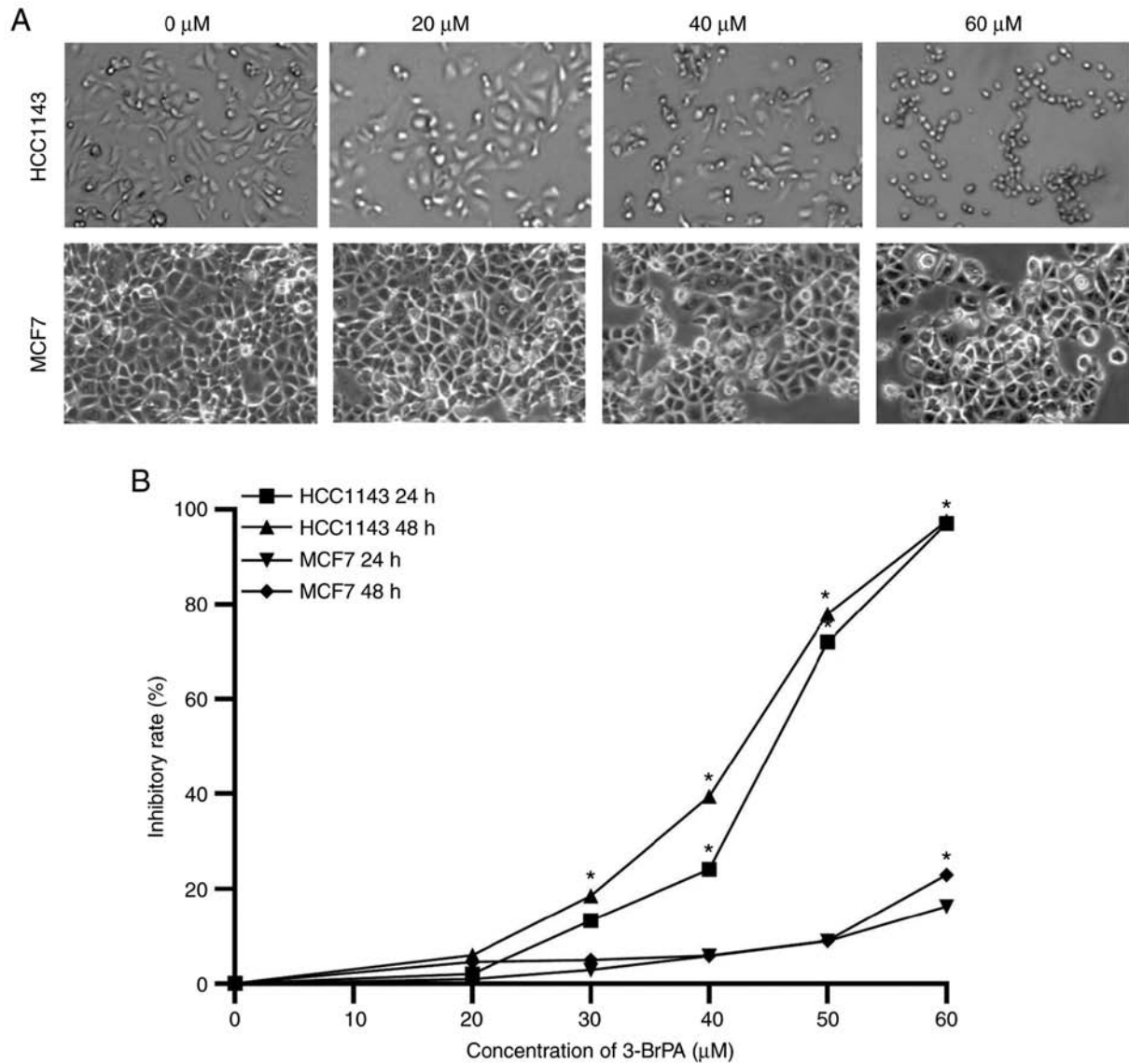
Figure 1. 3-BrPA inhibits TNBC cell viability. (A) Cell morphology changes were observed using an inverted microscope. Representative microscopy images showing the difference in size (magnification, x200) of untreated and 3-BrPA-treated HCC1143 and MCF-7 cells. Untreated cells attached closely to each other. However, the cells treated with 3-BrPA became round or floated up with larger zones between cells and the number of viable cells decreased. (B) Cell viability was measured using a Cell Counting Kit-8 assay. Cell viability was inhibited by 3-BrPA. Furthermore, the inhibitory rate in TNBC cells was higher than that in non-TNBC cells (*P<0.05 vs. non-TNBC group at the same time and dose). 3-BrPA, 3-bromopyruvic acid; TNBC, triple-negative breast cancer.

glycolytic metabolism in a TNBC cell line, the generation of lactate after treatment with 3-BrPA for 24 and 48 h was measured. Intracellular lactate generation was significantly suppressed at 30/40 μM at 24 h and 20/30/40 μM at 48 h in 3-BrPA-treated TNBC cells compared with control TNBC cells (HCC1143; Fig. 3A) and the results were dose- and time-dependent. In the non-TNBC group (MCF-7), 3-BrPA showed a similar significant effect only at highest concentration at 48 h, showing this effect was not as strong as that in the TNBC group.

3-BrPA reduces intracellular ATP generation in TNBC cells. The ATP generation level was considered to be a favorable indicator of the glycolysis rate in cells because it is the main ultimate product in glycolysis (24). Treatment with 3-BrPA reduced intracellular ATP generation TNBC cells (HCC1143) in a time- and dose-dependent manner (P<0.05; Fig. 3B). In

the non-TNBC cell line (MCF-7), the ATP level was not significantly affected (P>0.05).

3-BrPA decreases HK activity in TNBC cells. As a key enzyme in aerobic glycolysis, HK activity reflects the strength of glycolytic metabolism in tumor cells (25). The effect of 3-BrPA on HK in TNBC (HCC1143) and non-TNBC (MCF-7) cell lines was investigated. The present results demonstrated that HK activity was decreased in a dose-dependent manner in the TNBC cell line after incubation with 3-BrPA (P<0.05; Fig. 3C). The effect was prominent when the cells were treated with 30 and 40 μM 3-BrPA. However, no significant differences in the non-TNBC cell line group were noted compared with the control group.

Protein expression in TNBC cells is altered by 3-BrPA. To confirm the effect of 3-BrPA on c-Myc, TXNIP, HK2 and
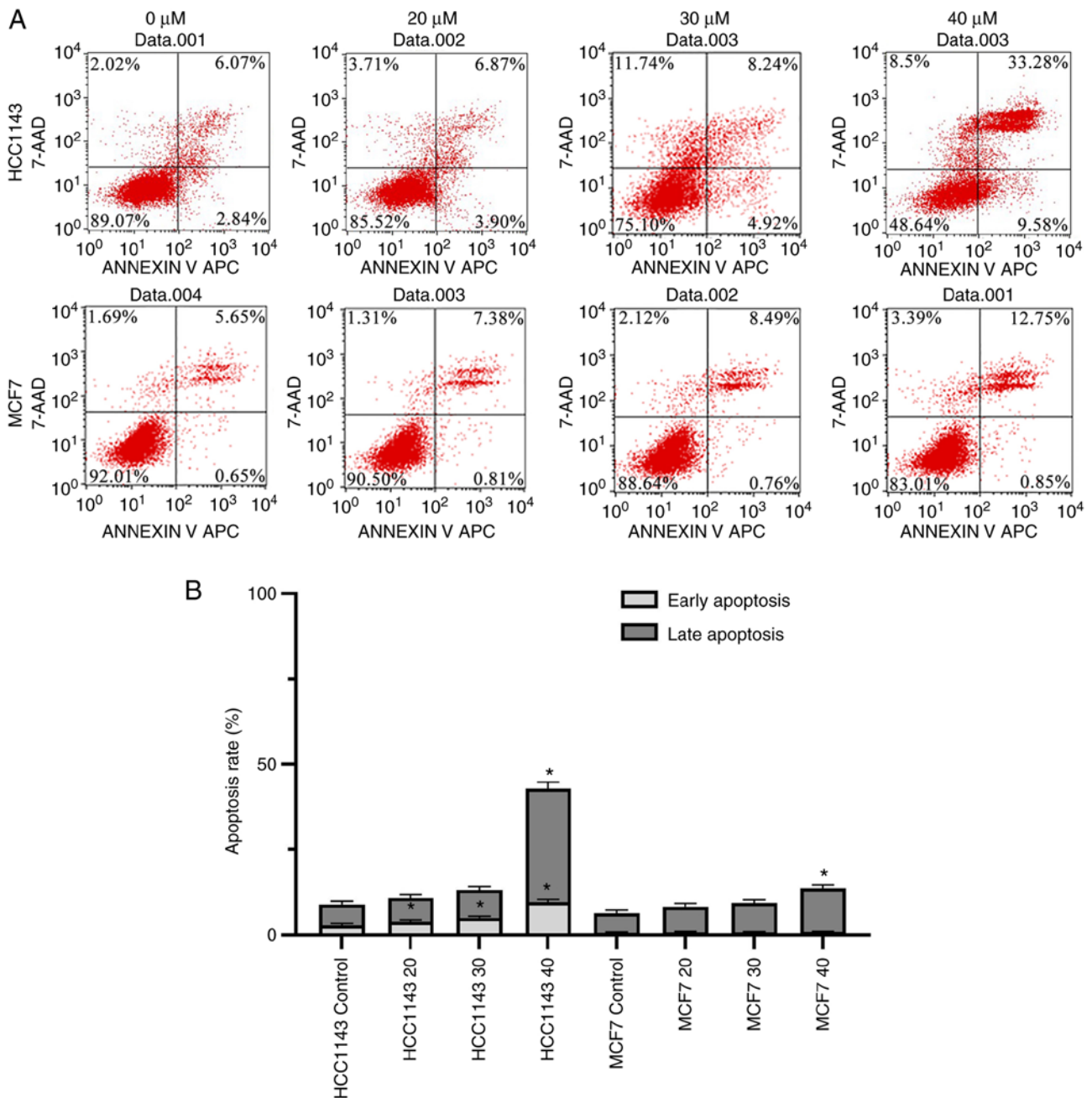
Figure 2. 3-BrPA induces TNBC cell apoptosis. (A) Cell apoptosis was analyzed using flow cytometry. The upper right quadrant shows late apoptotic cells, the lower right quadrant shows early apoptotic cells, the upper left quadrant shows necrotic cells and the lower left quadrant shows normal cells. (B) Percentages of the early apoptosis in TNBC cells induced by 3-BrPA were significantly increased. However, this trend was not identified in non-TNBC cells. The percentages of the late apoptosis in both TNBC cells and non-TNBC cells induced by 3-BrPA were only significantly increased at 40 μM. *P<0.05 vs. corresponding control group. 3-BrPA, 3-bromopyruvic acid; 7-AAD, 7-amino-actinomycin D; APC, allophycocyanin; TNBC, triple-negative breast cancer.

GLUT1 expression, TNBC (HCC1143) and non-TNBC (MCF-7) cells were treated with 3-BrPA at different doses for different durations. Subsequently, protein expression levels were detected through western blotting. The protein expression levels of c-Myc and HK2 in the TNBC group were dose and time-dependently decreased following treatment with 3-BrPA (Fig. 4A-C). Meanwhile, TXNIP protein expression was enhanced upon treatment with 3-BrPA. In the non-TNBC group, 3-BrPA did not exhibit a similar regulatory effect on c-Myc, HK2 and TXNIP. No evident changes were observed in the protein expression levels of GLUT1 in both the TNBC and non-TNBC groups.

Expression of mitochondria-regulated apoptosis pathway proteins is altered by 3-BrPA. To examine the effect of 3-BrPA on the mitochondria-mediated apoptosis pathway, the relative expression levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax, Cyt-C and Caspase-3 were analyzed. Both TNBC and non-TNBC cells were exposed to 3-BrPA at different doses and for different durations. Subsequently, the expression levels of mitochondria-mediated apoptosis pathway proteins were detected through western blotting. Treatment with 3-BrPA significantly decreased the protein expression levels of anti-apoptotic Bcl-2 at 40 μM at 24 h, and 20/40 μM at 48 h; while it increased the protein expression levels of
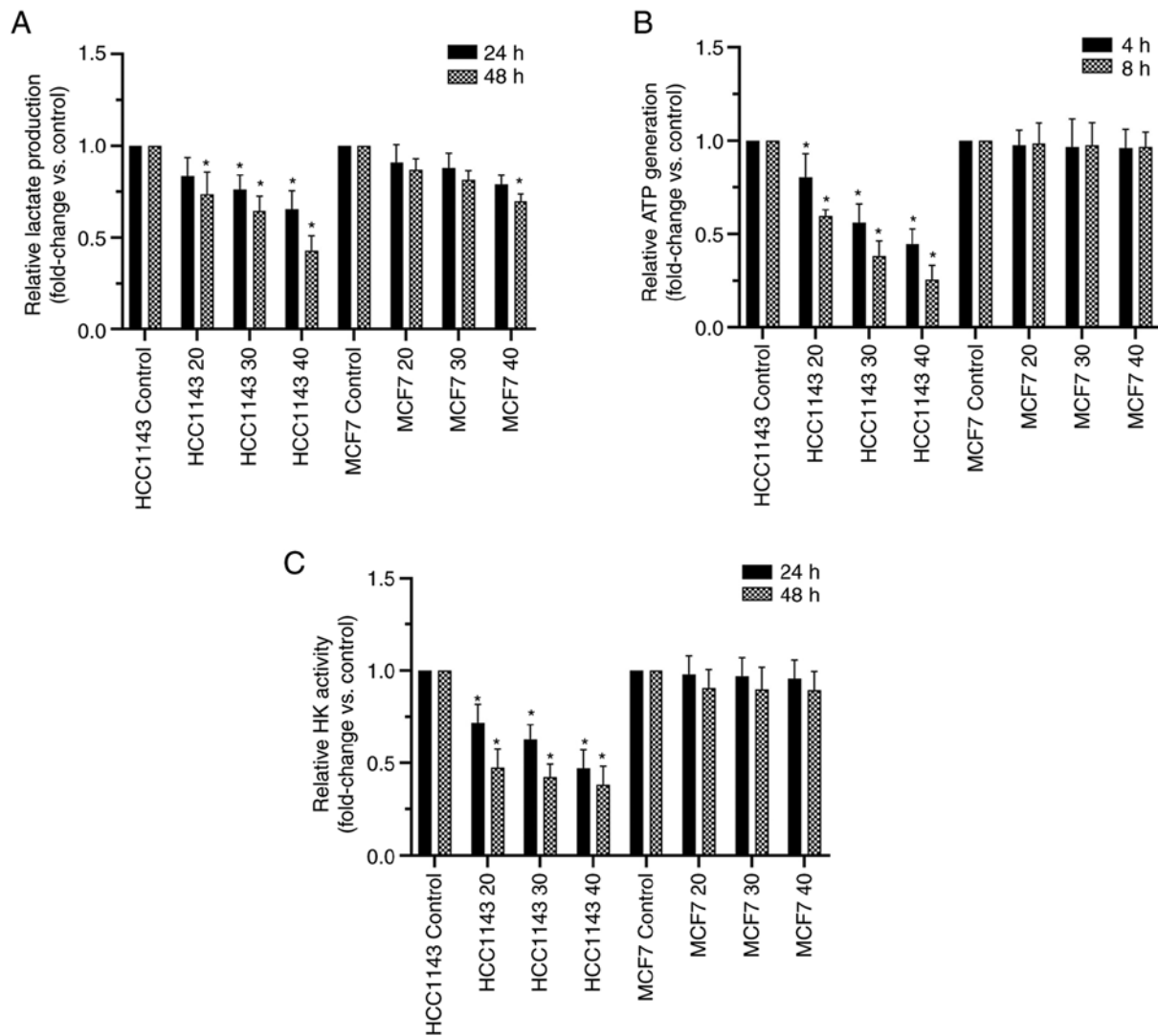
Figure 3. Effect of 3-BrPA on glycolysis-related indicators. (A) 3-BrPA induced a reduction in intracellular lactate production in TNBC cells. (B) TNBC cells treated with 3-BrPA exhibited reduced intracellular ATP production. (C) HK activity was significantly reduced by 3-BrPA treatment in TNBC cells. However, the glycolysis-related indicators were not affected by 3-BrPA treatment in non-TNBC cells. *$P<0.05$ vs. corresponding control group. 3-BrPA, 3-bromopyruvic acid; HK, hexokinase; TNBC, triple-negative breast cancer.

pro-apoptotic Bax and Caspase-3 at both 20/40 μM at 24/48 h; and Cyt-C at 40 μM at 24 h and 20/40 μM at 48 h in TNBC cells (Fig. 5A and B). The regulatory effect of 3-BrPA was dose-dependent. However, the same effect of 3-BrPA on these apoptosis-related proteins was not observed in non-TNBC cells (Fig. 5A-C).

Discussion

TNBC is a notable type of breast cancer because of its unique molecular type. The 5-year survival rate of patients with TNBC is poorer than that of patients with other types of breast cancer (26), and TNBC most commonly arises in premenopausal patients with a high recurrence rate (27). TNBC is extremely invasive and has a higher incidence of remote metastasis than non-TNBC subtypes; <30% of patients with metastatic TNBC survive for >5 years (27). Targeted therapies for TNBC are lacking. Compared with non-TNBC cells, TNBC cells possess unique metabolic characteristics: Higher glucose uptake, increased lactate

production and low mitochondrial respiration levels (4). These characteristics suggest that suppressing breast cancer cell proliferation by inhibiting the glycolytic pathway may represent a novel therapeutic mechanism for antitumor drugs.

3-BrPA is receiving increasing attention given the increasing interest in research on antitumor drugs. 3-BrPA is an analog of pyruvate with high tumor selectivity (28). This small alkylating compound can induce cell death through two mechanisms: One involves inhibiting HK2 to prevent the glycolysis process (29), and the other involves activating the mitochondrial pathway of apoptosis (30). 3-BrPA functions in a variety of tumor cells, including multiple myeloma cells (31) and hepatocellular carcinoma cells (32). However, normal cells avoid the damage induced by this drug (33). Therefore, 3-BrPA represents a promising antitumor drug that may be useful for clinical treatment in the future. The present study revealed that 3-BrPA inhibited the viability of TNBC cells and promoted their apoptosis; however, this effect was not significant in non-TNBC cells, suggesting a unique mechanism.
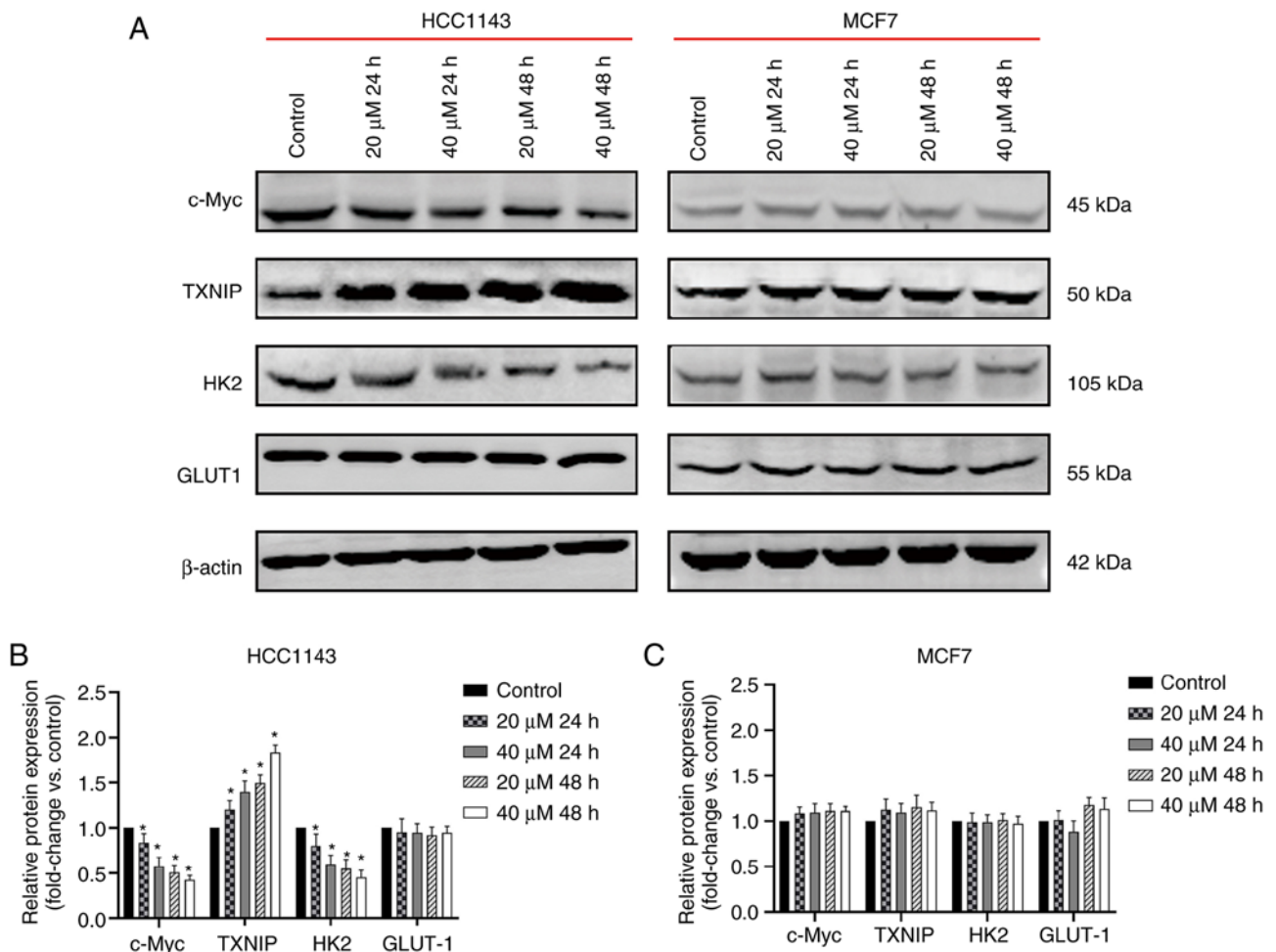
Figure 4. 3-BrPA regulates expression of c-Myc, TXNIP and HK2. (A) Expression levels of c-Myc, TXNIP, HK2 and GLUT1 were detected through western blot analysis. Grayscale values of the bands were determined using software. (B) 3-BrPA downregulated c-Myc and HK2 protein expression, whereas it upregulated TXNIP protein expression in TNBC cells ($^*P<0.05$ vs. control group, the same cell line with 0 μM 3-BrPA). (C) However, in non-TNBC cells, the expression levels of c-Myc, TXNIP, HK2 and GLUT1 were not significantly affected. The expression levels of GLUT1 were not significantly affected in both TNBC and non-TNBC cells. TXNIP, thioredoxin-interacting protein; HK2, hexokinase 2; GLUT1, glucose transporter 1; 3-BrPA, 3-bromopyruvic acid; TNBC, triple-negative breast cancer.

HK is a key rate-limiting enzyme in the first step of glycolysis in tumor tissues, and its expression and activity increase in tumor tissues to promote effective anaerobic glycolysis even under anaerobic conditions (7). In mammals, four HK subtypes, HK1, HK2, HK3 and HK4, are encoded by separate genes (34). HK2 is absent or expressed at low levels in the majority of adult normal cells but is widely upregulated in several cancer cells (35). Patra *et al* (36) found that HK2 expression is markedly elevated in tumors derived from mouse models of lung and breast cancer. Marini *et al* (37) revealed that HK1 and HK2 inhibition caused by metformin could modify glucose metabolism in TNBC both *in vitro* and in xenografted mice models. These results suggest that HK2 may represent an alternative target for cancer therapy. The present data demonstrated that 3-BrPA reduced the protein expression levels of HK2 in a dose-dependent manner in TNBC cells. However, the same inhibitory effect on HK2 protein expression was not found in non-TNBC cells.

An increasing number of researchers have recognized the prognostic and predictive power of TXNIP, which is a potent negative regulator of glucose uptake, in human breast cancer. Shen *et al* (21) demonstrated the unique molecular mechanism of TNBC cells: Increased c-Myc expression and reduced TXNIP and c-Myc expression promote glucose uptake and use in tumor cells, which subsequently accelerates cancer cell proliferation. Park *et al* (38) demonstrated that TXNIP operates to suppress the high proliferative activity and estrogen-dependent cell proliferation in breast cancer. Qu *et al* (39) demonstrated that ectopic TXNIP expression decreased glucose uptake induced by c-Myc and led to suppression of a broad range of glycolytic target genes in prostate cancer. The present data demonstrated that 3-BrPA upregulated TXNIP protein expression and that this upregulating effect was stronger in TNBC cells than in non-TNBC cells. Therefore, it was concluded that 3-BrPA inhibited TNBC cell proliferation by assisting TXNIP and inhibiting glycolytic enzymes, such as HK2.

Among various oncogenes, c-Myc, which is a strong transcription factor participating in several aspects of the oncogenic process, acts as a vital member of the Myc family (40). The idea that c-Myc serves an important role in cell proliferation and differentiation has been proposed and
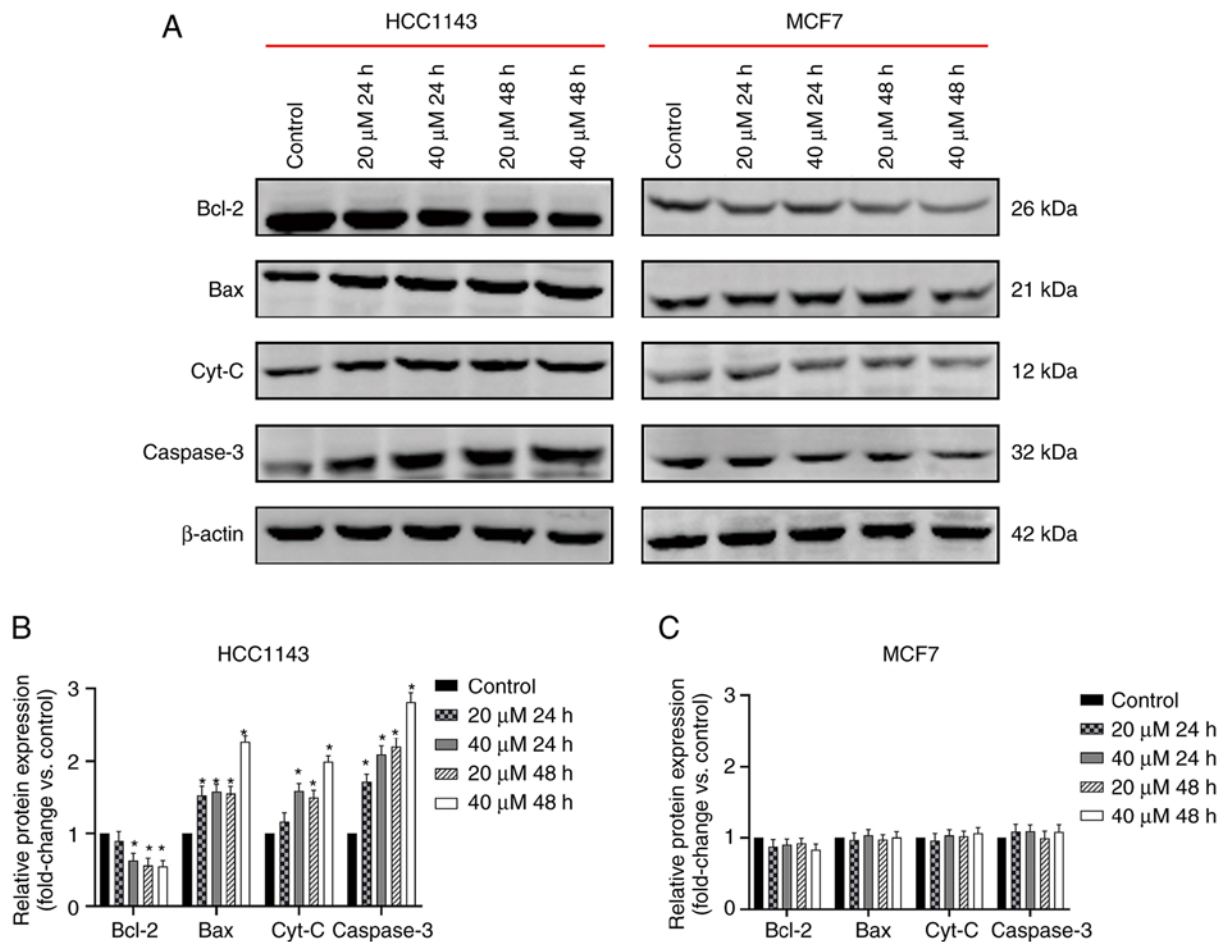
Figure 5. 3-BrPA regulates expression of mitochondria-regulated apoptosis pathway proteins. (A) Expression levels of mitochondria-regulated apoptosis pathway proteins, including Bcl-2, Bax, Cyt-C and Caspase-3 were detected through western blot analysis. (B) Bcl-2 protein expression was decreased, while Bax, Cyt-C and Caspase-3 protein expression was increased following 3-BrPA treatment in TNBC cells (*P<0.05 vs. control group, the same cell line with 0 μM 3-BrPA). (C) However, in non-TNBC cells, the expression levels of Bcl-2, Bax, Cyt-C and Caspase-3 were not significantly affected. 3-BrPA, 3-bromopyruvic acid; Cyt-C, cytochrome *c*; TNBC, triple-negative breast cancer.

confirmed (41). The relevant protein encoded by c-Myc affects cell proliferation (42). c-Myc functions to determine the entry of cells from G_1 into S phase and contributes to progression to the G_2 phase (43). Evidence also indicates that both low expression and overexpression of c-Myc lead to cell apoptosis (44). More than half of human cancers exhibit loss of the c-Myc oncogene, which results in a poor prognosis and survival rate (40). Additionally, other studies demonstrated that c-Myc can mediate other pathways to regulate cell metabolism. For example, the activation of the serine biosynthesis pathway mediated by c-Myc serves a critical role in cancer progression under nutrient-deficient conditions (45). Furthermore, enzymes in glucose metabolism, such as GLUT1, phosphofructokinase, M2 isoform of pyruvate kinase and HK2, are all targets of c-Myc (46-49). Based on these findings, it is reasonable to hypothesize that 3-BrPA affects the metabolism of TNBC cells mainly by targeting the c-Myc/TXNIP axis, down-regulating c-Myc and upregulating TXNIP, and this effect can only be fully displayed in cells with high c-Myc/low TXNIP. In the present study, the HCC1143 cell line was selected because of its high c-Myc/low TXNIP expression based on the dataset from Cancer Cell Line Encyclopedia (CCLE, portals.broadinstitute.org/ccle). The MCF-7 cell line does not

have this feature. In addition, although most TNBC cells have this feature, not all cells do, for example, MDA-MB-231 cells do not (10). In preliminary experiments performed by this research group, 3-BrPA was tested on different TNBC cells not having the same high c-Myc/low TXNIP characteristic, and it was revealed that the inhibitory effect was lower than that of TNBC cells with high c-Myc/low TXNIP expression (data not shown). The reason is that the upregulation of c-Myc is closely related to an increased tumor invasiveness, increased drug resistance and reduced survival of patients with TNBC (50). In terms of glucose metabolism, c-Myc can indirectly promote the expression of monocarboxylate transporter (MCT) 1 and MCT-2 (21). MCT-1 is ubiquitously expressed and involved in the uptake or efflux of lactate (51) and, due to the Warburg effect of tumor cells, it can reprogram the energy metabolism, including inducing high glucose consumption and increased lactate production. The channels that transport lactate play an important role in regulating the balance of lactate inside and outside of cancer cells (51). In response to a lack of glucose in cancer cells, lactic acid can be used as 'fuel' to be taken into cells by MCT-1 for energy production. As a lactate analog, 3-BrPA can be taken up by cancer cells through this channel, thereby entering the cells and binding to related targets (51,52).

In addition, high c-Myc expression can promote and encode key enzymes of glycolysis and GLUTs to increase glucose tumor cell uptake and utilization for proliferation (53).

The following mechanisms may be involved in the inhibition of TXNIP caused by c-Myc. Myc, which is a member of the basic region helix-loop-helix leucine zipper (bHLHZip) family of transcription factors, operates by binding to another member of the same family, MYC associated factor X (MAX) (54). TXNIP is a direct target of the MAX-like protein (Mlx):Mlx-interacting protein (MondoA) complex, another member of the bHLHZip family, which controls uptake and utilization of glucose and has an activation efficacy on the TXNIP promoter stronger than that of Myc:MAX complexes (17,55-57). Thus, the Myc:MAX complex competes with the MondoA:Mlx complex to bind and displace TXNIP, resulting in repression of TXNIP.

Apoptosis is a form of programmed cell death that occurs in multicellular organisms and is induced by a variety of physical and chemical factors. Although there are three predominant signaling pathways in apoptosis, apoptotic signaling usually occurs at the mitochondrial-mediated pathway level, which is mainly regulated by Bcl-2 family genes (58). A previous study has demonstrated that Bcl-2 is located at the mitochondrial outer membrane where it serves an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins (59). Furthermore, Bax alters mitochondrial outer membrane permeabilization antagonizing the function of Bcl-2, leading to the release of bioactive substances such as Cyt-C from the intermembrane space into the cytosol (59). Thereby, Cyt-C activates caspase-3/caspase-9 and initiates a caspase signaling cascade and induces apoptosis (59,60). The relationship between glycolysis and apoptosis was studied by a team (61), but the conclusions had some limitations, showing that related research needs to be further carried out. In the present study, apoptosis was closely related to glycolytic enzyme inhibition. By interacting with the outer membrane protein voltage dependent anion channel (VDAC), HK protects the normal function of the mitochondrial outer membrane and maintains the balance between pro-apoptotic proteins and anti-apoptotic proteins, prevents the release of Cyt-C, and enhances cell proliferation and inhibits apoptosis (62). Based on this, it is reasonable to hypothesize that 3-BrPA inhibited HK activity and dissociated it from the mitochondria, destroying the integrity of the mitochondrial outer membrane and breaking the balance between pro-apoptotic proteins and anti-apoptotic proteins, thereby enabling VDAC to open and release Cyt-C and ultimately inducing caspase-mediated apoptosis. Thus, there may be a link between glycolysis and the mitochondrial apoptotic pathway that can be targeted by 3-BrPA.

The results obtained by different teams were dissimilar despite the same drug and the same cell line being used. For example, Kwiatkowska *et al* (63) used 3-BrPA to act on MCF-7 for 24 h, and the IC_{50} value was 101 ± 28 μM. However, Wu *et al* (64) reported that if the cells were treated with 3-BrPA (100 μM) for 24 h, the inhibition rate of MCF-7 cells was as high as 95%. We hypothesized that the results of the experiment are affected by multiple factors, such as drug batch, cell state, culture conditions, laboratory environment and other comprehensive factors, which lead different research groups to obtain different results. Although the present results indicated

that the HK activity was not significantly affected by 3-BrPA in MCF-7 cells within the existing concentration range, which disagrees with the results from Wu *et al* (64), the HK activity in MCF-7 cells continued to decline while increasing the 3-BrPA concentration in our preliminary experiments (data not shown). However, in terms of a future clinical application of 3-BrPA, a high drug concentration could produce more toxic and side effects.

In conclusion, in the present study, 3-BrPA reduced the HK2 expression and promoted the TXNIP protein expression in TNBC cells by downregulating the expression of c-Myc, thereby inhibiting glycolysis including suppressing lactate generation, intracellular ATP generation and HK activity, inducing mitochondria-regulated apoptosis, and eventually limiting TNBC cell proliferation. These findings contribute to the field of breast cancer therapy, and 3-BrPA is expected to become an effective drug in breast cancer therapy in the future. The concept of using glycolysis inhibitors combined with chemotherapeutic drugs has already been proposed (24,65). However, whether targeting c-Myc and the glycolysis pathway could increase the sensitivity of cancer cells to chemotherapy and radiotherapy requires further studies. To verify the proposed mechanism, transfection and *in vivo* experiments will be performed in future research. Further research is required to provide basic data on novel therapeutic targets to improve the clinical treatment of TNBC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and JP participated in the preliminary experimental design, preliminary experiment, main experiment operation, data analysis, manuscript writing and revision. YL, XL, CY, WX and QL participated in data analysis, manuscript writing and revision. LY and XZ participated in early experimental design, including selecting drugs and designing possible signal pathways. JL and JP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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