Ppm1b Negatively Regulates 3-Bromopyruvate Induced Necroptosis in Breast Cancer Cells

Yiming Sun†, Qiong Pan‡, Linyan Ma‡, Chao Chen, Surong Zhao and Hao Liu*

† Department of Pharmacy, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China, ‡ School of Pharmacy, Bengbu Medical College, Bengbu, China

Up to 30% of breast cancer mortality is caused by cancer relapse despite primary clinical treatments due to distant metastases. Further research focusing on breast cancer mechanisms are needed for deeper understanding of disease prognosis. 3-bromopyruvate (3-BP), a glycolysis inhibitor, has been studied as one of the antitumor agents in recent years. In this report, we want to investigate the form of cell death induced by 3-BP and demonstrate the inhibitory effect of 3-BP on breast cancer cell proliferation and its mechanism in vivo and in vitro. We found that 3-BP could inhibit MDA-MB-231 and MCF-7 breast cancer cell proliferation, through energy metabolism inhibition. Further, necroptosis characters in MDA-MB-231 cells after 3-BP treatment were observed, which could be negatively regulated through Ppm1b by dephosphorylation of RIP3. In addition, 3-BP treatment in an MDA-MB-231 cell-transplanted mouse model showed a significant antitumor effect, which correlated with necroptosis-related protein Ppm1b. The findings demonstrate the potential for 3-BP in the treatment of breast cancer, providing impetus for further clinical studies.

Keywords: breast cancer cells, protein phosphatase 1b, necroptosis, dephosphorylation, 3-bromopyruvate

INTRODUCTION

Breast cancer is one of the dominant types of cancers, which affects public health, especially in women (1, 2). Previous studies suggest that, up to 30% of the patients die due to distant metastatic formation (3), and patients with metastatic breast cancer survive no more than 1 year (4). Hence, various strategies need to be explored to understand mechanisms involved in breast cancer and to provide better clinical treatment.

3-BP is a small reactive molecule formed by bromination of pyruvate, which is one of the widely studied compounds due to its antitumor properties (5). A glycolysis inhibitor, 3-BP can inhibit cancer cell proliferation through energy metabolism interruption (6), primarily through inhibition of the glycolytic enzymes and those related with mitochondrial function, including hexokinase II (HK II) (7), pyruvate dehydrogenase (PDH) (6), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), and by altering adenosine triphosphate (ATP) levels (8). We had previously demonstrated the antitumor properties of 3-BP both in vitro and in vivo which was achieved through energy metabolism alterations, in colon cancer cells SW480 and HT29 treated with 3-BP, by inducing their necroptosis and apoptosis (9). Further, 3-BP enhanced

OPEN ACCESS

Edited by: Swee Keong Yeap, Xiamen University, Malaysia
Reviewed by: Yew Woh Hui, Xiamen University, Malaysia; Yow-Ling Shiue, National Sun Yat-sen University, Taiwan
*Correspondence: Hao Liu liuhao6886@foxmail.com
†These authors have contributed equally to this work

Specialty section: This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology
Received: 25 April 2020
Accepted: 30 November 2020
Published: 14 January 2021

Citation: Sun Y, Pan Q, Ma L, Chen C, Zhao S and Liu H (2021) Ppm1b Negatively Regulates 3-Bromopyruvate Induced Necroptosis in Breast Cancer Cells. Front. Oncol. 10:555546. doi: 10.3389/fonc.2020.555546

Frontiers in Oncology | www.frontiersin.org January 2021 | Volume 10 | Article 555546

ORIGI NAL RESEARCH

Published: 14 January 2021
doi: 10.3389/fonc.2020.555546
antitumor activity of daunorubicin (DNR) in breast cancer cells through monocarboxylate transporter 1 (MCT-1) (10), suggesting that 3-BP could be an effective therapeutic target for breast cancer treatment.

Necroptosis is a type of programmed cell death with morphological characteristics of necrosis, associated with the kinase activity of caspase 8, receptor-interacting serine/threonine kinase 1 (RIP1), and receptor-interacting serine/threonine kinase 3 (RIP3) (11). When caspase 8 is inhibited or is deficient, RIP1 combines with RIP3 to form the RIP1/RIP3 complex via the-terminal RIP1 homotypic interaction motif (RHIM) domain, which initiates necroptosis (12). This confirms that the RIP1/RIP3 complex forms the core of necosome, however, understanding of molecular regulation of necroptosis remains limited. A recent study showed that protein phosphatase 1b (Ppm1b) selectively suppresses necroptosis through dephosphorylation of RIP3, which then prevents the recruitment of mixed lineage kinase domain-like protein (MLKL) on the necroosome (13).

In this study, we report the cell death forms induced by 3-BP, we found that 3-BP, a glycolysis inhibitor can induce cell death in breast cancer cells, MDA-MB-231 and MCF-7, respectively, especially in MDA-MB-231 cells by regulating Ppm1b and necroptosis, which are crucial for our understanding of breast cancer cell death signaling network.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

Breast cancer cells MDA-MB-231 and MCF-7 were obtained from the cell research institute of the Chinese Academy of Sciences (Shanghai, China). Both cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA), and supplemented with 10% fetal bovine serum (FBS), 80 U/ml penicillin and 100 U/ml streptomycin. Cells were grown in an atmosphere of 5% CO$_2$ at 37°C.

3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assay

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Beyotime Institute of Biotechnology, Wuhan) was used to determine cell viability. MDA-MB-231 and MCF-7 cells (3000 cells/well) were seeded onto 96-well plate and incubated with various concentrations of 3-BP (0, 20, 40, 80, 160, 320 μmol·l$^{-1}$) (Sigma-Aldrich, USA) when cells were attached completely. MTT solution (15 μl; 5 mg/ml in phosphate-buffered saline) were added into 96-well plate respectively at the 3-BP treatment of 24, 48, and 72 h. After 4 h, the solution was replaced with 150 μl dimethylsulfoxide (DMSO) (Beyotime Institute of Biotechnology, Wuhan) and 30 min later, cell viability was determined at a wavelength of 490 nm by a microplate reader (Synergy, BioTek, USA).

Flow Cytometry

Prior to 3-BP treatment, MDA-MB-231 and MCF-7 cells (1.2×10$^5$ cells/well) were seeded onto 12-well plate and then treated with 3-BP (0, 80, 160, 320 μmol·l$^{-1}$) for 24 h when cells were attached completely, then stained by PI solutions for 2 h, then detected by Accuri C6 flow cytometry (BD Biosciences, State of New Jersey, USA). To further analyze cell death, PI staining was used to detect cell death. Annexin-V FITC/PI staining (Nanjing keygen biotech, Nanjing) was used to detect the amounts of apoptosis cells by the method just described according to the manufacturer’s instructions.

**Nuclear Staining**

DAPI nuclear staining (Beyotime Institute of Biotechnology, Wuhan) assay was performed to examine the effects of 3-BP on cancer cell apoptosis. MDA-MB-231 and MCF-7 cells were seeded onto 6-well plates (2×10$^5$ cells/well). Cells were fixed with immunostaining setting for 30 min at 4°C after 3-BP treatment for 24 h, washed twice with PBS and then incubated with DAPI solution for 30 min at 4°C in the dark. The plates were then washed with PBS to remove the excess DAPI solution and the cell nuclei were observed under a laser confocal scanning microscope (LCSM, Olympus 1x71).

**Determination of ATP Level**

ATP levels were measured using a luminescence-based ATP Assay Kit (Beyotime Institute of Biotechnology, Wuhan). In this assay, luciferase catalyzes the production of a fluorescent signal from oxygen, ATP, and luciferin, and the fluorescence intensity is proportional to the amount of ATP. Cells (1.2×10$^5$ cells/well) were seeded onto 12-well plate and incubated with various concentrations of 3-BP for 5 h at 37°C. After 24 h, all cells were collected and homogenized in radio immunoprecipitation assay (RIPA) lysis buffer for 10 min on ice, and then centrifuged at 12000 g/min for 5 min at 4°C. Next, 100 μl nucleotide-releasing buffer and 1 μl ATP-monitoring enzyme were added to each well of a 96-well plate, and 30 μl cell suspension was transferred into each well. The plate was then incubated at 25°C for 60 s, and the fluorescence was measured using a Luminoskan luminometer (Thermo Scientific, Atlanta, GA, USA).

**Determination of Lactate Dehydrogenase Level**

Cells (6000 cells/well) were seeded onto a 96-well cell culture plate so that the cell density does not exceed 80%–90%. Aspirate the culture medium and wash it once with PBS. Replace with fresh culture medium, and divide each culture well into the background blank control wells, control wells and drug wells, and incubated at 37°C. Then add the LDH release reagent provided by the kit, mix by pipetting several times. After reaching the predetermined time, the cell culture plate was centrifuged with a multi-well plate centrifuge at 4°C. Next, 100 μl nucleotide-releasing buffer and 1 μl LDH-monitoring enzyme were added to each well of a 96-well plate, and 30 μl cell suspension was transferred into each well. The plate was then incubated at 25°C for 5 min. Take 120 μl of supernatant from each well, add Insert into the corresponding wells of a new 96-well plate, and then LDH levels were measured (Beyotime Institute of Biotechnology, Wuhan).

**Small Interfering RNA Transfection**

The Human Ppm1b siRNA (Gene Pharma, Shanghai, China) was transiently transfected into MDA-MB-231 cells in a 6-well
plate with Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer’s protocol. After 48 h of transfection, the cells were collected for immunoblot analysis. The sequences of siRNA used for experiments of human Ppm1b siRNA were as follows: Negative control sense, 5′- UUCUC CGAACCUGUCGAC UTT-3 and antisense, 5′-ACGU GACCUUUGCGAGATT-3′; Positive control sense, 5′-UGAC CUCAAAGACUUGUUGUTTT and antisense, 5′-AAACC AUGUAGUUGAGGUGATT-3′; Ppm1b-homo-1728 sense, GGAGACUUCUCUGGCGAGGATT and antisense UUCCU AACCGUGUGUGUGGTT; Ppm1b-homo-1129 sense, CCAACA GAACAACUUGUUUTT and antisense, AAACA AGUUGUUUCUGUUGGTTT; Ppm1b-homo-1458 sense, GGGAAGUCUGGCAGGAGATT and antisense UUCCU CGCCAGACUUCUCCCTT.

Western Blotting
MDA-MB-231 and MCF-7 cells were lysed in RIPA lysis buffer on ice for 30 min, then centrifuged (12000 g/min; 30 min) at 4°C. A bicinechonic acid (BCA) assay was used to detected protein concentrations. Equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (millipore, USA), and then incubated with primary antibodies overnight at 4°C after the membranes were blocked (5% skim milk in PBS with 0.1% Tween 20) for 4 h. The next day, the membranes were imaged with gel imaging equipment (Bio-Rad, USA) after the membranes were incubated with secondary antibodies for 2 h. β-actin was used as a loading control. The following antibodies were used: Bcl-2 and Bax (Cell Signaling technology, USA); anti-RIP1, anti-RIP3, and p-RIP3 (Santa Cruz Biotechnology, USA); TNF-α (Abcam, USA); Caspase 3 (Enzo, USA); Ppm1b (BETHYL, USA); anti-β-actin (Biosharp, China) All reagents were dissolved according to the manufacturer’s instructions.

Live Cell Imaging System
MDA-MB-231 and MCF-7 cells (1.2×10^5 cells/well) were seeded onto 12-well plate and then treated with 3-BP (0, 160 μmol·l^-1) combined with Annexin-V (5 μmol·l^-1), PI (5 μmol·l^-1), and DAPI (5 μg/ml) solution when cells were attached completely. The plate was placed under a live cell workstation microscope for 24 h persistent observation, then the final video was analyzed by The Live cell imaging system (ZEISS, Germany).

Evaluation of Cell Death Form by Electron Microscopy
MDA-MB-231 and MCF-7 cells (1.2×10^5 cells/well) were seeded onto 12-well plate and then treated with 3-BP (0, 160 μmol·l^-1), then collected cells, washed and fixed with 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C overnight. Then, the cells were post-fixed with 1% osmium tetroxide for 1.5 h, washed once by PBS and treated with 3% aqueous uranyl acetone for 1 h before being dehydrated with a graded series of ethanol and acetone and embedded in Araldite. Thin sections were cut using a Reichert ultramicrotome (Leica, Germany), post-stained with 0.3% lead citrate, and examined by TEM (Olympus JEOL, Peabody, MA, USA).

Xenograft Model
This study was performed according to guidelines approved by the Bengbu Medical College Institutional Animal Care and Use Committee (No. 2017003). Female nude mice (BALB/c) aged 4–5 weeks and weighted 18–20 g were used to perform the antitumor effect in vivo, which were obtained by Shanghai slac laboratory animal. Mice were grafted subcutaneously in the right flank with MDA-MB-231 cells resuspended in 0.2 ml PBS (10^7 cells/ml), then randomized into negative control (PBS), 3-BP (8 mg/kg), Nec-1 (40 mmol·l^-1) (Sigma-Aldrich, USA), 3-BP combined with Nec-1 (8 mg/kg; 40 mmol·l^-1), DNR positive control (5 mg/kg) (Sigma-Aldrich, USA) when tumor volume reached approximately 100 mm³ (n=6 per group). Tumors were measured with caliper and volume was calculated by the formula: 0.5×a×b², where a and b were tumor length and width. Tumors were stored in 4% formalin solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E) and used for immunohistochemistry.

Statistical Analysis
The experimental results were analyzed by SPSS 21.0 software. Data are expressed as the mean ± SD of three experiments. The differences between the groups were compared by one-way ANOVA. Mean differences were evaluated by t-test analysis of variance. P < 0.05 was considered statistically significant.

RESULTS

Cell Proliferation Were Inhibited by 3-Bromopyruvate in Breast Cancer Cells
To identify the cell proliferation inhibition effect of 3-BP in breast cancer cells, we examined the cell viability in MDA-MB-231 and MCF-7 cells, both cell viability is inhibited by 3-BP (Figures 1A, B). For further confirmation, we also determined that 3-BP induced apoptosis in both cell lines (Figure 1C), as 3-BP could reduce cellular ATP level (Figure 1D). And increase cellular LDH level in MDA-MB-231 cells, while no significant change of LDH level in MCF-7 cells (Figure 1E).

3-Bromopyruvate Induced Breast Cancer Cells Apoptosis and Necroptosis
To identify the apoptosis induced by 3-BP in breast cancer cells, we examined the apoptosis-related proteins, up-regulated expression of Bax and down-regulated expression of Bcl-2 mean that breast cancer cells could be induced apoptosis by 3BP (Figures 2A, B). Especially, cell viability in MDA-MB-231 increased in 3-BP combined with the Nec-1 group than the 3-BP group, which means that 3-BP may induce breast cancer cells MDA-MB-231 necroptosis (Figure 2C).

Results shown that plasma membranes of MDA-MB-231 cells were disrupted after incubated with 3-BP for 24 h, and cell fragments are visible, resulting in the release of cell contents.
Inhibition effect of proliferation in breast cancer cells by 3-BP. (A) Breast cancer cells MDA-MB-231 and MCF-7 cells were treated with 3-BP (0, 20, 40, 80, 160, 320 μmol·l⁻¹) for 24, 48, and 72 h. Cell viability was measured using the MTT assay. (B) The cell death rate in MDA-MB-231 and MCF-7 cells induced by 3-BP for 24 h. (C) Cell apoptosis induced by 3-BP in MDA-MB-231 and MCF-7 cells for 24h. (D, E) Reduction in ATP production and LDH level by 3-BP treatment, cells were cultured with 3-BP (0, 40, 80, 160, 320 μmol·l⁻¹). Cellular levels of ATP were measured after 5 h. LDH level was measured after 24 h. *P < 0.05, **P < 0.01.
FIGURE 2 | Cellular apoptosis and necroptosis induced by 3-BP in breast cancer cells. (A, B). Apoptosis-related proteins were detected after cells were treated with 3-BP for 24 h, and were analyzed by western blotting. β-actin served as the loading control. (C) Cells were treated with 3-BP or 3-BP combined with Nec-1 for 24 h, and cell viability was determined using MTT assay. (D) Cells were treated with 3-BP (0, 160 μmol·l⁻¹) for 24 h and then prepared for Electron Microscopy analysis. Scale bar: 2 μm; (E) Cells were treated with 3-BP (0, 160 μmol·l⁻¹), and observed under a live-cell workstation microscope for 24 h persistent observation. (F) Cells were treated with 3-BP (0, 80, 160, 320 μmol·l⁻¹) for 24 h, subjected to DAPI staining and then visualized under a fluorescence microscope. *P < 0.05, **P < 0.01.
and swollen nuclei (N). MCF-7 cells treated with 3-BP demonstrates apoptotic cell death characteristics, with typical apoptotic bodies and the number of apoptotic nuclei had significantly increased in both the cell lines treated with 3-BP. On the other hand, necrotic symptoms such as swelling of the cell body and rupture of the cell membranes could be observed in MDA-MB-231 cells treated with 3-BP (Figure 2D).

Live-cell observation also showed that necroptosis characteristics, including plasma membranes, were disrupted in MDA-MB-231 cells after incubation with 3-BP (Figure 2E, Supplementary Video 1). Cell membrane valgus Annexin V/ FITC combines with it and emits green light in cells early apoptosis, cell membrane swells and ruptures, and PI enters the cells, making it emits red fluorescence when 3-BP stimulates MDA-MB-231 cells for 240 min, and MCF-7 cells are mainly dominated by cell body shrinkage, which proves that 3-BP induced necrotic apoptosis in MDA-MB-231 cells and 3-BP induced MCF-7 apoptosis (Figure 2E, Supplementary Video 2). Further, cell permeability of MCF-7 cells increased, which is consistent with the Nec-1 results (Figure 2C). DAPI staining shows nuclear damage in both the cells indicating apoptosis (Figure 2F).

These observations reinforce the notion that 3-BP induces apoptosis in breast cancer cells MDA-MB-231 and MCF-7, while necroptosis in MDA-MB-231 cells.

**Ppm1b Negatively Induced Necroptosis by Dephosphorylation-Receptor–Interacting Serine/Threonine Kinase 3**

Previous researches have shown that Ppm1b negatively induced necroptosis by dephosphorylation-RIP3 (13). To identify the function of Ppm1b in necroptosis in different cancer lines, we tested the expression of Ppm1b in normal human colon cell line FHC, HT29 and SW480, and breast cancer cells MDA-MB-231, MCF-7, and MDA-MB-453, we found that 3-BP could inhibit the expression of Ppm1b in MDA-MB-231 cells, with up-regulated expression of TNF-α and p-RIP 3, while no significant change in expression of RIP3 in MCF-7 cells (Figures 3A, B). The expression of Caspase 3 shown that apoptosis induced by 3-BP in MCF-7 cells. Knockdown of Ppm1b in MDA-MB-231 cells shown that Ppm1b could negatively up-regulate the expression of p-RIP3 (Figures 3C, D).

These observations reinforce the notion that 3-BP induced breast cancer cells MDA-MB-231 necroptosis and Ppm1b negatively regulated 3BP-induced necroptosis through dephosphorylating RIP3.

**Anti-Tumor Effect of 3-Bromopyruvate in Breast Cancer Xenograft Model**

To examine the anti-tumor effect of 3-BP in breast cancer in vivo, we performed a mice model using the breast cancer cell lines MDA-MB-231 stimulated with 3-BP. 3-BP could reduce tumor volume and tumor weight (Figures 4A–C) compared with the control group, with no significant liver damage (Figure 4E). H&E staining results shown that 3-BP induced tissue damage with no significant liver and kidney damage, while 3-BP combined with Nec-1 could reduce the tissue damage (Figure 4F).

In the tissue, the expression of Ppm1b in the 3-BP group was down-regulated by 3-BP than the control group, which could be released by 3-BP combined with Nec-1, and no significant change in expression of RIP3 (Figure 5A). Electric Microscope results shown that 3-BP induced disruption of cellular membrane and release of cell contents (Figure 5B), shown that 3-BP induced necroptosis in vivo, which consistent with the results in vitro (Figure 2D).

**DISCUSSION**

Cellular necroptosis is a necrotic programmed cell death modality pathway in a caspase-independent fashion and is mainly mediated by RIP1 (14) and RIP3 (15). Accumulating evidence suggests that necroptosis serves a double-edged sword to the development of the cancer process, performs an anti-tumor effect in cancer, or plays a tumor-promoting role in cancer development (16, 17). While much remains to be fully elucidated about the mechanism and regulation of necroptosis, given that necroptosis can be potential for developing a novel approach in cancer therapies (18).

Ppm1b is another type 2C protein phosphatase (PP2C) family member in human, which controls numerous cellular functions by dephosphorylation of serine and threonine residues, and binds to the phosphorylated TBK1 and acts as a TBK1 phosphatase (19). On the other hand, Ppm1b was found in associating with the IKKβ/NF-κB pathway (20), overexpression of Ppm1b can block the activation of the IKKβ/NF-κB pathway induced by TNF-α treatment (21). Furthermore, Ppm1b plays an important role in the progression of cells.

Published data have provided evidence of the interaction between necroptosis and protein Ppm1b in vivo and in vitro (13). It was shown that gene deletion of Ppm1b regulates necroptosis through dephosphorylating RIP3. Our data support this conclusion in breast cancer cell lines. In this investigation, we found that the anti-tumor effect of glycolysis inhibitor 3-BP in breast cancer cells, which regulated apoptosis in MCF-7 cells and MDA-MB-231 cells, with novel cell proliferation inhibition effect. These observations reinforce the notion that 3-BP suppresses breast cancer cells proliferation. We also found that 3-BP induced necroptosis in MDA-MB-231 cells, which was consistent with our previous research (22). Especially, dephosphorylating RIP3 induced by the Ppm1b depletion pathway seems to be the more dominant mechanism of 3-BP induced necroptosis in MDA-MB-231 cells (Figures 2C, D). Although the interaction of Ppm1b with p-RIP3 in MDA-MB-231 cells (Figures 4A–C) explains the role of Ppm1b in preventing necroptosis in breast cancer cells and in vivo (Figures 5A, B), the mechanism of Ppm1b during necroptosis is still unclear.

In view of the data presented here and in previous reports, it will be valuable to test the function of Ppm1b in necroptosis in the future. Taken together, the present study has identified...
FIGURE 3 | Ppm1b negatively induced necroptosis by dephosphorylation-RIP3. (A, B) MDA-MB-231 cells were treated with 3-BP (0, 160 μmol·l⁻¹), Protein expression of Ppm1b, TNF-α, RIP3, p-RIP3, Caspase 3, and β-actin were analyzed by western blotting. β-actin served as the loading control. (C, D) MDA-MB-231 cells were transfected with siRNA against Ppm1b, then Ppm1b and p-RIP3 were analyzed by western blotting. β-actin served as the loading control. *P < 0.05; **P < 0.01.
FIGURE 4 | Anti-tumor effect of 3-BP in vivo. (A, B) Comparison of the transplanted tumor volume. (C, D) Bodyweight and tumor weight were monitored every 4 days and calculated using the formula: length x width²/2. (E) ALT and AST level in mice blood after 3-BP treatment. (F) H&E staining in vivo. *P < 0.05, **P < 0.01.
Ppm1b as a novel phosphatase of RIP3 and provides new insight into the molecular mechanism in breast cancer cell necroptosis.

CONCLUSION

In conclusion, our results reveal that Ppm1b negatively regulates 3-bromopyruvic acid induced necroptosis in MDA-MB-231 cells that correlates with enhanced dephosphorylation of RIP3. We found that 3BP was able to directly suppress its target Ppm1b, thereby impairing proliferation and inducing necroptosis in breast cancer cells. This mechanism involved in regulation of Ppm1b on necroptosis highlight the potential role of Ppm1b as a therapeutic target to treat breast cancers. Which part of the stage of the development of breast cancer cells does Ppm1b involved in is still clear, which needs to be investigated with further research.

FIGURE 5 | Necroptosis induced by 3BP in vivo. (A) Expression of Ppm1b and RIP3 in tumor tissue were detected by immunohistochemistry, then visualized under a fluorescence microscope. (B) Mice were treated 3-BP for 24 h, and necroptosis in tumor tissue were detected by Electron Microscopy analysis.
DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Bengbu Medical College Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

YS and QP provided intellectual contributions in the design of the study, generated all the results in this study, and wrote the manuscript. SZ and HL reviewed the manuscript. LM and CC helped in tumor measurements in mice and contributed to the study design including reviewing the manuscript. YS, QP, and HL are the principal investigators who designed the study and helped in tumor measurements in mice and contributed to the main draft of the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Koutras A, Tsilikis I, Syllaion A, Zouliati I, Zotos PA, Kalinterakis G, et al. Changes in the incidence of breast cancer due to the use of radioactive materials for warfare or nuclear and environmental accidents over the last 60 years in Europe and Asia. J BUON Of J Balkan Union Oncol (2019) 24(1):5–10.

2. Blok EJ, Bastiaanet E, van den Hout WB, Liefers GJ, Smit V, Kroep JR, et al. Systematic review of the clinical and economic value of gene expression profiles for invasive early breast cancer available in Europe. Cancer Treat Rev (2018) 62:74–90. doi: 10.1016/j.ctrv.2017.10.012

3. Early Breast Cancer Trials’ Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet (2005) 365 (9472):1687–717. doi: 10.1016/S0140-6736(05)65644-0

4. Cheng MH, Wong YH, Chang CM, Yang CC, Chen SH, Yuan CL, et al. B1, a novel HDAC inhibitor, induces apoptosis through the regulation of STAT3 and NF-kappaB. Int J Mol Med (2017) 39(5):1137–48. doi: 10.3892/ijmm.2017.2946

5. Ko YH, Niedzwiecka K, Casal M, Pedersen PL, Ulaszewski S. 3-Bromopyruvate as a potent anticancer therapy in honor and memory of the late Professor Andre Goffeau. Yast (2019) 36(4):211–21. doi: 10.1002/yea.3367

6. Fan T, Sun G, Sun X, Zhao L, Zhong R, Peng Y. Tumor Energy Metabolism and Potential of 3-Bromopyruvate as an Inhibitor of Aerobic Glycolysis: Implications in Tumor Treatment. Cancers (2019) 11(3):317. doi: 10.3390/cancers11030317

7. Gong L, Wei Y, Yu X, Peng J, Leng X. 3-Bromopyruvic acid, a hexokinase II inhibitor, is an effective autotumor agent on the hepatoma cells : in vitro and in vivo findings. Anti-cancer Agents Med Chem (2014) 14(5):771–86. doi: 10.2174/1871520614661401053009

8. Ganapathy-Kanniappan S, Geschwind JF, Kunjithapatham R, Buijs M, Vossen JA, Tchernyshyov I, et al. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is pyruvylated during 3-bromopyruvate mediated cancer cell death. Anticancer Res (2009) 29(12):4909–18.

9. Sun Y, Liu Z, Zou X, Lan Y, Sun X, Wang X, et al. Mechanisms underlying 3-bromopyruvate-induced cell death in colon cancer. J Bioenerget Biomembr (2015) 47(4):319–29. doi: 10.1007/s10863-015-9612-1

10. Liu Z, Sun Y, Hong H, Zhao S, Zou X, Ma R, et al. 3-bromopyruvate enhanced daunorubicin-induced cytotoxicity involved in monocarboxylate transporter 1 in breast cancer cells. Am J Cancer Res (2015) 5(9):2673–85.

11. Zhang YY, Liu H. Connections between various trigger factors and the RIP1/ RIP3 signaling pathway involved in necroptosis. Asian Pac J Cancer Prev APJCP (2013) 14(12):7609–74. doi: 10.7314/apjcp.2013.14.12.7609

12. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell (2009) 137 (6):1112–23. doi: 10.1016/j.cell.2009.05.037

13. Chen W, Wu J, Li L, Zhang Z, Ren J, Liang Y, et al. Ppm1b negatively regulates cancer cell death. Anticancer Res (2009) 29(12):4909–18.

14. Fan T, Sun G, Sun X, Zhao L, Zhong R, Peng Y. Tumor Energy Metabolism and Potential of 3-Bromopyruvate as an Inhibitor of Aerobic Glycolysis: Implications in Tumor Treatment. Cancers (2019) 11(3):317. doi: 10.3390/cancers11030317

15. Ko YH, Niedzwiecka K, Casal M, Pedersen PL, Ulaszewski S. 3-Bromopyruvate as a potent anticancer therapy in honor and memory of the late Professor Andre Goffeau. Yast (2019) 36(4):211–21. doi: 10.1002/yea.3367

16. Fan T, Sun G, Sun X, Zhao L, Zhong R, Peng Y. Tumor Energy Metabolism and Potential of 3-Bromopyruvate as an Inhibitor of Aerobic Glycolysis: Implications in Tumor Treatment. Cancers (2019) 11(3):317. doi: 10.3390/cancers11030317

17. Goffeau A, Chen H, Yuan J. Necroptosis and Cancer. Trends Cancer (2017) 3(4):294–301. doi: 10.1016/j.trecan.2017.03.002

18. Seifert L, Werba G, Tiwari S, Gao Ly NN, Aloehman S, Alqaunait D, et al. The necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. Nature (2016) 532(7598):245–9. doi: 10.1038/nature17403

19. Geng Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer (2019) 18(1):100. doi: 10.1186/s12943-019-1029-8

20. Zhao Y, Liang L, Fan Y, Sun S, An L, Shi Z, et al. PPM1B negatively regulates TRAIL-induced cell death from apoptosis to necrosis. Science (2009) 325(5938):332–6. doi: 10.1126/science.1172308

21. Najafov A, Chen H, Yuan J. Necroptosis and Cancer. Trends Cancer (2017) 3(4):294–301. doi: 10.1016/j.trecan.2017.03.002

22. Seifert L, Werba G, Tiwari S, Gao Ly NN, Aloehman S, Alqaunait D, et al. The necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. Nature (2016) 532(7598):245–9. doi: 10.1038/nature17403

23. Geng Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Mol Cancer (2019) 18(1):100. doi: 10.1186/s12943-019-1029-8

24. Zhao Y, Liang L, Fan Y, Sun S, An L, Shi Z, et al. PPM1B negatively regulates antiviral response via dephosphorylating TBK1. Cell Signal (2012) 24 (11):2197–204. doi: 10.1016/j.cellsig.2012.06.017

25. Varricchio CG, Jassak FF. Acute pulmonary disorders associated with cancer. Semin Oncol Nurs (1985) 1(4):269–77. doi: 10.1016/0749-2081(85)90007-5

26. Sun W, Yu Y, Dotti G, Shen T, Tan X, Savoldo B, et al. PPM1A and PPM1B act as IKKbeta phosphatases to terminate TNFalpha-induced IKKbeta-NF-kappaB activation. Cell Signal (2009) 21(1):95–102. doi: 10.1016/j.cellsig.2008.09.012

FUNDING

Funding support for this work was provided by The National Science Foundation of China (81372899), The Provincial Science and Technology Cooperation Project of Anhui province (1503062024), The key University Natural Science Project of Anhui Province (KJ2016A486), The Natural Science Foundation of Anhui province (1508085MH166), and The Foundation of Bengbu Medical College (Byyczxz1635, BYKY2019024ZD).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.555546/full?supplementary-material

SUPPLEMENTARY VIDEO 1 | Live-cell observation of MDA-MB-231 cells after incubation with 3-BP. Results shown that disruption of plasma membranes of MDA-MB-231 cells after incubation with 3-BP for 24 h and cell fragments are visible, resulting in the release of cell contents, swollen nuclei (N) and cytoplasmic material are visible, which mean that 3-BP may induce breast cancer cells MDA-MB-231 necroptosis.

SUPPLEMENTARY VIDEO 2 | Live-cell observation of MCF-7 cells after incubation 24h with 3-BP. Results shown that the characteristics of apoptotic cell death, with typical apoptotic bodies, and the number of apoptotic nuclei was significantly increased, which mean that 3-BP may induce breast cancer cells MCF-7 apoptosis.
22. Zhang Q, Zhang Y, Zhang P, Chao Z, Xia F, Jiang C, et al. Hexokinase II inhibitor, 3-BrPA induced autophagy by stimulating ROS formation in human breast cancer cells. *Genes Cancer* (2014) 5(3-4):100–12. doi: 10.18632/genesandcancer.9

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.