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13. SUPPLEMENTARY NOTES

14. ABSTRACT

It has been well established that tumor cells escape immune detection through immunosuppressive networks and is one of the hallmarks of cancer. Down-regulation of surface antigen is one of the immunosuppressive mechanisms that enable cancer cells to escape immune detection. Among several antigens, shedding or cleavage of MIC [MHC class I Chain-related]-A or MIC-B surface antigens is frequently witnessed in cancer cells. Shedding of MICA and/ or MICB has been implicated in tumor progression and immune evasion. Since MICA/B shedding is an energy-dependent process, we hypothesized that targeting energy metabolism of cancer cells could delay or prevent the shedding process resulting in an increased expression of MIC-A/B and better immune detection. This will enable us to enhance the antitumor immune-response. Data from the current research investigation demonstrate that human breast cancer cells pre-treated with low, non-toxic dose of the glycolytic inhibitor, 3-bromopyruvate (3-BrPA) show a better response to antitumor immunotherapy. One of the major implications of the current finding is that at low, non-cytotoxic dose antiglycolytic agent, 3-BrPA induces minimal perturbation of metabolism which is sufficient to block the shedding or cleavage of MICA and/ or MICB. The outcome of our study suggests that antiglycolytic pre-treatment sensitizes breast cancer cells to enhance the effectiveness of immunotherapeutics in the treatment of human breast cancer.

15. SUBJECT TERMS
Breast cancer, Energy metabolism, 3-bromopyruvate, immune response.

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INTRODUCTION

The ability to evade immune surveillance is one of the hallmarks of cancer *(1, 2)*, and it has been well established that tumor cells escape immune detection through immunosuppressive networks. The factors that challenge the functional efficiency of antitumor-immune cells (e.g. T-cells, NK cells) have been broadly classified as lymphocyte or T cell-associated factors and tumor-associated factors *(3)*. The former includes immune suppressor elements such as regulatory-T cells, myeloid derived suppressive dendritic cells among others. The tumor-associated factors include alteration in the expression of antigens (e.g. MHC) that enables cancer cells to escape immune detection, and the acidic microenvironment which is hostile to the invading antitumor immune cells *(4)*. One of the immunosuppressive processes, the shedding or cleavage of *M*HC class *I C*hain-related (MIC) *A* or MICB, is an energy-dependent process. Cancer cells have been known to exhibit an increased rate of glycolysis irrespective of the oxygen availability (aerobic glycolysis) and normal mitochondria *(5, 6)*, and it has also been recognized that this metabolic phenotype provides selective advantages to cancer cells *(7)*. Thus the altered energy metabolism (e.g. increased glucose utilization and aerobic glycolysis) enables the cancer cells to meet their energy demands and has recently been recognized as one of the hallmarks of cancer *(2)*. Thus, the uninterrupted generation and supply of cellular energy (e.g. ATP) could facilitate the shedding of MICA or MICB. On the other hand, a decrease in cellular energy will affect the rate of MICA or MICB cleavage or shedding which in turn will facilitate an effective immunesurveillance resulting in an improved antitumor immune response. *Thus, we hypothesized that if the glycolytic capacity of cancer cells is decreased or challenged it could increase the surface expression of MICA and/ or MICB, to promote the efficacy of immuno-therapeutics.* Data from the current research investigation demonstrate that human breast cancer cells pre-treated with low, non-toxic dose of the glycolytic inhibitor, 3-bromopyruvate (3-BrPA) show an improved-response to antitumor immunotherapy. The outcome of our study suggests that antiglycolytic pre-treatment sensitizes breast cancer cells to enhance the effectiveness of immunotherapeutics in the treatment of human breast cancer.

BODY

Statement of Work (SOW) for the project entitled, *"Targeting tumor metabolism to enhance the effectiveness of antitumor immune response in the treatment of breast cancer"*.

I. *In vitro* studies
(**1**) Characterization of the effect of antiglycolytic treatment on MICA in breast cancer cell lines.
(**2**) Evaluation of NK cell cytotoxicity on breast cancer cells treated with antiglycolytic agents   using NK cells.

II. *In vivo* studies
Determination of antitumor and antimetastatic effects of breast cancer cells treated with antiglycolytic agents.

SOW – I. (1) Characterization of the effect of antiglycolytic treatment on MICA in breast cancer cell lines.

(a) Cell culture, chemicals and reagents:

Human breast cancer cell lines, MDA-MB-231 and T47D, and mouse breast cancer cell line 4T1, and human NK 92mi cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured as per the supplier's instructions. In brief, MDA-MB-231 cells were cultured in L-15 medium supplemented with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Thermoscientific, USA) and 50 U/ml penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere without CO_2. T47D and 4T1 were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Invitrogen) supplemented with 10% FBS (Hyclone) and 50 U/ml penicillin and streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO_2. Highly potent cytotoxic human NK cell line NK-92mi, originally isolated from a patient with non-Hodgkin lymphoma has the ability of continuous in vitro growth, and unlike its parental cell line NK-92, this cell line does not require IL-2 supplement. It has a CD2+/CD3−/CD4−/CD8−/CD56+ phenotype characteristic of activated killer cells. These cells were cultured in alpha[α]-Minimum Essential Medium, [α-MEM], obtained from ATCC, supplemented with 2 mM L-glutamine, 0.2 mM Inositol, 0.02 mM Folic acid, 0.1mM β-mercaptoethanol, 12.5% fetal bovine serum (FBS) and 12.5% horse serum. Unless otherwise indicated all the necessary chemicals were purchased from Sigma Chemical Co.
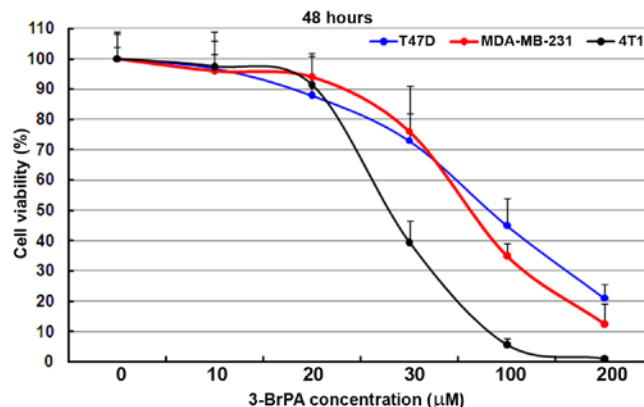
(b) Cell viability:

Effect of various concentrations of the pyruvate analog, 3-bromopyruvate (3-BrPA), a glycolytic inhibitor on different breast cancer cell lines was determined by the Trypan-blue staining method as described *(8)*.

Figure-1: Effect of the glycolytic inhibitor, 3-BrPA on the viability of breast cancer cell lines
The loss of viability is evident >30μM concentration. The maximum tolerated dose which is non-toxic is ~20μM.
Note: At 20μM the error bars overlap indicating no significant loss of viability at this concentration. Data represent mean ± S.E.M. of triplicates.

As evident from Fig.1, 3-BrPA treatment induces significant loss viability from 30μM concentration, and until 20μM the cells remain viable. Thus for further studies, 20μM concentration was determined as the maximum tolerated dose.
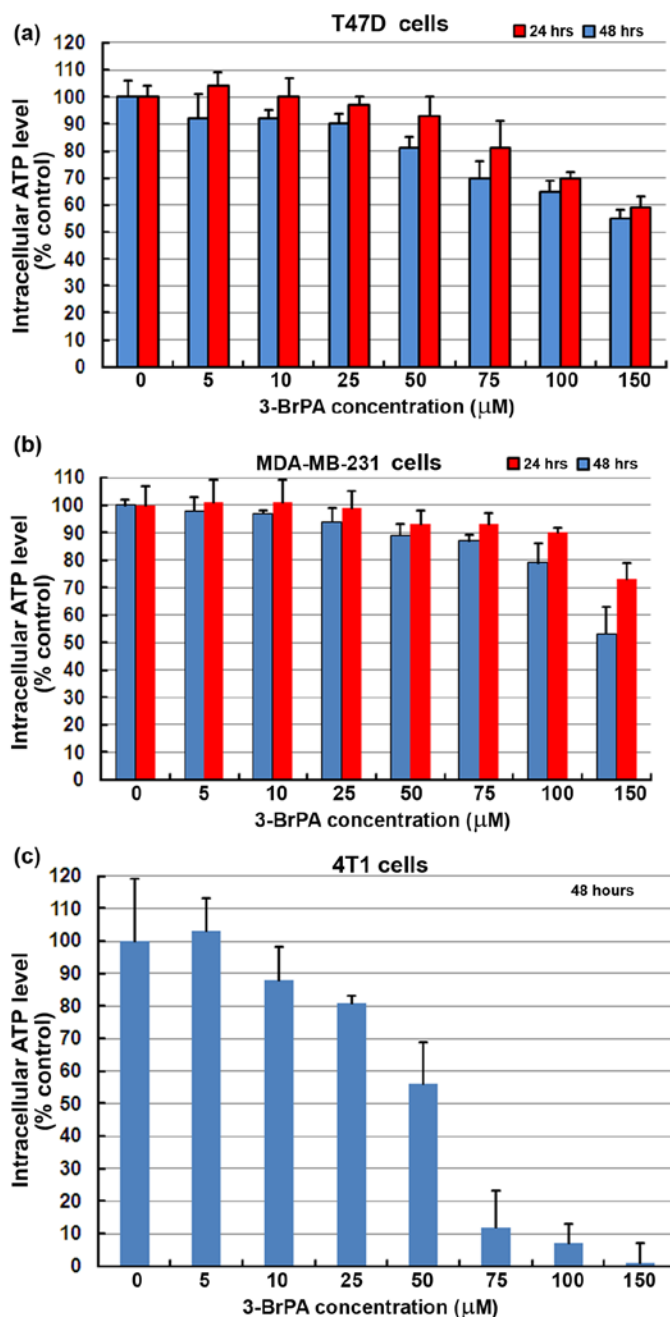
(c) Intracellular ATP:

Although, at 20μM concentration the 3-BrPA did not induce cell death, we investigated if there is any effect on the level of intracellular ATP. The quantification of intracellular ATP was performed using

Cell-Titer Glo Assay kit (Promega Inc.) in a Victor[3] multilabel plate reader. Cells were seeded in 96-well, flat-bottomed opaque (white) plates the day before 3-BrPA treatment and the experiment was carried out as per the manufacturer's protocol. Figure-2 shows that at non-lethal dose the intracellular ATP level (< 20 µM) remains almost unaffected except the 4T1 cells.

Figure-2: Effect of 3-BrPA on the level of intracellular ATP in breast cancer cell lines

In both T47D and MDA-MB-231 breast cancer cell lines, until 50 µM concentration 3-BrPA did not have any effect on the level of intracellular ATP at 24 hours of treatment. However, after 48 hours of treatment at 50µM there was a decrease in the level of intracellular ATP, but no effect at lower concentration (~25µM) of 3-BrPA. Thus, the intracellular ATP measurement as well as the Trypan-blue viability assay corroborated that the maximum tolerated dose of 3-BrPA without significant loss of viability is < 25µM. In the mouse mammary tumor cell line, 4T1, the intracellular ATP level was unaffected up to 10µM, beyond which a decrease in ATP level was evident.
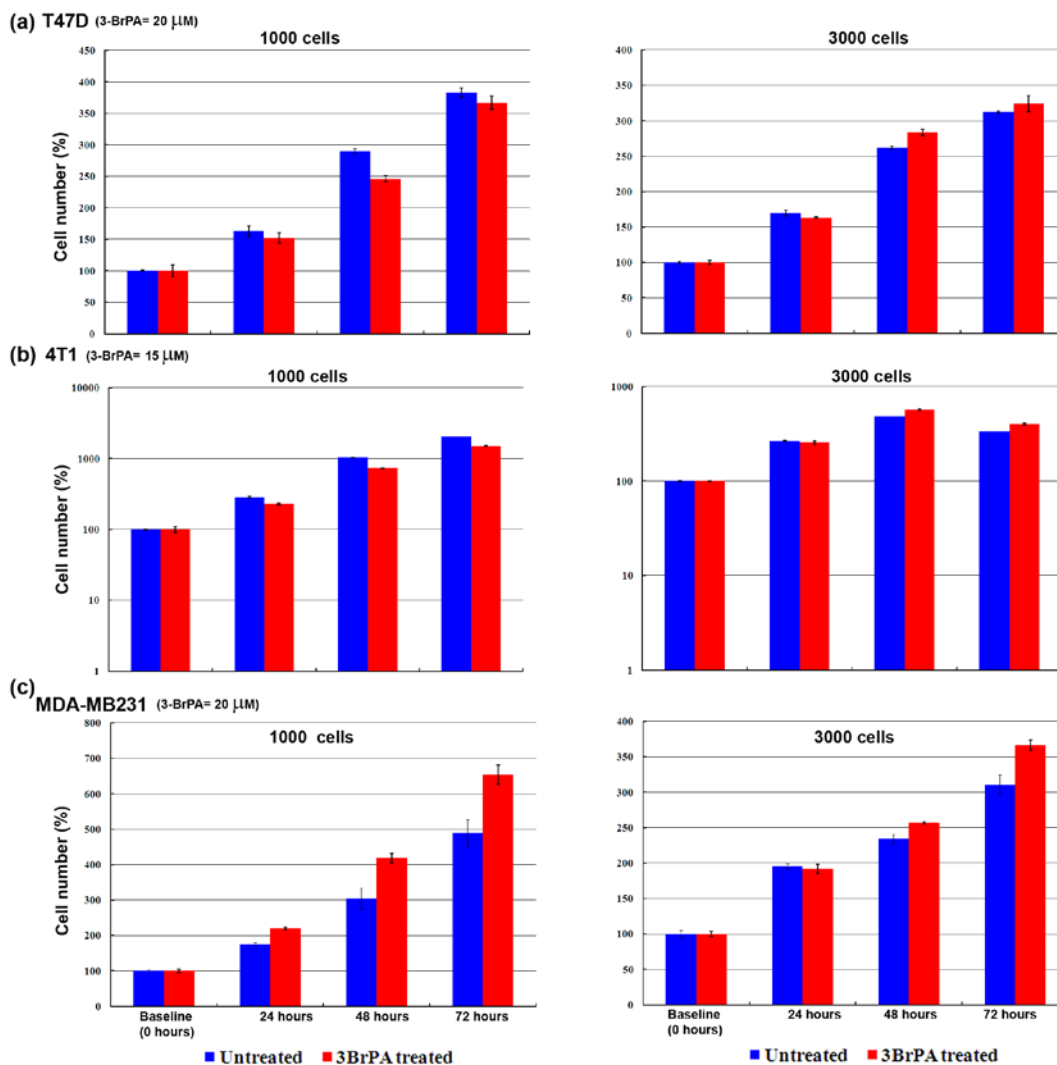
Thus, for subsequent experiments 10 µM 3-BrPA was used for 4T1 cells, whereas 20 µM 3-BrPA was used for both T47D and MDA-MB-231 cells. Data represent mean ± S.E.M. of triplicates.

(d) Effect of low dose 3-BrPA on cell proliferation and chemosensitivity:

In order to determine if the non-toxic, maximum tolerated dose of 3-BrPA affects the rate of proliferation of breast cancer cells, a proliferation assay was performed. Different cell numbers were used to confirm if the rate of proliferation is similar irrespective of the cell number. In brief, a known number of cells either 1000 or 3000 cells were seeded on to a 96-well plate (opaque) and cultured either in the presence of absence of 3-BrPA (20 µM for T47D and MDA-MB-231cells; 15 µM for 4T1 cells). Cell numbers were quantified as percentage of the control (baseline) using the Cell-Titer Glo Assay kit (Promega Inc.) in a Victor^3 multilabel plate reader.
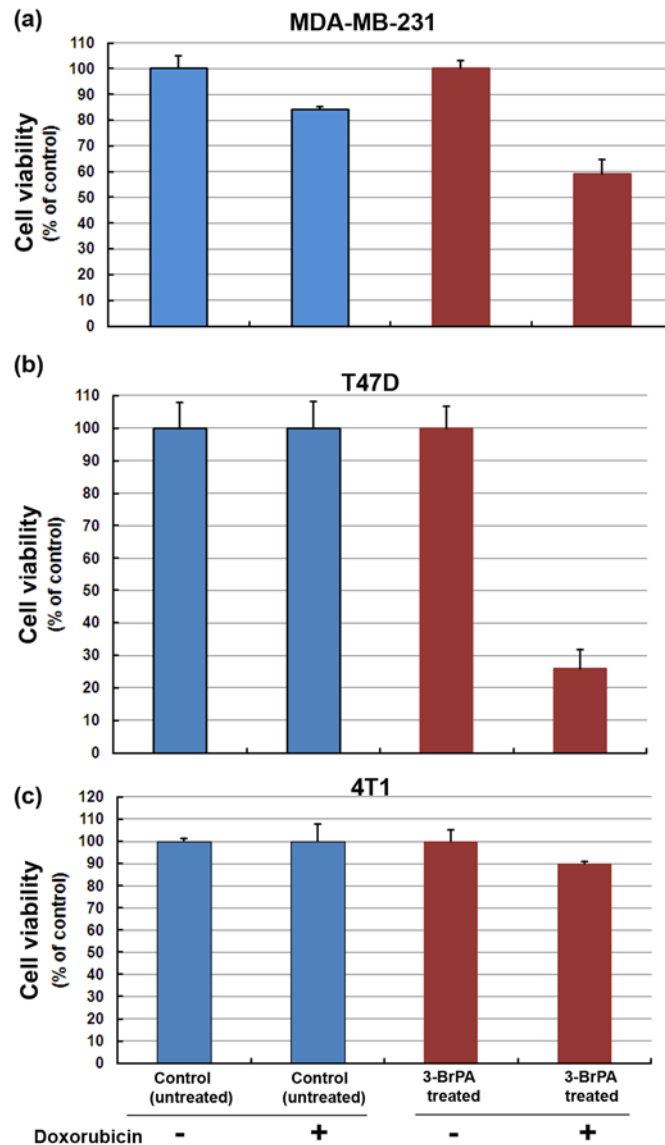

Figure-3: Proliferation of breast cancer cell lines in the presence or absence of low dose 3-BrPA

As evident in Figure-3, although the overall rate of proliferation remained unaffected in the presence of low dose 3-BrPA, interestingly, a slight increase in the rate of proliferation was observed in MDA-MB-231 cells treated with 3-BrPA, the causes which remains to be known.

Next we investigated if the low-dose 3-BrPA treatment affects the chemosensitivity of breast cancer cells. Breast cancer cells pre-treated with low dose 3-BrPA or the vehicle (saline) were tested for the effect of the chemotherapeutic, doxorubicin. Figure-4 shows that human breast cancer cell lines T47D and MDA-MB-231 pretreated with low dose 3-BrPA exhibit increased sensitivity to doxorubicin treatment than their control (no 3-BrPA treatment) groups. Among the three cell lines tested, T47D, the human breast cancer cell line was the most sensitive while the 4T1 (mouse mammary tumor cell line) was found to be the least responsive.

Figure-4: Effect of 3-BrPA pretreatment on the chemosensitivity of breast cancer cell lines

(e) Expression of MICA or MICB in breast cancer cell lines pretreated with low dose 3-BrPA-Analysis by flow cytometry

In brief, breast cancer cells were washed in PBS, collected with EDTA (Sigma-Aldrich), and analyzed for cell surface expression of proteins, MICA/B, MICA, and MICB, by direct or indirect immunofluorescence using specific monoclonal antibodies (mAbs), including PE-labeled MICA/B (6D4; BioLegend, San Diego, CA, USA), PE-labeled MICA (159227; R&D Systems, Minneapolis, MN, USA), or PE-labeled MICB (236511; R&D Systems) as first Ab, with PE-labeled anti-mouse IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA) as secondary Ab, and FITC-, or PE-labeled anti-mouse IgG (Santa Cruz Biotechnology Inc.) as isotype controls. Stained cells were acquired on a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software 6.4.7 (Tree Star, Ashland, OR).

> **Figure-5: FACS analysis of MICA and MICB expression in breast cancer cell lines treated with low dose 3-BrPA or the vehicle (saline)**
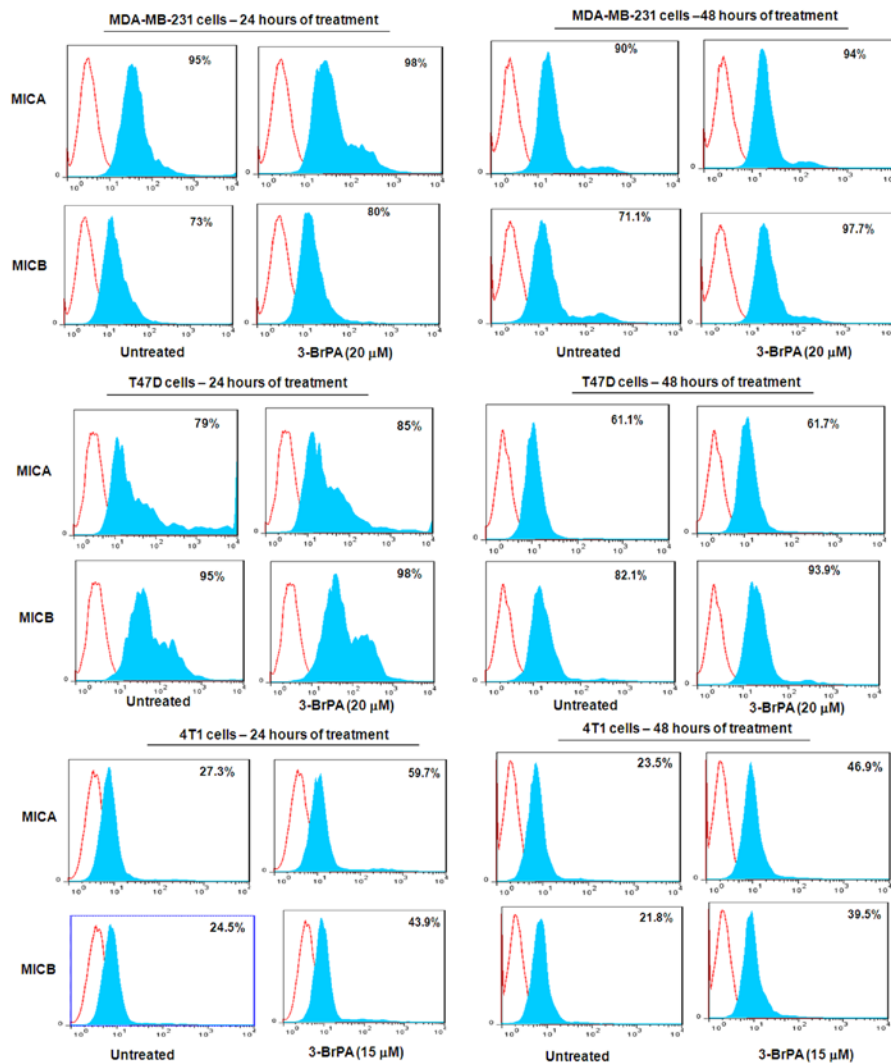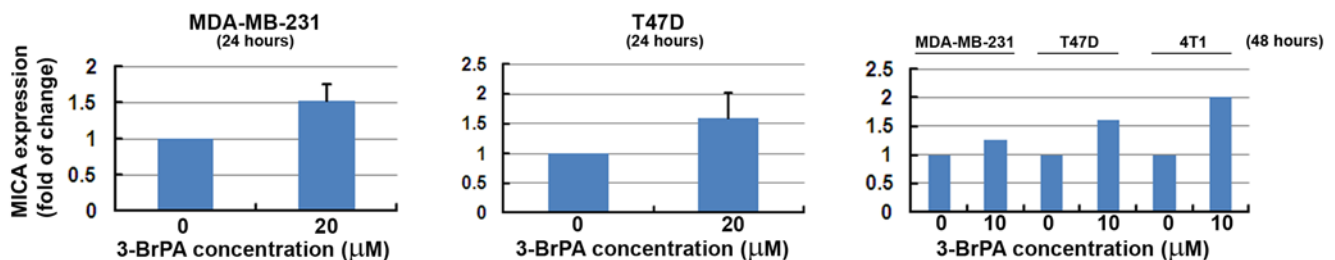
Figure-5 shows that 3-BrPA treatment (at low, non-toxic concentration) enhanced the expression level of both MICA and MICB in all the three breast cancer cell lines. The increased expression was evident at 24 hours as well as 48 hours of 3-BrPA treatment. However, the level of increase varied among cell lines and also between the MICA and MICB proteins. In both MDA-MB-231 and T47D cell lines, at 48 hours of treatment, the MICA showed no significant increase however the MICB level showed a marked increase, >25% and >10% respectively. Interestingly, in 4T1 mouse mammary cancer cells, both MICA and MICB showed a significant increase, ~22% and ~18%, respectively. Collectively, the treatment of breast cancer cells with low, non-toxic dose of 3-BrPA increases the expression of either MICA or MICB or both in different breast cancer cell lines.

(f) Expression level of MICA or MICB in breast cancer cell lines pretreated with low dose 3-BrPA- Analysis by enzyme-linked immunosorbent assay (ELISA) and western blot

For the ELISA analysis, breast cancer cells were washed in PBS, lysed by 15 min shaking in RIPA lysis buffer (Sigma-Aldrich) with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both from Sigma-Aldrich) at 4°C. Cell debris was cleared at 12,000 × g for 15 min. The supernatants were collected while cell lysates and the protein concentrations were analyzed using a 2-D Quant protein assay Kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's protocol. MICA and MICB concentrations were determined by using DuoSet ELISA according to the manufacturer's instructions (ab59569 and ab100593, Abcam Co., MA, USA).
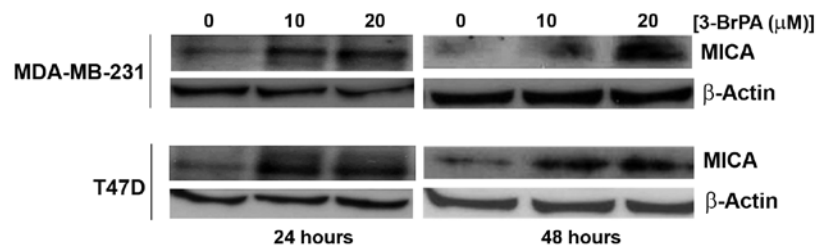
For the western blot analysis, breast cancer cells were washed in PBS, lysed during 15 min shaking in RIPA lysis buffer (Sigma-Aldrich) with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both from Sigma-Aldrich) at 4°C . Cell debris was cleared at 12,000 × g for 15 min. The supernatants were collected while cell lysates and the protein concentrations were analyzed using a 2-D Quant protein assay Kit (GE Healthcare) as described above. Equal amounts of protein samples were separated on 4–12% NuPAGE Bis-Tris acrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were blocked for 30 min in TBS (Tris Buffered Saline) with 0.05% Tween 20 (Sigma-Aldrich) buffer (TBS-T) with 5% nonfat dry milk, followed by incubation overnight at 4°C in TBS-T with 2% nonfat dry milk and primary Abs against MICA, MICB , MICA/B, (Santa Cruz) and β-actin (Sigma-Aldrich). After two washes in TBS-T, membranes were incubated with HRP-linked goat anti-rabbit or anti-mouse IgG Abs (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature, followed by washing in TBS-T and detection using ECL reagent (GE Healthcare).

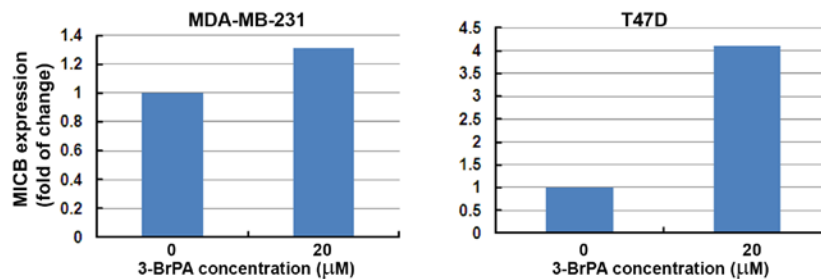**Figure-6: ELISA showing the expression of MICA in 3-BrPA-treated breast cancer cell lines**

Data from ELISA (Figure-6) show that 3-BrPA treatment elevated the expression level of MICA in both the human breast cancer cell lines even at 24 hours of treatment, while at 48 hours all the three breast cancer cell lines (including the mouse mammary tumor cell line) showed a prominent increase in the expression level of MICA. However, in contrast to the FACS analysis data (Figure-5) where no significant increase in the level of MICA expressing cells was observed, the ELISA showed at least 50% (0.5 fold) increase in MICA in MDA-MB-231 and T47D cells. Interestingly, western blot (immunoblot) analysis (Figure-7) revealed that MICA level increased in both MDA-MB-231 and T47D cell lines correlating with the data from ELISA analysis. Both ELISA and immunoblot consistently demonstrate 3-BrPA-dependent increase in the MICA expression.

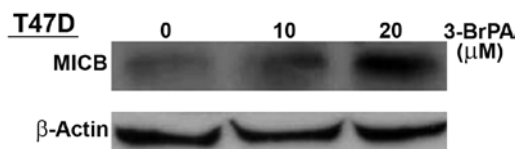**Figure-7: Immunoblot showing the expression of MICA in 3-BrPA-treated breast cancer cell lines**

Data from ELISA (Figure-8) show that 3-BrPA treatment elevated the expression level of MICB in both the human breast cancer cell lines. However, in contrast to the FACS analysis data (Figure-5) where the MICB expression was seen elevated in MDA-MB-231 cells compared to T47D, in the ELISA, the T47D showed higher increase than the MDA-MB-231. The immunoblot also confirmed a prominent increase in the expression of MICB in T47D (Figure-9).

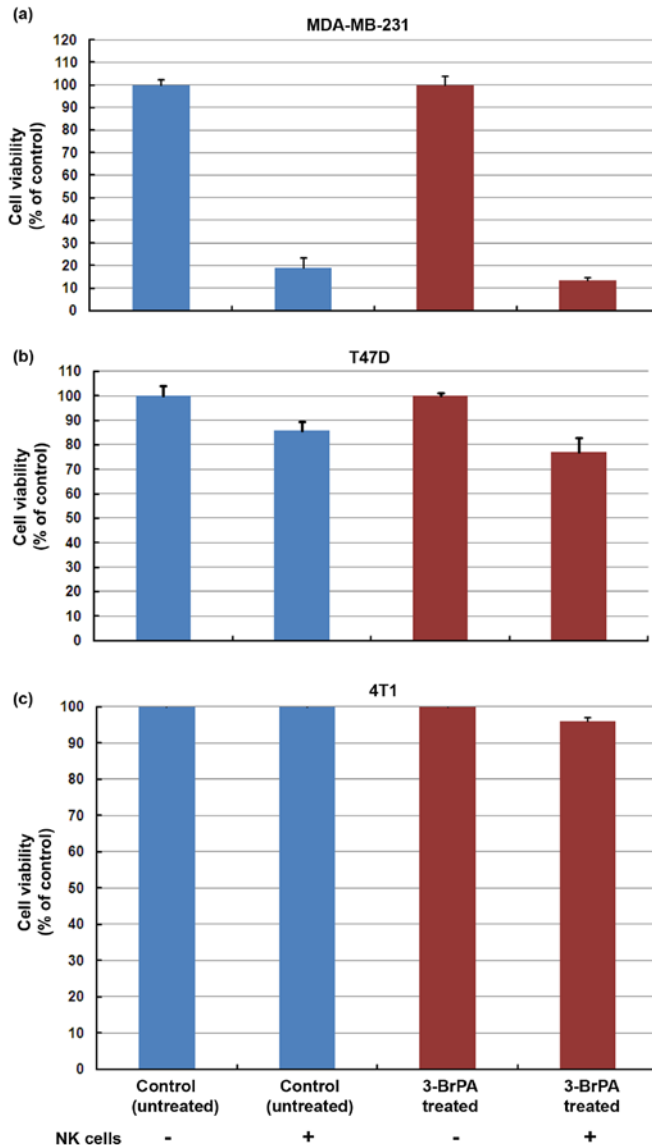**Figure-8: ELISA showing the expression of MICB in 3-BrPA-treated breast cancer cell lines**

Figure-9: Immunoblot showing the expression of MICB in 3-BrPA-treated T47D cells

SOW-I. (2): Evaluation of NK cell cytotoxicity on breast cancer cells treated with 3-BrPA.

Effect of low dose 3-BrPA-treatment on NK-cell mediated cytotoxicity

Figure-10: Effect of NK-cell mediated cytotoxicity on 3-BrPA-treated breast cancer cell lines

As effector cells, NK 92mi were cultured in α-MEM medium [as described in section I-(1)]. The cytolytic assay was performed as previously described with modifications *(8)*. In brief, either 3-BrPA treated or untreated breast cancer cells were used as target cells. In brief, 5000 target cells were seeded in 96-well plates and next day effector cells (NK 92mi) were added at an effector: target ratio of 5:1. After 96 h incubation, the cells were tested for sensitivity to NK cell-mediated lysis using Cell-Titer Glo Assay kit (Promega Inc.) which relies on the intracellular ATP level.

Figure-10 shows that 3-BrPA-treated MDA-MB-231 and T47D cells showed improved sensitivity to NK-cell mediated cytotoxicity. However, due to the innate expression or background expression of MICA/ MICB in both MDA-MB-231 and T47D, the untreated cells also showed response to NK-cell treatment. In 4T1 cells there was no effect in the untreated cells, where as a minimal effect was seen in 3-BrPA-treated cells. Noteworthy, 4T1 cells are mouse mammary tumor cells where as the NK-cells are human, and their efficacy and specificity will be pronounced on human breast cancer cells.

SOW-II. *In vivo* studies

NK-cell therapy in a mouse model of human breast cancer:

For *in vivo* experiments, female athymic (Foxn1/nu)) nude mice, aged 4-6-wk old and weighing 20-25 g, were used (Crl:NU-Foxn1nu strain; Charles River Laboratory, Germantown, MD, USA). Tumor xenografts were initiated with subcutaneous injection of breast cancer cells (4–5 x 10^6 cells) growing in log phase, either pretreated with 3-BrPA or vehicle (Saline). Untreated (control) cells were injected onto the right of the mouse whereas the 3-BrPA treated cells were injected onto the left side of the mouse.

Tumors were allowed to grow for 3 wk up to about 2-5 mm in largest diameter. A total of 20 nude mice with cancer xenografts were allocated randomly into different groups and subjected to intra venous (i.v.) injection with $1x10^6$ NK cells or phosphate buffered saline (PBS). After one week, the mice were injected again with NK cells or PBS.

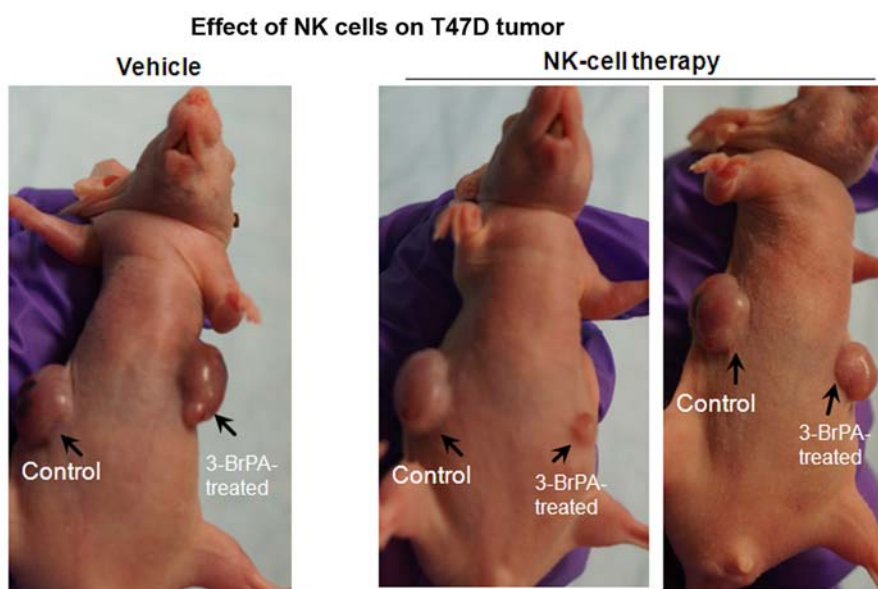### Figure-11: Effect of NK-cells on T47D tumor growth

Figure 11 shows representative animal images from vehicle and NK-cell treated tumors of T47D xenografts. Figure-12 shows the effect of NK-cell therapy on tumor growth in T47D, MDA-MB-231 and 4T1 cells pretreated with either saline or 3-BrPA.

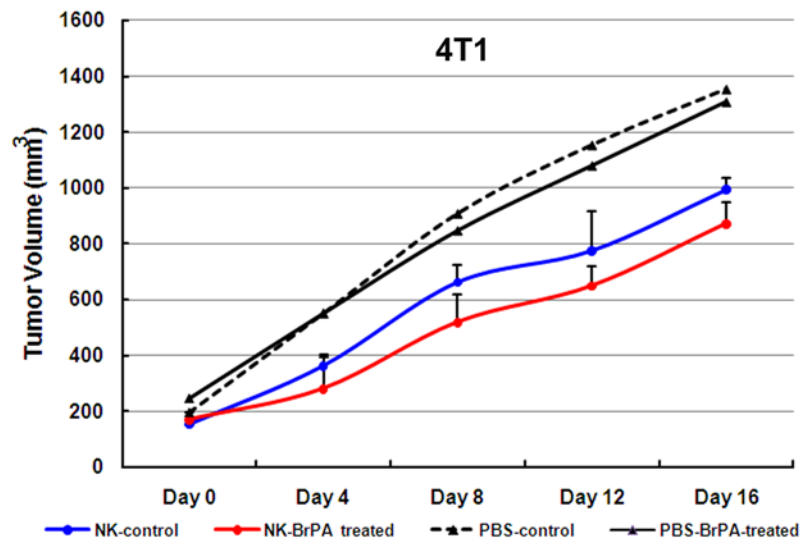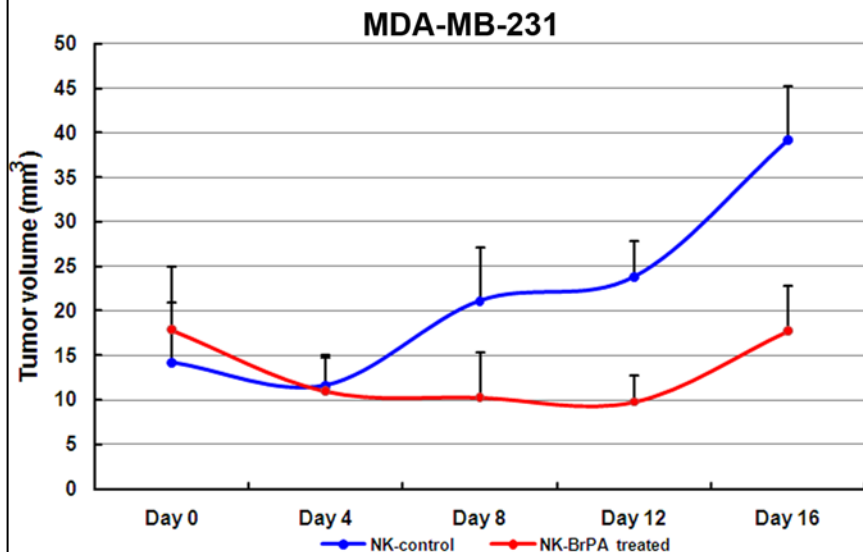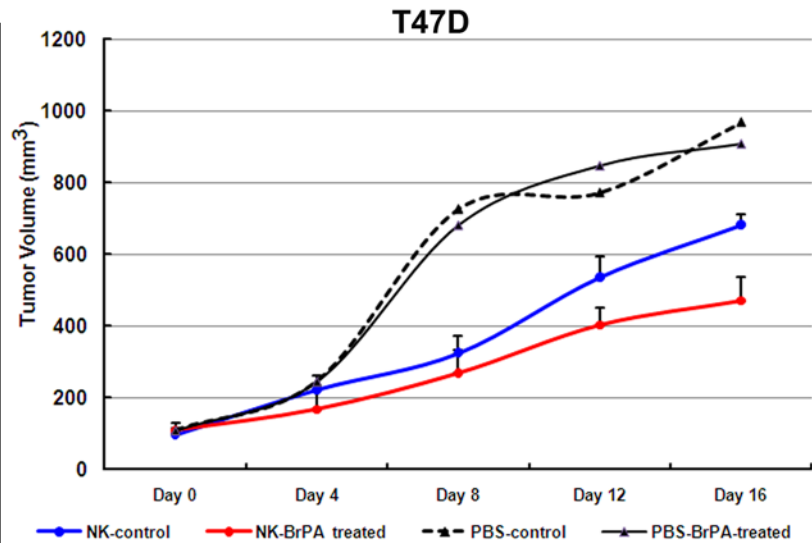**Figure-12: Effect of NK-cell mediated cytotoxicity on tumor growth in vivo.**

Mice were subjected to NK-cell injection once in 7 days for a total of 2 injections. Data represent mean± S.E.M. of 5 mice per group (for NK cell treatment) and 2 or 3 mice per group (for PBS control).

NK-control: NK cells' effect on control (no 3-BrPA pretreatment) tumor cells.

NK-3BrPA-treated: NK cells' effect on 3-BrPA pre-treated tumor cells.

PBS-control: Effect of vehicle (phosphate buffered saline, PBS) on control tumor cells.

PBS-3-BrPA-treated: Effect of vehicle (PBS) on 3-BrPA-pretreated tumor cells.

The effect of NK-cell therapy was much pronounced in T47D tumor [~200 mm^3 difference in tumor size between control (untreated) and 3-BrPA-treated T47D cells]. In MDA-MB-231 tumor, the effect of NK-cell therapy was witnessed and the difference in tumor size was ~20 mm^3 between control (untreated) and 3-BrPA-treated cells. In 4T1 cells, the effect was not different between the control (untreated) and 3-BrPA-treated cells. However, the NK-cells are of human origin hence their specificity and cross reactivity against mouse mammary tumor cells is unknown and remains to be elucidated.

KEY RESEARCH ACCOMPLISHMENTS

- Treatment of human breast cancer cells with low, non-cytotoxic dose of the glycolytic inhibitor, 3-bromopyruvate (3-BrPA) sensitizes them for chemotherapy (e.g. Doxorubicin).

- Human breast cancer cells treated with a low, non-cytotoxic dose of 3-BrPA increases the expression level of MICA and MICB.

- 3-BrPA treatment dependent increase in the level of MICA or MICB in human breast cancer cells enhances the efficacy of natural killer (NK)-cell mediated immunotherapy in vitro.

- In vivo, systemic administration of NK cells affected the growth of 3-BrPA-pretreated T47D tumor in a mouse model of human breast cancer. Thus, an increase in the level of MICA or MICB by pretreatment with a low, non-lethal dose of 3-BrPA, enhances the antitumor immune response of human breast cancer cells.

- This study demonstrates that antiglycolytic pretreatment improves the outcome of immunotherapy, suggesting a novel framework of combing antiglycolytic and immunotherapeutic approaches for the better treatment of human breast cancer.

REPORTABLE OUTCOMES

Manuscript under preparation.

CONCLUSION

Our study demonstrates that perturbation of energy metabolism using antiglycolytic agent such as 3-BrPA could alter the expression profile of cancer cell surface antigens such as MICA and/ or MICB. Significantly, such alteration involves an elevation in the level of MICA and/ or MICB. An increase in the expression of MICA or MICB enhances the antitumor immune response, as indicated by NK-cell mediated therapy. In addition, modifying the energy metabolism of breast cancer cells also sensitizes them to chemotherapy.

In breast cancer cells HER2/HER3 pathways has been known to regulate the MICA and MICB expression (9). Shedding of MICA and/ or MICB has been implicated in tumor progression and immune evasion (10). Further, targeting MICA or MICB using NK cells have also been shown to be effective in

targeting cancer *(11)*. However, interference with energy metabolism has never been demonstrated to impact the MICA or MICB expression and sensitize cells for NK mediated cell therapy.

One of the major implications of the current finding is that at low, non-cytotoxic dose antiglycolytic agent, 3-BrPA induces minimal perturbation of metabolism which is sufficient to block the shedding or cleavage of MICA and/ or MICB. Thus a combinatorial approach involving antiglycolytic treatment followed by immunotherapy could be effective in treating breast cancer.

<u>"So what section"</u>: Targeting energy metabolism in cancer cells has recently gained considerable momentum owing to the therapeutic implications. Particularly, inhibition of glycolysis has been of great interest due to cancer cell's reliance or preference for glycolytic pathway that provides it selective advantage *(7)*. Efforts on the clinical translation of glycolytic inhibitors as potent chemotherapeutics are underway with several agents at different phases of clinical trials. Our study suggests for the first time that a low dose treatment with glycolytic inhibitor (which is non-toxic or non-lethal) could enhance the efficacy of immunotherapy. The fine-tuning or perturbation of energy metabolism by a glycolytic inhibitor to facilitate and improve immune surveillance for breast cancer treatment is a new, previously unknown therapeutic approach. Thus we provide a novel framework for improving the effectiveness of immunotherapy for breast cancer. Nevertheless, this therapeutic strategy can be successfully adopted/ or extended to any solid malignancy that shows a glycolytic phenotype.

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APPENDICES: NONE

SUPPORTING DATA: NONE