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Pharmacological Double Inhibition of Glycolysis in glioblastoma Multiforme Cells Maximizes Cancer Cell Killing: A Synergism between Citrate and 3-bromopyruvate

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Abstract Glioblastoma multiform (GBM) tumors are the most aggressive and diffusely infiltrating brain tumors in human patients. Despite surgery, modern chemotherapy and anti-angiogenesis treatments, GBM tumors regrow rapidly and end patients' lives in a relatively short time. GBM tumors aggressively invade and infiltrate adjacent normal brain tissues. GBM cells are driven by glycolysis (glucose oxidation to produce ATP and lactate in cancer cells). In this study, we cultured rat C6 GBM cells and human GBM cells (U373MG) to be treated using glycolysis inhibitors. Citrate is a natural product enormously available in citrus fruits and has many pharmacological uses e.g. to treat urate renal stones. Citric acid was recently reported to exert cardioprotective effects on myocardial ischemia/reperfusion injury. Serial doses of citrate (glycolytic inhibitor of phosphofructokinase) were lethal to human GBM cell lines at relatively high doses (in millimolar range). Using the combination drug index, low effective doses of citrate (1, 3 and 5 mM) exerted a synergistic anticancer effect with low effective doses of 3-bromopyruvate (15 and 25 µM). Combination index was < 1 and denoted strong synergism between citrate and 3-bromopyruvate. As 3-bromopyruvate is another glycolysis inhibitor (hexokinase II inhibitor) in addition to citrate itself, double inhibition of glycolysis was evident when combining both citrate and 3-bromopyruvate. Both drugs benefited from tumor biology as citrate is acidic in solution and does better in acidic tumor microenvironment and same thing applies to 3-bromopyruvate that is also acidic in solution. In addition, 3-bromopyruvate is a structural analog and competitive antagonist of lactate (The Warburg effect) to deprive cancer cells of vital benefits of lactate. Lactate-based benefits to cancer cells include enhancing angiogenesis, metastasis, invasion, proliferation, migration, chemoresistance, radioresistance and acidic tumor microenvironment. Our data confirmed that pharmacological

glycolysis double inhibition significantly, maximally and synergistically distorted GBM cells morphology and reduced cellular viability. This may carry a lot of hope for treating the dismal outcome in those patients.

Keywords: *Glycolysis, double inhibition, hexokinase, pharmacological antagonist, phosphofructokinase, glioblastoma, viability*

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1. Introduction

Glioblastoma multiform (GBM) tumors are the most aggressive and diffusely infiltrating gliomas in human patients. Despite surgery, modern chemotherapy and anti-angiogenesis treatments, glioblastoma tumors regrow rapidly and end patients' lives in a relatively short term. GBM tumors aggressively invade and infiltrate adjacent normal brain tissues [1].

Citrate is both a natural product and a drug (Figure 1A-B) that carries a lot of hope in modern oncology in both diagnostic as well as therapeutic purposes. Citric acid was recently reported to exert cardioprotective effects on myocardial ischemia/reperfusion injury. Diagnostically, citrate is an *in vivo* marker that can differentiate benign from malignant prostate tumors [2]. Moreover, citrate makes the diagnosis of prostate cancer easier [3]. Citrate concentrations in human seminal fluid were reported to be better than using the traditional prostate specific antigen in diagnosing prostate cancer [4]. Moreover, citrate level was reported to be significantly decreased in metastatic prostate cancer [2]. Citrate levels significantly decreased with time in advanced brain stem gliomas [5]. Moreover, ^{52}Fe-citrate and positron emission tomography can measure iron uptake in brain tumors [6].

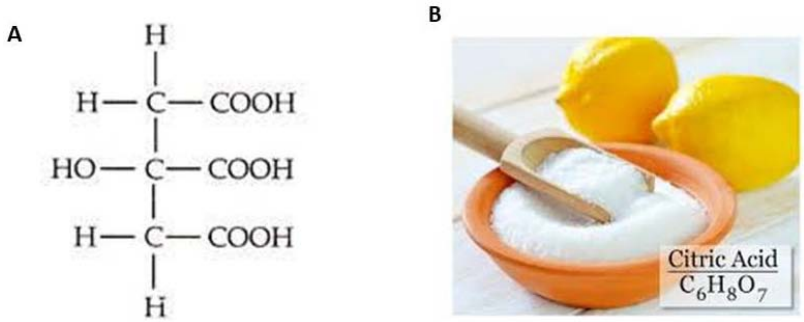

Figure 1. Citric acid is a natural organic acid. A. Molecular structure of citric acid. B. Citric acid in enormously present in citrus fruits

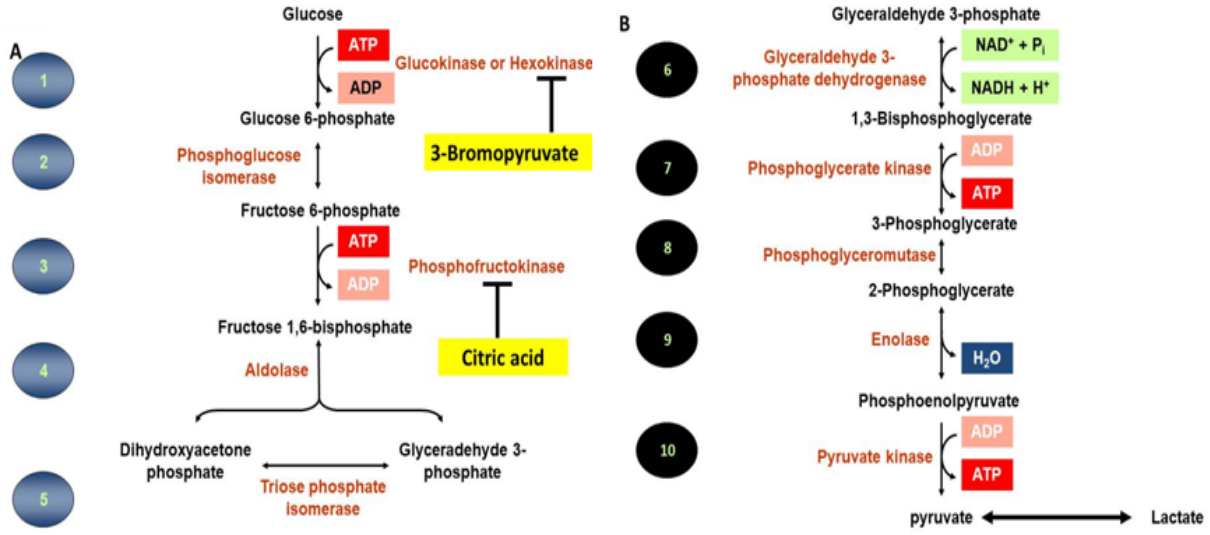

Figure 2. Glycolysis is the basic and essential primary source of energy in body cells. It is a ten-step metabolic pathway. Glycolysis is more vital for cancer cells than for normal cells. **A.** 3-bromopyruvate inhibits hexokinase enzyme while citrate inhibits phosphofructokinase. Simultaneous inhibition of both creates glycolysis double inhibition. **B.** Warburg effect occurs in cancer cells due to permanent conversion of pyruvate into lactate through activity of lactate dehydrogenase

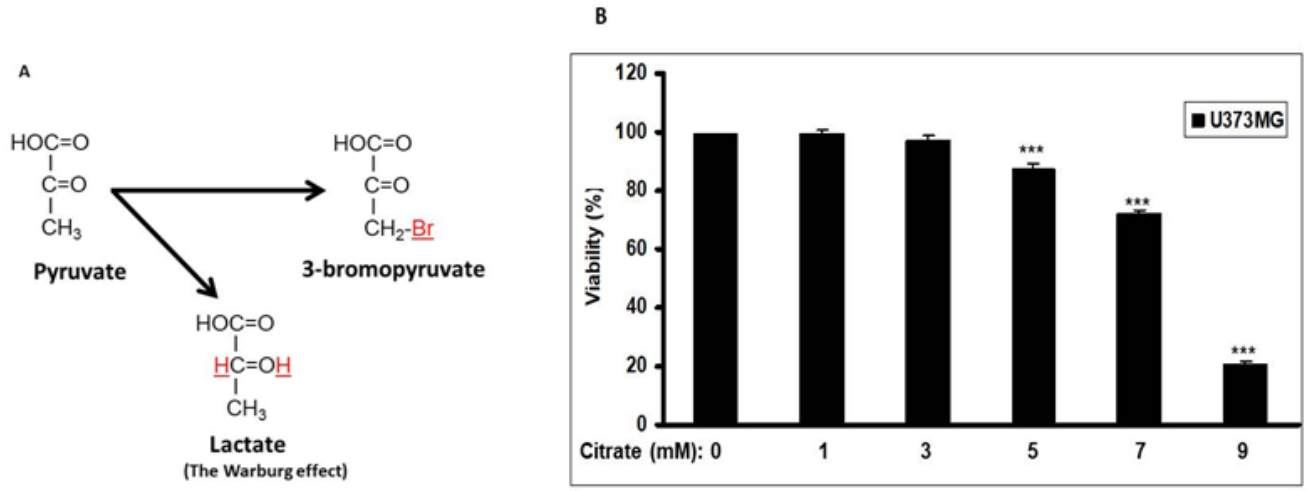

Figure 3. Glycolysis inhibitors decrease cancer cells viability. A. Pyruvate, 3-bromopyruvate and lactate are structural analogs. Lactate-based benefits to cancer cells include enhancing angiogenesis, metastasis, invasion, proliferation, migration, chemoresistance, radioresistance and acidic tumor microenvironment. B. Effects of serial citrate concentrations on viability of human glioblastoma cells (U373MG)

Therapeutically, citrate was reported to exhibit lympholytic activity in neoplastic cell lines [7]. Citrate was efficient in the management of diarrhea-induced metabolic acidosis where sodium citrate did similar to sodium bicarbonate in oral rehydration therapy for childhood diarrhea [8]. In nephrology, citrate is popular in use for treating urate stones as it inhibits stone formation and retention [9] and urinary stone deposition [10]. Moreover, citrate proved effective in treating hyperuricosuric calcium oxalate nephrolithiasis [11].

In previous reports, El Sayed and co-researchers reported a novel antiglioma effect of the glycolysis inhibitor 3-bromopyruvate (hexokinase II inhibitor) (Figure 2A) [12]. Serial doses of 3-bromopyruvate exerted synergistic antiglioma effects with low effective dose of citrate [13]. Practical problems facing 3-bromopyruvate in modern clinical oncology and relevant solutions were previously discussed [14]. Moreover, El Sayed and co-researchers reported a novel anti-angiogenesis effects of both citrate and 3-bromopyruvate [15] that strongly minimized the glycolysis-induced lactate (Warburg effect) (Figure 2B) and related benefits to cancer cells e.g. enhancing angiogenesis, metastasis, invasion, proliferation, migration, chemoresistance, radioresistance and acidic tumour microenvironment [16]. Moreover, serial doses of 3-bromopyruvate were antagonistic to both lactate and pyruvate [13] that occurs due to structural similarity (Figure 3A).

In this study, we introduced citric acid as a promising natural drug treatment for human GBM and investigated a possible synergistic effect between serial low effective doses of citrate when combined with a low effective dose of 3-bromopyruvate.

2. Materials and Methods

Citrate (citric acid) was purchased from Elnasr pharmaceutical company (Cairo, Egypt). 3-bromopyruvate, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylyltetrazolium bromide (MTT) were purchased from Sigma (St. louis, MO, USA). C6 rat glioma cells and U373MG human glioblastoma cell line were also purchased from Sigma (CA, USA). DMEM/F12, Geniticin (G418) and penicillin-streptomycin antibiotic mixture were from Invitrogen life technologies (Carlsbad, CA, USA).

2.1. Cell Culture

U373MG human glioblastoma cell line was maintained in DMEM containing 10% (v/v) FBS and 1% penicillin-streptomycin at 37°C under a humidified atmosphere containing 5% CO_2.

2.2. MTT Assay

C6 rat glioblastoma cells (Dainippon Pharmaceutical Co., Osaka, Japan) were maintained in DMEM/F12 containing 15% (v/v) horse serum, 2.5% (v/v) FBS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere containing 5% CO2. U373MG cells were seeded into 96-well plates for 24 h until cells reached 80% confluency. Medium aspiration and stimulating medium (DMEM/F12 containing 1% (v/v) FBS) addition was done. Cells received treatment in the form of serial doses of citrate (0, 1, 3 and 5 mM), 15 µM 3-bromopyruvate or (0, 1, 3 and 5 mM citrate combined with 15 µM 3-bromopyruvate) followed by incubation for 21 h. MTT (50 µl of 1 mg/ml) solution was added and followed by incubation for an additional 3 h. Centrifugation, supernatant aspiration and DMSO addition (150 µl/well) were done. After complete dissolution of the insoluble formazan crystals, absorbance was measured at 550 nm using absorption function of Infinite™ M200 microplate reader.

2.3. Glycolysis Double Inhibition Effect

U373MG cells seeded in 96 well plates were treated at 80% confluency with serial doses of citrate (1, 3 and 5 mM) and 25 µM 3BP for 24 h. U373MG received treatment in the form of 25 µM 3BP with and without same doses of citrate. Effect of treatment on viability of U373MG was investigated. Microscopic photographs

were captured to investigate the effects of the given treatments on morphology of U373MG.

2.4. Combination Index

To estimate if the combination between small mildly effective doses of citrate and 3-bromopyruvate are synergistic or antagonistic, combination index was calculated as previously reported [17,18] through using the equation:

Combination index = a/A + b/B

- If Combination index (a/A + b/B) = 1, both drugs are additive
- If Combination index (a/A + b/B) is < 1, both drugs are synergistic
- If Combination index (a/A + b/B) is >1, both drugs are antagonistic

Where: (a) is the small effective dose of the 1st drug to be combined with (b, the small effective dose of the 2nd drug) to get a desirable effect. (A) is the large dose of 1st drug the gets the same effect. (B) is the large dose of 2nd drug the gets the same effect.

2.5. Statistics

Results shown are (Mean ± S.E.M) of the values obtained from the indicated number of experiments. Differences between groups were analyzed by Student's t test. Significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$ versus control are indicated by *, ** and ***, respectively.

3. Results

3.1. Serial Doses of Citrate (in millimolar Range were CYTOTOXIC to GBM Cells)

Serial doses of citrate (in millimolar range were cytotoxic to GBM cells: Although 1-3 mM citrate doses were almost ineffective in killing glioblastoma cells, larger doses (5-9 mM) were cytotoxic to such cells and significantly decreased GBM cellular viability (Figure 3B).

3.2. Effect of Combination of Serial Doses of Citrate and 3BP (Glycolysis Double Inhibition) on Glioma Viability

As both citrate and 3BP can induce glioma cell death through inhibiting different glycolytic enzymes, we investigated if a combination of 3BP and serial doses of citrate may have a synergistic effect as an antiglioma treatment. We investigated effect of combination of dose 15 µM 3BP with serial doses of citrate (1, 3 and 5 mM). Our data revealed that a significant synergistic effect ($p<0.001$) was observed at all the previously mentioned citrate doses on C6 glioblastoma cells (Figure 4A) where maximal cancer cells killing was achieved. Same synergistic effect was obtained when 25 µM 3BP was combined with serial doses of citrate (Figure 4B). Marked damage and loss of fibroblast shape of U373MG was observed with glycolysis double inhibition treatment (Figure 4C).

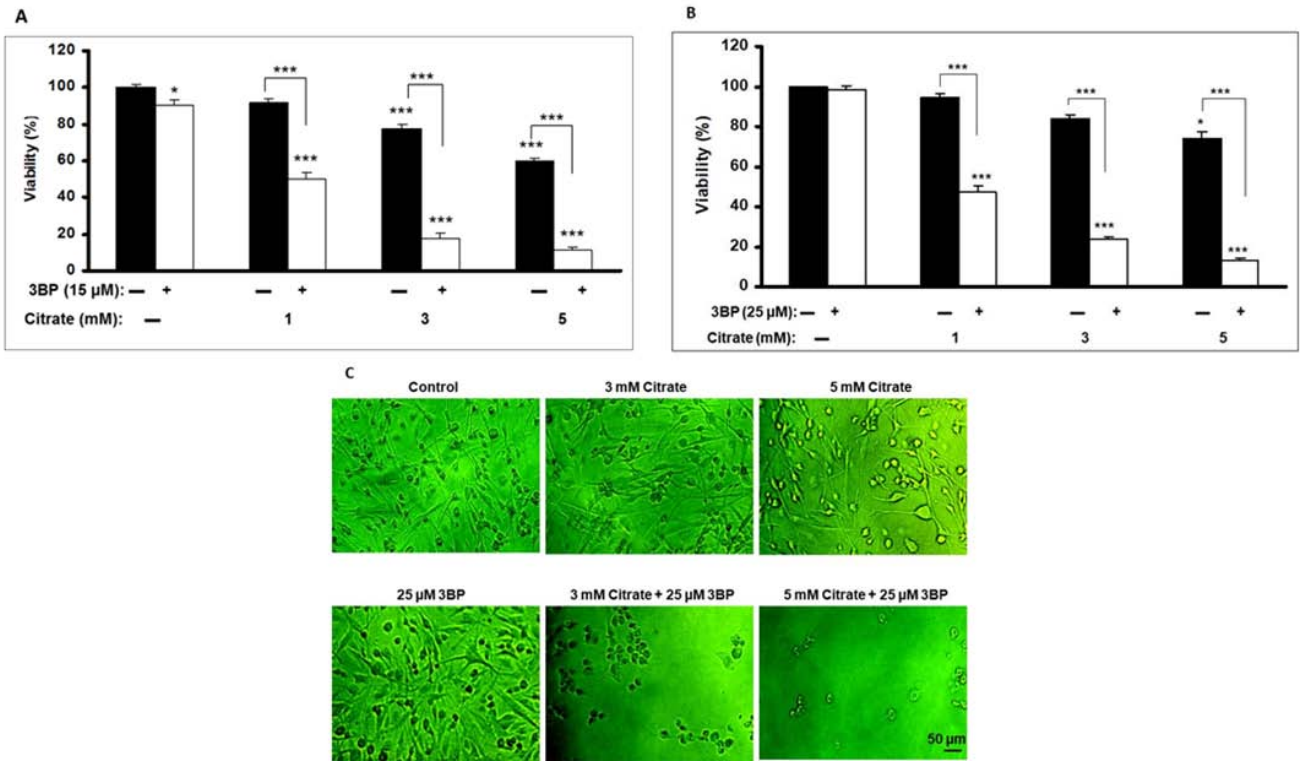

Figure 4. Citrate exerts a synergistic effect with 3-bromopyruvate (A) Glycolysis double inhibition through combining serial doses of citrate with 3-bromopyruvate in C6 glioma cells. (B) Effect of glycolysis double inhibition on U373MG human GBM cells. (C) Effect of glycolysis double inhibition on morphology of U373MG. Data are (Mean ± SEM) of the percentages of the control values of three independent experiments. *p<0.05, ** p<0.01 and ***p<0.001 indicate significance between control and different treatment conditions and also between treatment conditions within the same group

3.3. Calculating the Combination Index (Citrate + 3-bromopyruvate)

- In a previous report [12], LD50 (lethal dose that decreased viability of C6 GBM cells was 30 µM 3-bromopyruvate. So, A=30.
- LD50 (lethal dose that decreased viability of C6 GBM cells was 5 mM citrate. So, B = 5
- 15 µM 3-bromopyruvate killed 10% of C6 GBM (Figure 4A). So, a = 15
- 1 mM citrate killed 15% of C6 GBM cells (Figure 4A). So, b = 15

The combination index = a/A + b/B = (15/30) + (1/5) = 0.5 + 0.2 =0.7 i.e. <1

Based on that, both citrate and 3-bromopyruvate are synergistic.

As for the effect of glycolysis double inhibition on viability of U373MG human GBM cells:

- Lethal dose that killed 80%-90% of U373MG human GBM cells was 100 µM 3-bromopyruvate. So, A=100 [13].
- Lethal dose that killed 80%-90% of U373MG human GBM cells was 9 mM citrate (Figure 3B). So, B = 9
- 25 µM 3-bromopyruvate killed almost 3% of U373MG human GBM cells (Figure 4B-C). So, a = 25
- 5 mM citrate killed 25% of U373MG human GBM cells (Figure 4A). So, b = 5

The combination index = a/A + b/B = (25/100) + (5/9) = 0.25 + 0.45 =0.7 i.e. <1.

Based on that, both citrate and 3-bromopyruvate are synergistic.

3.4. Glycolysis Double Inhibition Markedly Distorted the Morphology of Human GBM Cells (Figure 4C)

Serial doses of citrate (3 and 5 mM) were not evident in destroying the cellular morphology of human GBM cells. Adding a small effective dose of 3-bromopyruvate to serial doses of citrate (glycolysis double inhibition) markedly damaged the cells. U373MG cells lost their spindle shape and the cells became involuted with total loss of the fibroblast morphology that corresponds to rapid, massive and group cell death (necrosis).

4. Discussion

Extracellular pH in tumor microenvironment is strongly acidic (6.2 to 6.9) [20]. This renders tumors cells chemoresistant and able to neutralize basic (alkaline) drugs e.g. mitoxantrone and doxorubicin. On the other hand, the anticancer effects of acidic chemotherapeutics as 5-fluorouracil and chlorambucil are maximized by such acidic tumor pH [21]. As both citric acid and 3-bromopyruvate are acidic in reaction, their anticancer effects are expected to be maximized inside tumor tissues. This potentiates prescribing citric acid as a future cancer chemotherapeutic. Interestingly, in vitro citrate caused a decrease in pH of the culture medium (DMEM) in a dose-dependent manner [22].

Citrate is both a natural product and a drug (Figure 1A-B). Citric acid is not reported to be a carcinogen or a teratogenic agent. On the other hand, citrate may be promising in managing some special difficulties in medical practice e.g. citrate effectively managed antibiotic-resistant postoperative wounds in cancer patients [23]. Citrate effectively improved sperm motility [24]. Unfortunately, few research studies are there to describe the role of citrate as an anticancer agent.

Citrate is also a proved glycolysis inhibitor drug where low dose of citrate (< 1 mM) was reported to be inhibitory of the key glycolytic enzyme phosphofructokinase-1 (PFK-1) (Figure 2A) [25]. This may make citrate a suitable anti-neoplastic drug targeting glycolysis and Warburg effect, a common metabolic alteration in most cancer cells in which cancer cells use glycolysis as a major energy source even in the presence of oxygen [26] as citrate can inhibit glycolysis pathway upstream of the lactate formation step (Figure 2A). This can maximally reduce the amounts of lactate formed i.e. decreases lactate-based benefits to cancer cells e.g. angiogenesis, metastasis, chemoresistance and radioresistance [16].

Serial doses of citrate significantly decreased viability of human glioblastoma cells (U373MG) in a dose-dependent manner (Figure 3B). As a novel combination, we reported recently that combination of antiglycolytics targeting different key enzymes of glycolysis pathway may have a synergistic effect on decreasing glioma cell death. Low dose of citrate combined with serial doses of 3BP were synergistic in decreasing viability of C6 glioma [12]. Our data revealed that combination of 3BP and citrate had a strong synergistic effect on decreasing glioma viability using minimally effective doses of 3BP and serial doses of citrate. A dose of 15 µM 3BP exerted a synergistic effect with serial doses of citrate (Figure 4A). Both 3BP and citrate exert antiglycolytic effects by inhibiting the two key enzymes of glycolysis i.e. 3BP inhibits hexokinase II while citrate inhibits PFK. This glycolysis double inhibition proved effective in decreasing glioma viability (Figure 4A). The chemoresistant and radioresistant human GBM (U373MG) was quite responsive to glycolysis double inhibition treatment. Marked loss of viability of U373MG was obtained when an almost non-effective dose of 3BP was combined with serial doses of citrate (Figure 4B). Marked loss of fibroblast shape of U373MG was observed when 3BP was combined with serial doses of citrate (Figure 4C).

Conflict of Interest

Authors declare that there is no conflict of interest.

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