Effects of the Glycolysis substrates, (glucose and glucose-6-phosphate on glioma cell death induced by the glycolysis inhibitors 3-bromopyruvate and citrate

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Abstract

Background: Grade IV astrocytoma, or glioblastoma multiforme (GBM), is among the most commonly aggressive gliomas resistant to treatment approaches. There is a high expression of glycolytic genes in clinical glioblastoma patients that was reported to correlate with decreased patients' survival. Both 3-bromopyruvate (3BP) and citrate are promising glycolysis inhibitors and anticancer agents with many wonderful mechanisms of anticancer effects. Glucose is the substrate of hexokinase while glucose-6-phosphate is the substrate of phosphofructokinase, PFK. 3BP is a hexokinase inhibitor while citrate is a PFK inhibitor.

Objectives: This study aims at investigating the effects of adding the first two substrates of glycolysis (glucose and glucose-6-phosphate) affecting the survival of C6 glioma cells, which were reported to be maximally killed using either 9 mM citric acid or 60 µM 3BP.

Materials and methods: MTT viability assay was performed to C6 glioma cells.

Results: Glioma cell death was significantly and maximally induced by 3BP and citrate. The addition of glucose or glucose-6-phosphate in successive doses did not prevent the mortality of C6 glioma cells caused by 3BP. Adding serial doses of glucose did not protect against citrate-induced C6 glioma cell death. However, the most interesting finding in this study was that citrate-induced glioma cell death was quite inhibited via adding serial doses of glucose-6-phosphate.

Conclusion: Glucose-6-phosphate antagonized citrate effects on glioma cell viability possibly via activating the glycolytic enzyme PFK. PFK is well-known to be inhibited by citrate and that citric acid is a structural analog of glucose-6-phosphate. This finding is quite important as citrate toxicity can be inhibited via adding glucose-6-phosphate.

Keywords: Glycolysis substrates; Glucose; Glucose-6-phosphates; Glioma cell death; 3-bromopyruvate and citrate

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Introduction

Grade IV astrocytoma, or glioblastoma multiforme (GBM), is among the most commonly aggressive gliomas that are resistant to treatment approaches. Malignant gliomas are among the deadliest malignancies that usually result in death **(Reardon and Wen, 2006)**. GBM is the most common primary brain tumor and the deadliest, with 3–5 occurrences per 100,000 persons annually in North America and Europe. Ten percent or less people survive five years, and the median life expectancy is only fifteen months **(Weller et al., 2013)**. Malignancy criteria that set GBMs apart from lower grade gliomas include significant angiogenesis, hypoxia, and necrosis **(Carmeliet and Jain, 2011)**.

There is a high expression of glycolytic genes in clinical glioblastoma patients that was reported to correlate with decreased patients' survival **(Stanke et al., 2021)**. In the glycolysis pathway, phosphofructokinase (PFK), the second important irreversible enzyme, catalyzes the conversion of glucose to glucose-6-phosphate, whereas hexokinase, also known as glucokinase, is the first irreversible enzyme in the process. (**Fig.1**). PFK is inhibited by citrate (citric acid), an abundant organic acid in citrus fruits as oranges and lemon.

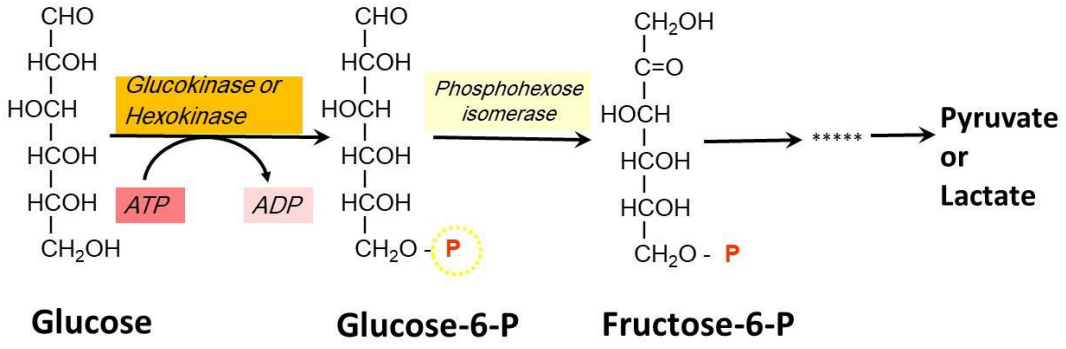

Fig.1. First stages of glycolysis. Following the conversion of glucose to glucose-6-phosphate and fructose-6-phosphate, lactate and pyruvate are produced after a series of successive biochemical reactions.

3-bromopyruvate (3BP) is a promising anticancer agent with many wonderful mechanisms of anticancer effects. 3BP-induced anticancer mechanisms in killing cancer cells include generating hydrogen peroxide, antagonizing lactate (the Warburg effect), inhibiting hexokinase and GAPDH, inhibiting angiogenesis and depleting the bioenergetics of glioma cells **(El Sayed et al., 2012a; El Sayed et al., 2012b; El Sayed et al., 2012c; El Sayed et al., 2013; El Sayed et al., 2014; El Sayed et al., 2017a; El Sayed et al., 2017b; El Sayed, 2023).** The lethal effects of chloroethylnitrosoureas on human glioma cells and the production of DNA interstrand cross-links were enhanced by 3BP's glycolytic inhibition. **(Sun et al., 2020)**.

Citrate itself is a harmless natural chemical and is formed in every mitochondrion from a combination of acetyl CoA and oxaloacetate during Krebs cycle that is also termed citric acid cycle. There had been many research studies on citrate's anticancer properties. Citrate has been found to be used in the treatment of mesothelioma **(Zhang et al., 2009),** gastric cancer **(Lu et al., 2011),** medullary thyroid carcinoma (Bucay, 2009), and postoperative wounds that are resistant

to antibiotics in cancer patients **(Nagoba et al., 2011).** Furthermore, leukemia and lymphoma cell lines demonstrated a dose-dependent lympholytic activity caused by citrate, either in combination with or without chemotherapeutic agents. Interestingly, citrate had very little effect on mesenchymal cells in normal human peripheral blood **(Yousefi et al., 2004)**. Citrate shares has many features in common with resveratrol, another promising anti-tumoral medication. The viability of cancer cells and glucose catabolism were both lowered by either resveratrol or citrate. A strong PFK inhibitor, resveratrol lowers glucose consumption, ATP levels, and glioma viability. Resveratrol inhibits pure PFK biochemically by dissociating the enzyme from its active form (tetramers) into a less active state (dimers). The mechanism by which resveratrol exerts its anticancer effects is comparable to that of ATP and citrate **(Gomez et al., 2013)**.

It was reported that a novel combination of the PFK inhibitor, citrate, and successive doses of 3BP blocked the first two important enzymes in the glycolysis pathway (glycolysis double inhibition), which may have fewer adverse effects and better therapeutic advantages than either agent acting alone **(El Sayed et al., 2012b)**. In this study, the author investigated the effects of adding the glycolysis substrates, (glucose and glucose-6-phosphate on glioma cell death induced by the glycolysis inhibitors 3-bromopyruvate and citrate.

Materials and Methods

El-Nasr Pharmaceutical Company (Cairo, Egypt) provided the citrate (citric acid). Sigma (St. Louis, MO, USA) provided fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-bromopyruvate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylyltetrazolium bromide (MTT). Sigma (CA, USA) also provided the C6 rat glioma cell line, glucose, and glucose-6-phosphate. DMEM/F12, geneticin (G418), and the penicillin-streptomycin antibiotic mixture were supplied by Invitrogen Life Technologies (Carlsbad, CA, USA).

MTT Assay

C6 rat glioblastoma cells (Dainippon Pharmaceutical Co., Osaka, Japan) were cultured at 37 °C in a humidified environment with 5% CO2 in DMEM/F12 supplemented with 15% (v/v) horse serum, 2.5% (v/v) FBS, and 1% penicillin-streptomycin. After 24 hours, cells were planted into 96-well plates and allowed to grow to 80% confluency. The procedures involved medium aspiration and stimulating medium addition (DMEM/F12 with 1% (v/v) FBS). Treatment for the cells included repeated doses of 9 mM citrate and millimolar doses of glucose (0, 0.1, 0.5,1, 2, 4, 6, 10 or 20 mM), or glucose-6-phosphate (0, 0.1, 0.5,1, 2, 4, 6, 10 or 20 mM). Another C6 glioma cells were treated with 60 µM 3BP or and serial millimolar doses of either glucose, or glucose-6-phosphate at the same doses. That was followed by incubation for 21 h. After adding 50 µl of a 1 mg/ml MTT solution, the mixture was incubated for an extra three hours. The steps involved centrifugation, aspiration of the supernatant, and addition of DMSO (150 µl/well). Using a BioTeck Multimode microplate reader, absorbance was measured at 550 nm following the full dissolution of the insoluble formazan crystals.

Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM©, Armonk, NY, USA). Quantitative parametric data were expressed as mean and standard error of mean (Mean ± SEM) and analyzed through unpaired Student t-tests. *** denotes p

≤ 0.001 in same groups under same treatments. ## and ### denote p< 0.01 and p ≤ 0.001 among different treatment groups, respectively.

Results

This study investigated the effects of adding the first two substrates of glycolysis (glucose and glucose-6-phosphate) (**Fig.1**) on the viability of C6 glioma cells

Successive doses of glucose did not protect against 3BP-induced glioma cell death

A dose of 60 µM 3BP significantly (p< 0.001) and maximally killed C6 glioma cells. Adding serial doses of glucose dissolved in DMEM medium did not add any survival protective benefit to glioma cells killed by 3BP (**Fig.2**).

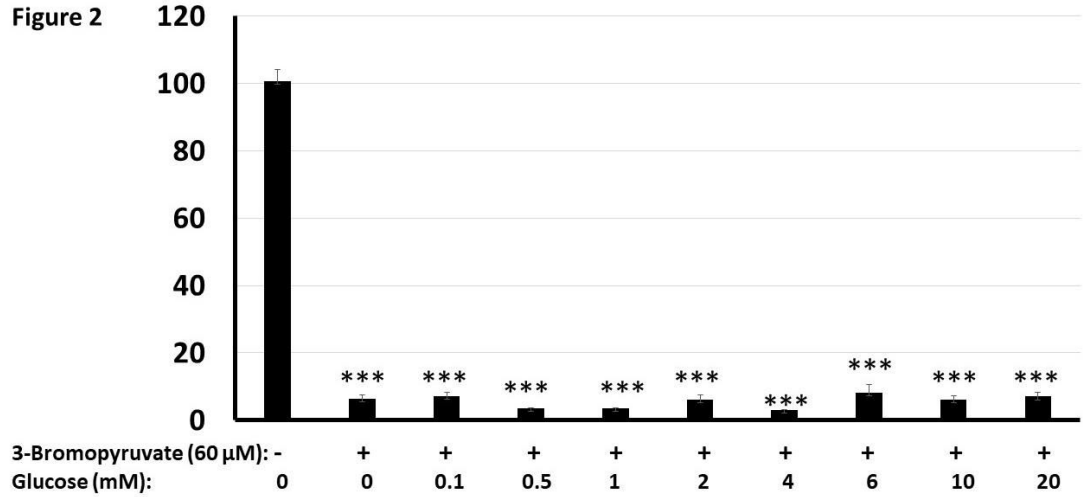

Fig. 2. Serial doses of glucose (in millimolar range) did not protect rat C6 glioma cells against cell death induced by 3BP.

Successive doses of glucose-6-phosphate did not protect glioma cells against 3BP-induced death

A dose of 60 µM 3BP significantly (p< 0.001) and maximally killed C6 glioma cells. Adding serial doses of glucose-6-phosphate dissolved in DMEM medium did not add any survival protective benefit to glioma cells killed by 3BP (**Fig.3**).

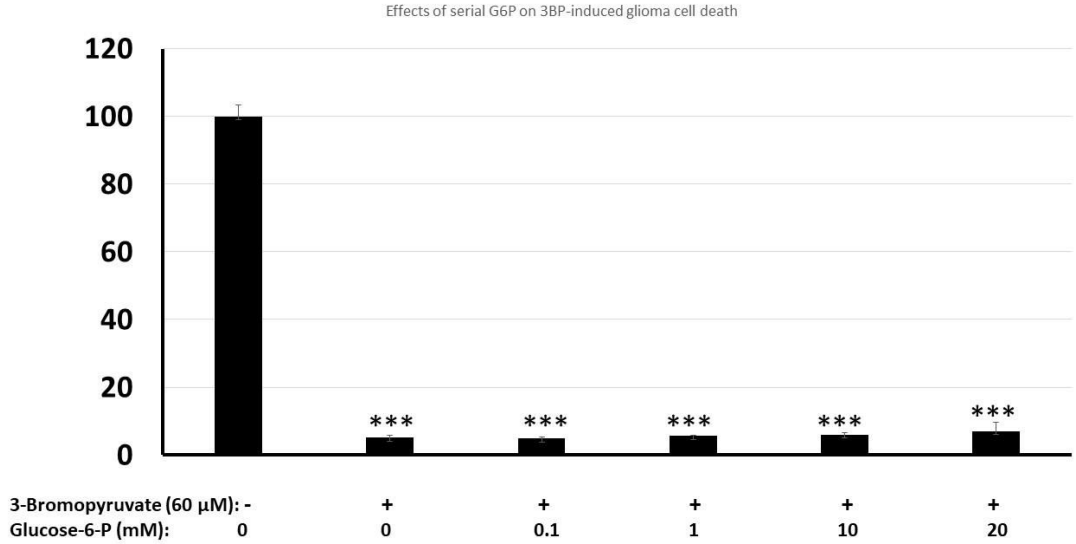

Fig.3. Serial doses of glucose-6-phosphate (in millimolar range) did not protect rat C6 glioma cells against cell death induced by 3BP.

Successive doses of glucose did not protect glioma cells against citrate-induced death

A dose of 9 mM citric acid (**Fig.4**) significantly (p< 0.001) and maximally killed C6 glioma cells. Adding serial doses of glucose dissolved in DMEM medium did not add any survival protective benefit to glioma cells killed by citrate (**Fig.5**).

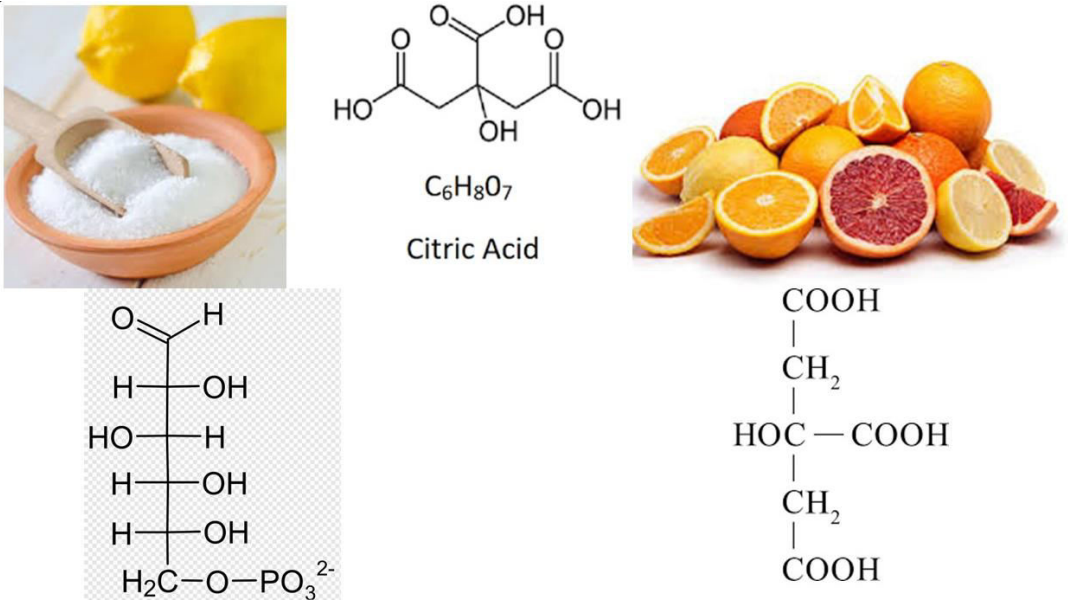

Fig.4. Citric acid is a natural organic acid present enormously in citrus fruits.

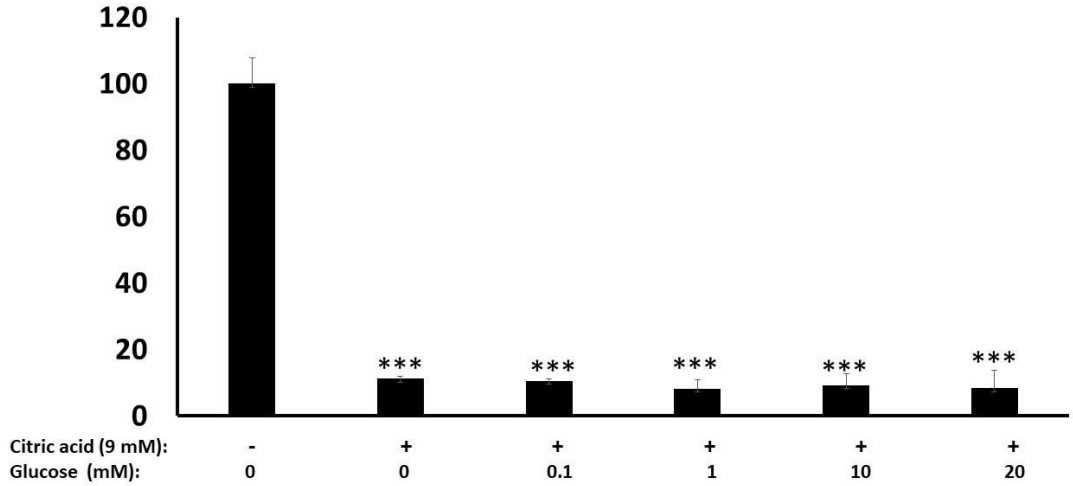

Fig.5. Serial doses of glucose (in millimolar range) did not protect rat C6 glioma cells against cell death induced by citrate.

Successive doses of glucose-6-phosphate significantly protected glioma cells against citrate-induced death

A dose of 9 mM citric acid significantly (p< 0.001) and maximally killed C6 glioma cells. In a dose-dependent manner, C6 glioma cells were substantially protected against citrate-induced cell death by the addition of successive doses of glucose-6-phosphate dissolved in DMEM media (p < 0.001) (**Fig.6**).

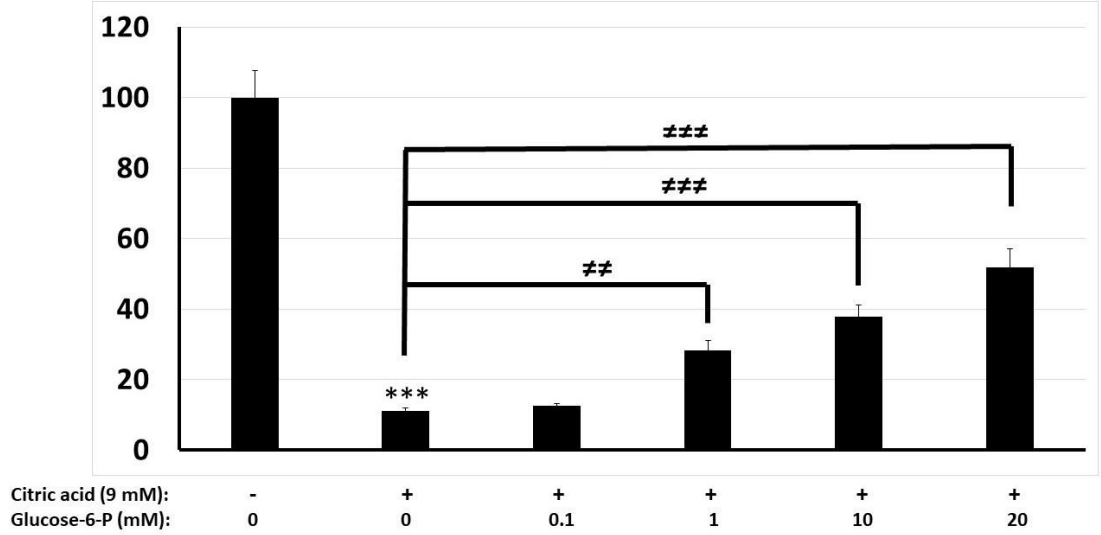

Fig. 6. Serial doses of glucose-6-phosphate significantly protected against citrate-induced glioma cell death

Discussion

The viability of glioma cells was reported to be impaired maximally using either 9 mM citric acid or 60 µM 3BP (El Sayed et al., 2012a; El Sayed et al., 2012b; El Sayed et al., 2012c). Normal cells (astrocytes) and cancerous cells (glioma cells) have different metabolic energy patterns because respiration (oxidative phosphorylation), which produces a large amount of ATP, is the primary source of astrocytic energetics **(Hertz et al., 2007; El Sayed et al., 2020)** that differs wholly from glycolysis, the major energy supply of glioma cells **(El Sayed et al., 2012a; El Sayed et al., 2012b; El Sayed et al., 2012c; El Sayed et al., 2013)**. Furthermore, in the developing rat brain, citrate aids in the synthesis of acetyl units at the synaptosomes **(Szutowicz et al., 1982)**. According to many reports, gliomas are fueled by glycolysis **(Oudard et al., 1997),** and they display the Warburg effect **(Wolf et al., 2010)**, which, even in the presence of oxygen, is the metabolic transition from oxidative phosphorylation to glycolysis., as well as enhanced glycolysis to produce ATP. The results of this investigation demonstrated that citrate-induced glioma cell death resulted in both apoptotic and necro-apoptotic cell death, which was dose- and time-dependent. It's interesting to note that citrate treatment caused the cleavage of caspase-3 to take place during citrate-induced glioma cell death **(El Sayed et al., 2018)**. This is consistent with the findings of Kruspig et al.'s report **(Kruspig et al., 2012)**, which suggested a connection between the citrate's prospective anticancer effects and the activation of caspase-3, apical caspases-8 and -2, poly-ADP-ribose polymerase breakage, and cytochrome c release. This study sheds light on the effects of the two early glycolysis substrates: glucose (substrate of hexokinase) and glucose-6-phosphate (substrate of PFK) on antagonizing cancer cell death induced by 3BP, a hexokinase inhibitor and citrate, a PFK inhibitor (figure 1). In this study, both 3BP (in micromolar range) and citrate (in millimolar range) exerted significant and maximal glioma cell death (figures 2-6), which was consistent to previously reported findings **(El Sayed et al., 2012a; El Sayed et al., 2012b; El Sayed et al., 2012c; El Sayed et al., 2017a; El Sayed et al., 2018)**. Figure 2-3 shows

that adding glucose or glucose-6-phosphate in successive dosages did not prevent 3BP-induced C6 glioma cell death. As a structural analogue of glucose-6-phosphate, citric acid is found naturally in citrus fruits (figure 4). Adding glucose in successive dosages could not prevent citrate-induced C6 glioma cell death (figure 5). However, the most interesting finding in this study was that citrate-induced glioma cell death was quite inhibited via adding serial doses of glucose-6-phosphate. In other words, glucose-6-phosphate antagonized citrate effects on glioma cell viability possibly via activating the glycolytic enzyme PFK. PFK is well-known to be inhibited by citrate (figure 6). This finding is quite important as citrate toxicity can be inhibited via adding glucose-6-phosphate.

Conflict of interest: The author declares that there is no conflict of interest with anyone.

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