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Biochemical and Pharmacological Effects of 3-bromopyruvate, Related Analogs and Some Antioxidants on Viability of Experimental Glioblastoma Cells: Towards Better Anticancer Effects (An Original Article)

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Abstract 3-bromopyruvate (3BP) is a promising anticancer drug that killed glioblastoma cells using different biochemical and pharmacological mechanisms e.g. inhibiting the glycolytic enzyme hexokinase II and inducing oxidative stress e.g. hydrogen peroxide generation. Glycolysis inhibition inactivates ABC transporters in cancer cells to restore the drug sensitivity in malignant cells. Moreover, the author and Japanese co-researchers proved that 3BP acts as an antimetabolite via antagonizing lactate (Warburg effect) and pyruvate and synergizing the anticancer effects of citrate. Interestingly, the author and Japanese co-researchers were the first to report that 3BP exerted potent anti-angiogenic effects. In this study, 3BP is structurally related to many different compounds that may affect its anticancer effects as pyruvate, lactate, acetic acid, L-alanine and beta-alanine. Author's experimental data revealed that administering L-alanine alone caused a significant dose-dependent decrease ($p<0.001$) in C6 glioblastoma cells' survival. This can be explained in light of the antioxidant merits exerted by the amino acid L-alanine. Unexpectedly, the structural analogs acetic acid, L-alanine and beta-alanine did not protect against 3BP-induced C6 glioma cell death. Treatment of C6 glioblastoma cells with hydrogen peroxide caused a significant glioblastoma cell death ($p<0.001$) that was significantly antagonized using the natural antioxidants e.g. pyruvate, reduced glutathione and N-acetyl L-cysteine (NAC) ($p< 0.001$). Lactate did not prevent or antagonize hydrogen peroxide-induced C6 glioma cell death. A combination of small doses of 3BP and citrate was synergistic in suppressing C6 spheroids viability. Moreover, 3BP induced C6 glioma protein depletion in a dose-dependent and time-dependent manner. Protein depletion was evident using SDS-PAGE and is thought to deprive the cancer cells from the necessary protein machineries for synthetic purposes. However, 3BP-induced protein depletion effects should be further studied on normal cells to prevent possible 3BP-induced side effects. Further studies are needed in this respect.

Keywords: *3-bromopyruvate, C6 glioblastoma, lactate, pyruvate, oxidative stress*

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1. Introduction

The most aggressive and widely invading brain cancers in human patients are glioblastoma multiform (GBM) tumors. Despite surgery, contemporary chemotherapy, and anti-angiogenesis therapies, GBM tumors quickly recur and end the lives of patients within a relatively short period of time. GBM tumors aggressively infiltrate and invade surrounding healthy brain tissue. Glycolysis (glucose oxidation to produce ATP and lactate in cancer cells) drives GBM cells [1,2,3]. Oxidative stress is a hallmark of cancer cells in addition to the Warburg effect (permanent conversion of glucose into lactate even in the presence of oxygen). In an early report, the author and Egyptian co-researchers stated that Warburg effect increases steady-state ROS condition in cancer cells through decreasing their antioxidant capacities. Based on that, a further explanation of the anticancer effects of

3-bromopyruvate (3BP) was reported [4]. Recently, the author reported that the origin of the Warburg effect to result from mitochondrial damage resulting in increased cytoplasmic NADH.H that drives lactate dehydrogenase enzyme to permanently convert pyruvate into lactate constituting the origin of the Warburg effect [5].

The hexokinase II inhibitor, 3-bromopyruvate (3BP) has strong anticancer properties. In terms of structure, lactate and pyruvate are structurally similar to 3BP and are transported by the same monocarboxylate transporters (MCT) as 3BP [6]. Structural similarity between pyruvate and lactate (glycolysis end products) and 3BP guided the author and Japanese co-researchers to report that that 3BP acts as an antimetabolite (antagonist) for both pyruvate and lactate as a novel mechanism of action of 3BP [6]. According to Otto Warburg's early, groundbreaking research, the end result of glycolysis in tumors, lactate, is a crucial molecule for cancer cells and is produced in large amounts. Even in the presence of oxygen, glycolysis continues to create ATP and lactate in cancer cells (Warburg effect) [7]. MCT1 is found to be expressed in C6 glioma [8] that is an experimental model for GBM growth and invasion [9]. Since lactate interferes with T lymphocytes' cell-mediated immunity, cancer cells are able to evade the immune system. Lactic acid significantly reduces the proliferation and cytokine synthesis of human cytotoxic T cells (95% suppression of cytokine production), which results in a loss of about 50% of cytotoxic T lymphocyte's cytotoxic activity [10]. According to Brizel et al. (2001), lactate increases the ability of cancer cells to metastasize by activating the interleukin-23-dependent and independent pathways [11], which significantly increases chronic inflammation in tumor's microenvironments [12].

Moreover, elevated lactate levels appear to be reliable predictors of the likelihood of tumor's spread and recurrence. In human cancer patients, high serum lactate severely reduces patient's survival [13]. The aggressive tumor microenvironment is mediated by lactate in order to continue glycolysis and live in this milieu so that they can spread within the host tissue, tumor cells reduce pyruvate to lactate and oxidize NADH to NAD+ [14]. Pyruvate significantly reduced the steady-state ROS situation in the C6 glioma, acting as an antioxidant in both cell-free and cell-based systems. The steady-state ROS situation in C6 glioma cells was unaffected by lactate. The author and Japanese co-researchers were the first to report that 3BP induces the production of H_2O_2. Due to the impact of 3BP, pyruvate is a powerful scavenger of endogenous and exogenous H_2O_2. In our previous reports, lactate lacked any antioxidant activity. In addition, lactate did not protect C6 glioma cells from the effects of H_2O_2 while pyruvate significantly protected C6 glioma cells from H_2O_2-induced glioma cell death [1].

According to Marin-Hernández et al. (2006), citrate inhibits phosphofructokinase (PFK), the second important enzyme in the glycolysis process. Citrate is a naturally occurring, harmless chemical found in citrus fruits [15]. Citrate's anticancer properties have been the subject of very little research. Citrate has recently been reported to be effective in treating many cancers such as mesothelioma, medullary thyroid carcinoma, gastric cancer, and antibiotic-resistant postoperative wounds in cancer patients [1]. Moreover, leukemia and lymphoma cell lines treated with citrate alone or in combination with chemotherapeutic agents exhibited a dose-dependent lympholytic activity. It's interesting to note that citrate had a little impact on healthy human peripheral blood mesenchymal cells [16].

In this study, the author aims to investigate new mechanisms of anticancer action of 3BP and its effects on protein depletion in cancer cells (GBM cells). The author also investigated the effects of glycolysis double inhibition on viability of C6 spheroids cells.

2. Materials and Methods

All chemicals were purchased from Sigma (St. louis, MO, USA) unless stated otherwise Dimethyl sulfoxide (DMSO), citrate, hydrogen peroxide, fetal bovine serum, 3-bromopyruvate, sodium L-lactate, sodium pyruvate, L-alanine, beta-alanine, citric acid and Dulbecco's modified Eagle's medium (DMEM), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylyltetrazolium bromide (MTT) were all obtained from Sigma (St. louis, MO, USA). Antibiotic solution of penicillin and streptomycin were provided by Invitrogen Life Technologies (Carlsbad, CA, USA). Bio-Rad laboratories (Hercules, CA) produced the Precision plus protein TM standard. Reduced glutathione originated in Kyoto, Japan's Nacalai tesque. Dojindo Molecular Technologies produced EDTA (Kumamoto, Japan). Sodium dodecyl sulfate, poly acrylamide and Immobilon-P Transfer Membrane were obtained from Millipore (Bedford, MA, USA).

2.1. C6 glioblastoma Culture

C6 rat glioma cells were maintained in DMEM containing 15% (v/v) horse serum, 2.5% (v/v) FBS, and 1% penicillin-streptomycin at 37°C in a humid environment containing 5% CO_2.

2.2. MTT Viability Assay

That was done using 96-well plates with C6 glioma cells seeded for 24 hours or until C6 cells reached 80% confluency. Then, fresh stimulating media (DMEM with 1% FBS) was used. The addition of pharmacological treatments to the stimulating media was done as indicated in the results section. A 21-hour incubation period was permitted using the CO_2 incubator. After adding MTT (50 µl of 1 mg/ml solution), the mixture was incubated for an additional 3-4 hours. DMSO addition (150 µl /well), centrifugation, and supernatant aspiration were immediately carried out. In order to ensure the best possible dissolution, the microplates were shaken in a microplate shaker until all of the insoluble formazan crystals had been completely dissolved. Readings of the absorbance values of all the wells were taken at 550 nm using a Biotek Synergy multimode microplate reader (VT, USA).

2.3. SDS-PAGE for Detecting Protein Depletion

In 100 mm Petri dishes, C6 cells were seeded and given time to confluence. Serial dosages of 3BP were administered to the cells for 24 hours in a fresh medium

(DMEM/F12 with 1% FBS). Cell pellets were aspirated, centrifuged, and added to the pellets of the corresponding dishes that had been scrapped. Pellets were maintained cold and lysed in a solution containing Complete Mini Protease Inhibitor Cocktail Tablets (20 mM Tris.Cl, pH 7.4, 137 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100, and 2 mM EDTA (Roche Diagnostics, Mannheim, Germany). Sonication was used to dislodge the cells, which were subsequently removed and centrifuged at 14,000 g for 20 min. SDS-PAGE analysis (blot analysis) was carried out under a lowering denaturing environment. The author used protein samples (40 g per lane). Protein samples were first put into a 12.5% polyacrylamide gel containing 0.1% SDS after being added to 4 X denaturing sample buffer, which contains the following ingredients: 200 mM Tris.Cl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, and 8% (v/v) 2-mercaptoethanol. SDS-PAGE was then carried out. An Immobilon-P Transfer Membrane (Millipore, Bedford, MA) was used to transfer the proteins. The membrane was photographed using a digital camera.

2.4. *In vitro* Glioblastoma Tumor Model (spheroids)

As previously reported [17], C6 glioma spheroids were created as *in vitro* tumor models in 96-well plates. The author prepared and heated 1.5% agar in autoclaved distilled water in a lab setting. Under strictly aseptic conditions, 100 µl of agar was pipetted onto each plate using a multichannel pipette. A suspension of about 10,000 C6 glioma cells was placed in 200 µl of nutrient medium. C6 glioma cells were grown in 96-well plates that had been covered in an agar layer before being incubated for 96 hours. A fresh nutrient medium with the same amount of treatment in the form of citrate (3 and 5 mM) and/or 3BP (15 and 30 µM) was used to replace half of the medium in each well. Treatment-containing medium was incubated for 72 hours. Spheroid viability was assessed after treatment by adding MTT solution (1 mg/ml) and incubating that for an additional 3-4 hours. After centrifugation, 150 µl of DMSO/plate was added to dissolve the insoluble formazan crystals. The supernatant was then slowly and carefully aspirated from each well (without touching the spheroids). By measuring the absorbance at 550 nm using a Biotek Synergy multimode microplate reader (VT, USA), the viability of tumor spheroids cells was calculated.

3. Results

3.1. Biochemical Analogs of Pyruvate May Exert Certain Pharmacological Effects (Figure 1)

Pyruvate is an analog of both the anticancer drug 3BP and lactate, the end product of glucose catabolism. Substituting one hydrogen atom in (–CH3) side chain of pyruvate by one bromine atom gives the anticancer drug 3BP. Adding two hydrogen atoms to pyruvate (usually via the activity of the glycolytic enzyme LDH) gives lactate (responsible for Warburg effect) in tumors. Subtracting two hydrogen atoms from lactate (usually via the activity of the glycolytic enzyme LDH) gives pyruvate. Acetate (a natural organic acid in vinegar), alanine (an amino acid) and beta alanine are also structural analogs of pyruvate. I herein investigated the possible effects of such analogs against 3BP-induced anticancer effects on C6 glioblastoma cells.

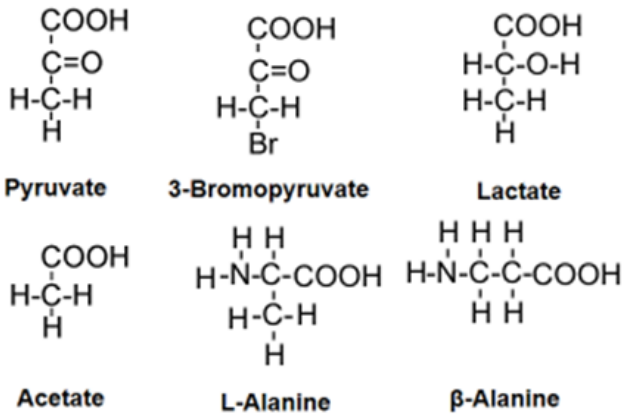

Figure 1. Some important structurally-related compounds to pyruvate and lactate

3.2. Effects of L-alanine on the Viability of C6 Glioblastoma Cells (Figure 2)

Using an MTT viability assay, serial doses of L-alanine (in millimolar range) exerted a significant decrease in the viability of C6 glioblastoma cells. 10 mM L-alanine caused a significant reduction in C6 glioblastoma cells' viability ($p < 0.05$) that was dose-dependent till reaching about 30% reduction on viability upon using a high dose of L-alanine (100 mM) ($p<0.001$).

3.3. Effects of Acetate, L-alanine and β-alanine on C6 glioma Cells Death Induced by 3-bromopyruvic Acid (Figure 3)

A high dose of 3BP (100 µM) caused a significant ($p< 0.001$) and maximal C6 glioblastoma cell death (>90%). A relatively high concentration of acetate (20 mM) did not protect C6 glioma cells against 3BP effects. The same effect was obtained upon using serial doses (20 and 100 mM) of both L-alanine and beta alanine. Both did not protect C6 glioma cells against 3BP-induced death.

3.4. Effects of Pyruvate, Lactate, Reduced Glutathione (GSH) and N-acetyl L-cysteine on C6 Glioma Cells Death Induced by Hydrogen Peroxide (Figure 4)

Exogenous hydrogen peroxide treatment (500 mM) was given to cultured C6 glioblastoma cells causing decreased viability by about 70%. Adding serial concentrations (10, 50 and 100 mM) of sodium pyruvate significantly protected the viability of C6 glioma cells ($p < 0.001$). On the contrary, adding serial concentrations (10, 50 and

100 mM) of sodium lactate did not protect the viability of C6 glioma cells. Adding a relatively low concentration of GSH and NAC significantly and maximally protected the viability of C6 glioma cells ($p < 0.001$).

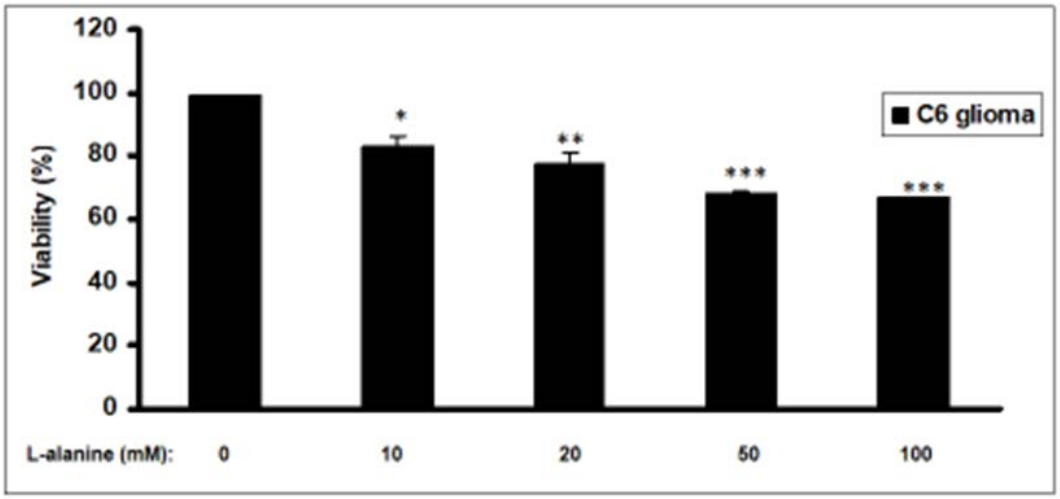

Figure 2. L-alanine induces a significant but weak dose-dependent loss of C6 glioma viability

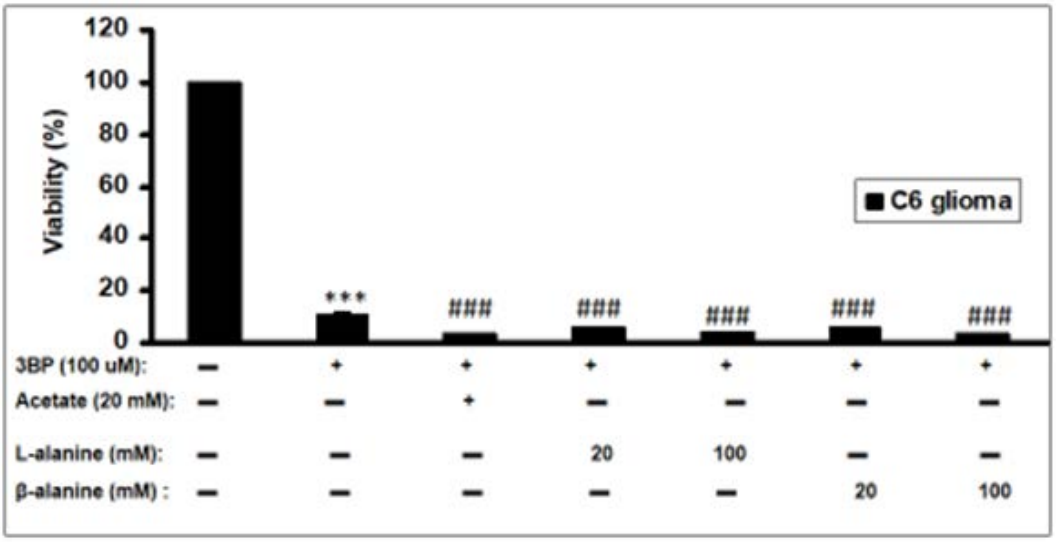

Figure 3. Acetate, L-alanine and β-alanine did not protect C6 glioma cells' viability against the anticancer effects of 3-bromopyruvic acid

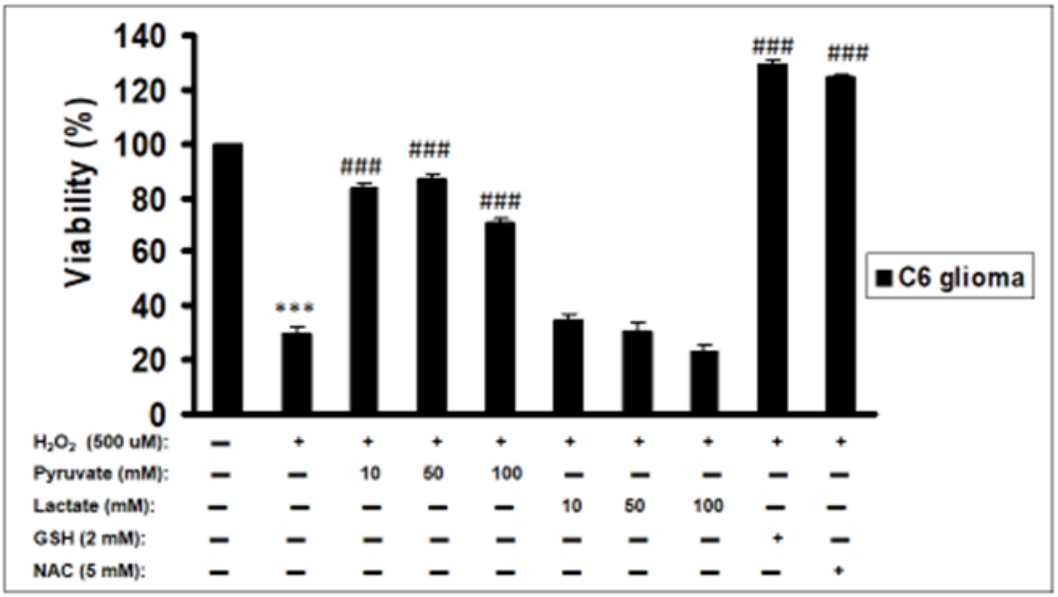

Figure 4. Pyruvate, not lactate, significantly protected the viability of C6 glioma cells treated with exogenous H_2O_2

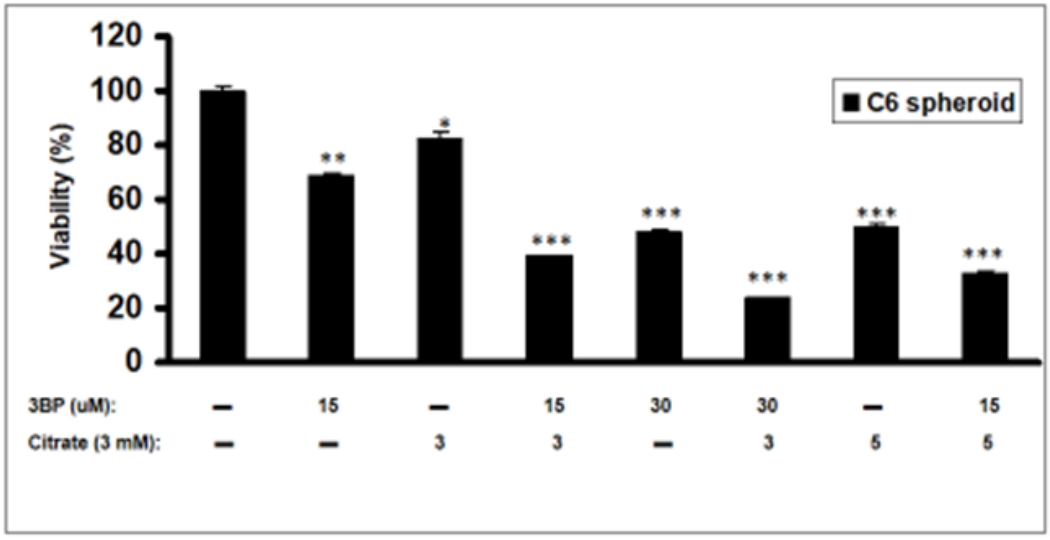

Figure 5. Effects of glycolysis double inhibition (3BP+ citrate) on glioma spheroid cells' viability

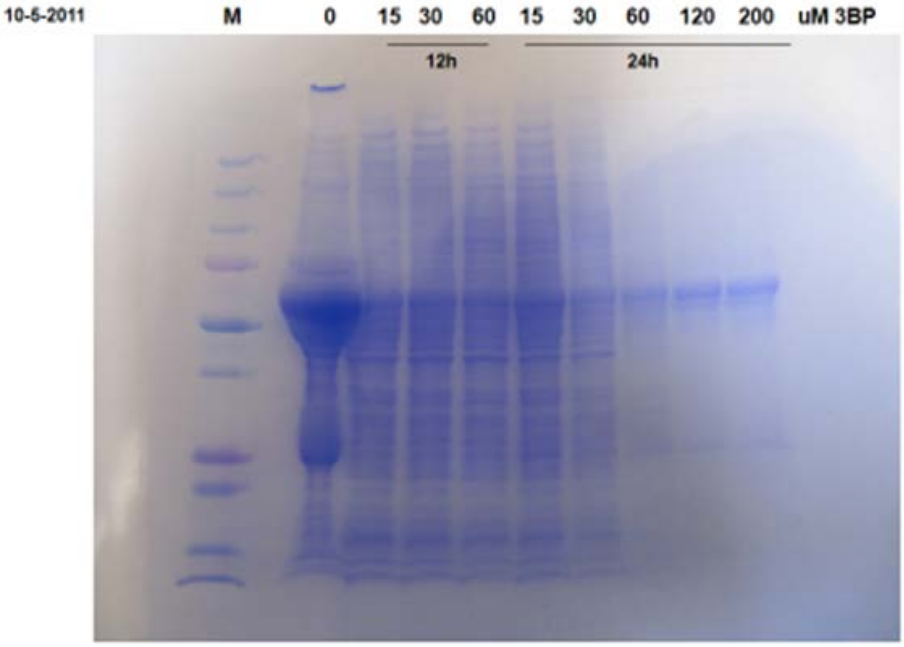

Figure 6. 3BP induced-glioma protein depletion is dose and time-dependent

3.5. Effects of Glycolysis Double Inhibition on C6 Spheroids Viability (Figure 5)

C6 glioma spheroids were allowed to grow in vitro and were allowed to receive serial doses of 3BP (15 and 30 µM) and citric acid (3 and 5 mM) alone and in combination. 3BP alone (15 and 30 µM) caused a 30% and 50% decrease in C6 glioma spheroids viability ($p<0.01$ and $p<0.001$), respectively. Citric acid alone (3 and 5 mM) caused a 20% and 50% decreases in C6 glioma spheroids viability ($p< 0.05$ and $p< 0.001$), respectively.

3.6. 3BP Induced Dose-dependent and Time-dependent Protein Depletion (Figure 6)

Using SDS-PAGE, 3BP induced a dose-dependent and time-dependent protein depletion in C6 glioblastoma cells. C6 glioma cells treated with serial doses of 3BP (15, 30 and 60 µM) exhibited a notable decrease in protein content run on SDS-PAGE. The higher the 3BP dose, the more the protein depletion. C6 glioma cells treated with serial doses of 3BP (15, 30 and 60 µM) for 24 hours exhibited more protein depletion than C6 glioma cells treated with serial doses of 3BP (15, 30 and 60 µM) for 12 hours.

3.7. Statistical Analysis

Results are presented as the mean ± standard error of the mean of the values obtained from at least three different experiments. Student's t test was used to examine the significance of groups' differences. *, **, and ***, respectively denote significant differences at $p<0.05$, $p<0.01$, and $p<0.001$ versus the control. #, ## and ### indicate significance of differences levels of $p<0.05$, $p<0.01$, and $p<0.001$ among different treatment conditions.

4. Discussion

3BP is a promising anticancer drug that killed glioblastoma cells using different biochemical and pharmacological mechanisms e.g. inhibiting the glycolytic enzyme hexokinase II and inducing oxidative stress e.g. hydrogen peroxide generation [2]. Glycolysis inhibition inactivates ABC transporters to restore the drug sensitivity in malignant cells [18]. Moreover, the author and Japanese co-researchers proved that 3BP acts as an antimetabolite via antagonizing lactate (Warburg effect) [19], pyruvate and synergizing the anticancer effects of citrate [1]. In addition, 3BP exerted potent anti-angiogenic effects and impaired the blood vasculature formation that is highly needed by cancer cells [3]. Other important anticancer antimetabolites include also dichloroacetate [19,20].

Interestingly, 3BP is structurally related to many different compounds that may affect its anticancer effects as pyruvate, lactate, acetic acid, L-alanine and beta-alanine (Figure 1). However, the most important are pyruvate (end product of glucose metabolism in normal cells in the presence of oxygen) and lactate (end product of glucose metabolism in normal cells in the absence of oxygen). Lactate is also the end product of glucose metabolism in cancer cells even in the presence of oxygen (Warburg effect). Lactate was reported to exert many beneficial effects to cancer cells e.g. enhancing angiogenesis, metastasis, chemoresistance, radioresistance, and invasion [4]. Unfortunately, administering 3BP to human patients using intravenous injection is very painful and was associated with venous phlebitis [21]. Another practical problems facing 3BP in clinical oncology is its fast catabolism using glutathione conjugation that impairs its use [22]. In a recent expert opinion, the author suggested formulating 3BP inside nanocarriers as liposomes to facilitate a non-painful intravenous intake of 3BP and to avoid 3BP loss during enhanced permeability and retention effect (PER effect) [23].

Administering L-alanine alone caused a significant dose-dependent decrease in C6 glioblastoma cell survival (Figure 2). This interesting outcome carries a lot of hope in guiding the nutritional management to patients having GBM. This can be explained in light of the antioxidant merits exerted by the amino acid L-alanine. Unexpectedly, the structural analogs acetic acid, L-alanine and beta-alanine did not protect against 3BP-induced C6 glioma cell death (Figure 3). Again, this is of quite practical importance in that glioblastoma patients receiving 3BP treatment can also eat diets rich in acetic acid, L-alanine and beta-alanine without facing any antagonistic effects to 3BP.

Treatment of C6 glioblastoma cells with hydrogen peroxide caused a significant glioblastoma cell death ($p<0.001$) that was significantly antagonized using natural antioxidants e.g. pyruvate, reduced glutathione and N-acetyl L-cysteine (NAC) ($p<0.001$). Lactate did not prevent or antagonize hydrogen peroxide-induced C6 glioma cell death (Figure 4). This confirms a significant biochemical difference between pyruvate and lactate i.e. pyruvate is an antioxidant while lactate is a pro-oxidant (lacking antioxidant functions). This is in quite agreement with our early report [1] and our recent report [5]. The author and Japanese co-researchers reported that pyruvate scavenged exogenous hydrogen peroxide in a cell-free system while lactate did not [1]. Figure 4 confirms that finding also in a cell-containing system. Both reports confirm the expert opinion recently reported by the author where lactate (Warburg effect) increases the steady-state ROS condition in cancer cells [4] and the published work that antioxidants are the guardians and gatekeepers against oxidative stress-induced hepatocarcinogenesis [24]. Glycolysis double inhibition and triple inhibition were novel terms introduced by the author's research group and were reported in a published work with Japanese co-researchers [1].

Spheroids are 3-dimensional tumor model but lacking a vasculature. A combination of small doses of 3BP and citrate was synergistic in suppressing spheroids viability (Figure 5). In this study, C6 GBM cells were grown and subjected to glycolysis inhibitor treatment. Citrate is a naturally occurring substance that is widely found in citrus fruits and has several medicinal purposes [1,2,3], such as the treatment of urate kidney stones. Recently, it was discovered that citric acid can prevent myocardial ischemia/reperfusion injury. Citrate, a glycolytic inhibitor of phosphofructokinase, was fatal to human glioblastoma cell lines when administered in series at comparatively high dosages (in millimolar range). Utilizing the combination drug index, low effective dosages of 3-bromopyruvate (15 and 30 µM) and citrate (3 and 5 mM) had a synergistic anticancer effect. Citrate and 3BP exhibited considerable synergism, as indicated by the combination index, which was less than 1 (Figure 5).

Interestingly, this study confirmed a relatively recent anticancer mechanism of action of 3BP that is protein depletion in a dose-dependent and time-dependent manner (Figure 6). Protein depletion was evident using SDS-PAGE and deprives cancer cells from necessary protein machineries for synthetic purposes. 3BP-induced protein pyruvylation [22,25] may be the underlying mechanism beyond 3BP-induced protein depletion. However, 3BP-induced protein depletion effects should be further studied on normal cells to prevent possible 3BP-induced side effects. Further studies are needed in this respect.

5. Conclusion

3BP is a promising anticancer agent that synergizes with citric acid for killing glioblastoma spheroids for better anticancer effects at lower doses using both drugs. 3BP is structurally similar to both lactate and pyruvate. Pyruvate (but not lactate) significantly prevented hydrogen peroxide-induced C6 glioblastoma cell death on "antioxidant versus oxidant" bases but not on structural bases. Likewise, structural similarity of 3BP to acetate, L-alanine and beta-alanine did not influence 3BP-induced cancer cells' death. 3BP induces a dose-dependent and time-dependent protein depletion in cancer cells.

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Conflict of Interest

The author reported no conflict of interest with anyone.

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