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3-Bromopyruvate antagonizes effects of lactate and pyruvate, synergizes with citrate and exerts novel anti-glioma effects

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Abstract Oxidative stress-energy depletion therapy using oxidative stress induced by D-amino acid oxidase (DAO) and energy depletion induced by 3-bromopyruvate (3BP) was reported recently (El Sayed et al., Cancer Gene Ther., 19, 1–18, 2012). Even in the presence of oxygen, cancer cells oxidize glucose preferentially to produce lactate (Warburg effect) which seems vital for cancer microenvironment and progression. 3BP is a closely related structure to lactate and pyruvate and may antagonize their effects as a novel mechanism of its action. Pyruvate exerted a potent H_2O_2 scavenging effect to exogenous H_2O_2, while lactate had no scavenging effect. 3BP induced H_2O_2 production. Pyruvate protected against H_2O_2-induced C6 glioma cell death, 3BP-induced C6 glioma cell death but not against DAO/D-serine-induced cell death, while lactate had no protecting effect. Lactate and pyruvate protected against 3BP-induced C6 glioma cell death and energy depletion which were overcome with higher doses of 3BP. Lactate and pyruvate enhanced migratory power of C6 glioma which was blocked by 3BP. Pyruvate and lactate did not protect against C6 glioma cell death induced by other glycolytic inhibitors e.g. citrate (inhibitor of phosphofructokinase) and sodium fluoride (inhibitor of enolase). Serial doses of 3BP were synergistic with citrate in decreasing viability of C6 glioma cells and spheroids. Glycolysis subjected to double inhibition using 3BP with citrate depleted ATP, clonogenic power and migratory power of C6 glioma cells. 3BP induced a caspase-dependent cell death in C6 glioma. 3BP was powerful in decreasing viability of human glioblastoma multiforme cells (U373MG) and C6 glioma in a dose- and time-dependent manner.

Keywords D-amino acid oxidase · 3-bromopyruvate · Pyruvate · Lactate · Citrate · Glycolysis double inhibition

Abbreviations

3BP	3-bromopyruvate
ABC transporters	ATP-binding cassette transporters
ATP	Adenosine triphosphate
DAO	D-amino acid oxidase
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
LDH	Lactate dehydrogenase
MCTSs	Multicellular tumor spheroids
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylyltetrazolium bromide
NAC	N-acetyl-L-cysteine
NADH	Reduced nicotinamide adenine dinucleotide

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NAD+	Oxidized nicotinamide adenine dinucleotide
PFK	Phosphofructokinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis gel

Introduction

Oxidative stress-energy depletion therapy was recently reported by our laboratory as a new modality for cancer treatment using oxidative stress induced by D-amino acid oxidase (DAO) and energy depletion induced by antiglycolytic agents e.g. 3-bromopyruvate (3BP) (El Sayed et al. 2012). DAO is a promising therapeutic protein with potent anticancer effects. DAO induces oxidative stress through generation of hydrogen peroxide (H_2O_2) (Kawazoe et al. 2007). Our laboratory reported other important roles of DAO in disease pathophysiology through using wild-type DAO (Abou El-Magd et al. 2010a) and the mutant DAO (Abou El-Magd et al. 2010b).

3BP is a hexokinase II inhibitor with potent anticancer effects. Lactate and pyruvate are closely related in structure to 3BP and are also transported via the monocarboxylate transporters (MCT) as 3BP (Zorzano et al. 2000). Lactate is a vital metabolite for cancer cells that is produced enormously in tumors as an end product of glycolysis as reported in the early pioneering work of Otto Warburg (Warburg 1956). We hypothesize here that the close structural similarity between 3BP and lactate together with their common transporters (MCT), may antagonize lactate effects and deprive tumor cells of benefits of lactate e.g. lactate influx through the endothelial cell monocarboxylate transporter (MCT1) is reported to enhance tumor angiogenesis through an NF-κB/IL-8 pathway (Végran et al. 2011). Angiogenesis is critical for tumor growth, metastasis and prognosis for patient survival. Increased survival was reported in patients with glioblastoma multiforma (GBM) responding to anti-angiogenesis treatment (Sorensen et al. 2011). MCT1 is reported to be expressed in C6 glioma (Mac and Nałecz 2003) that is an experimental model for GBM growth and invasion (Grobben et al. 2002).

Lactate allows cancer cell evasion from the immune system as lactate disturbs cell-mediated immunity exerted by T lymphocytes. Lactic acid markedly lowers the function of human cytotoxic T lymphocytes (CTLs) through decreasing their proliferation and cytokine production (95% suppression of cytokine production) and leads to loss of about one half of the cytotoxic activity of CTLs (Fischer et al. 2007). Lactate enhances the metastatic potential in cancer cells (Brizel et al. 2001), activates IL-23-dependent and independent pathways, leading to marked increase in chronic inflammation in tumor microenvironments (Yabu et al. 2011). Moreover, high lactate levels seem to be good prognostic factors for possibility of metastasis and recurrence of tumors. High lactate significantly restricts patient survival in human cancer (Walenta et al. 2004). Lactate mediates the aggressive microenvironment of tumors. Tumor cells use reduction of pyruvate to lactate to oxidize NADH to NAD+to maintain glycolysis to allow tumor cells to survive in this microenvironment and metastasize within the host tissue (Gatenby and Gawlinski 2003; Gatenby et al. 2006; Mathupala et al. 2006; Fantin et al. 2006). Although production of pyruvate from glucose is a step immediately before production of lactate, metabolism in cancer cells continues to produce lactate which carries many advantages for cancer cells.

The altered metabolism in tumors is under the control of hypoxia, oncogenes and tumor suppressor genes. Human cancers express an upregulated *myc* oncogene which leads to upregulation of glycolytic enzymes like lactate dehydrogenase (LDH-A) (Shim et al. 1997). Frequent mutations in cancer cells lead to genetic alterations e.g. loss of p53 which causes decreased oxygen consumption and increased lactate production (Matoba et al. 2006). Pyruvate is also an end product of glycolysis and a scavenger of free radicals which exerts a cytoprotecting antioxidant effect on neurons (Desagher et al. 1997).

Citrate is an inhibitor of phosphofructokinase (PFK), the 2nd key enzyme of glycolysis pathway (Marín-Hernández et al. 2006). Citrate is a safe natural substance available in citrus fruits. Research related to the anticancer effects of citrate is very few. Recently, citrate was reported in treating mesothelioma (Zhang et al. 2009), medullary thyroid carcinoma (Halabe Bucay 2009), gastric cancer (Lu et al. 2011) and treatment of antibiotic-resistant postoperative wounds of cancer patients (Nagoba et al. 2011). Moreover, citrate alone or combined with chemotherapeutic drugs induced a dose-dependent lympholytic activity in leukemia and lymphoma cell lines. Interestingly, citrate had minimal effects on human normal peripheral blood mesenchymal cells (Yousefi et al. 2004). A novel combination between PFK inhibitor, citrate and serial doses of 3BP (glycolysis double inhibition) is expected to block the first 2 key enzymes of glycolysis pathway and may carry better therapeutic benefits and lower side effects than either alone.

In this study, we investigated the possibility of novel mechanisms of action of 3BP related to functional effects of 3BP versus lactate especially as it regards cancer cell survival and migration. 3BP effects versus pyruvate, differences between effects of lactate and pyruvate on cancer cells, and the effect of glycolysis double and triple inhibition on the viability of the C6 glioma were also studied.

Materials and methods

Reagents and chemicals

Sodium acetate was purchased from Katayama Chemical Industries (Osaka, Japan). Citrate, H_2O_2, D-serine, Dimethyl sulfoxide (DMSO) and agar were purchased from Wako (Osaka, Japan). Sodium L-lactate, pyruvic acid (sodium salt), sodium fluoride (NaF), 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 from Sigma (St. louis, MO, USA). DMEM/F12 and penicillin-streptomycin antibiotic mixture were from Invitrogen life technologies (Carlsbad, CA, USA). Precision plus protein™ standard was from Bio-Rad laboratories (Hercules, CA). Reduced glutathione was from Nacalai tesque (Kyoto, Japan). EDTA was from Dojindo molecular technologies (Kumamoto, Japan). Rabbit polyclonal anti-caspase-3 antibody was from Santa Cruz biotechnology (CA, USA).

Cell culture

Maintenance of C6 rat glioma cells (Dainippon Pharmaceutical Co., Osaka, Japan) was 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% CO_2. C6/DAO cells were established as previously reported (Park et al. 2006). Maintenance of C6/DAO cells was in DMEM/F12 containing 15% (v/v) horse serum, 2.5% (v/v) FBS and 1% penicillin-streptomycin with 250 μg/ml G418. U373MG human glioblastoma cell line was maintained in DMEM containing 10% (v/v) horse serum, and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2.

MTT assay

C6 glioma was seeded into 96-well plates for 24 h until cells reached 80% confluency. Nutrient medium was replaced with fresh stimulating medium (DMEM/F12 containing 1% FBS). Treatment was added on stimulating medium. Incubation in a CO_2 incubator was allowed for 21 h. MTT (50 μl of 1 mg/ml) solution was added and followed by incubation for an additional 3–4 h. Centrifugation, supernatant aspiration and DMSO addition (150 μl/well) were done. Complete dissolution of insoluble formazan crystals was achieved and plates were shaken in a microplate shaker for optimal dissolution. Absorbance at 550 nm using an Infinite™ M200 microplate reader was monitored.

SDS-PAGE and protein immunobloting

C6 cells were seeded in 100 mm Petri dishes and allowed to reach confluency. Cells received treatment in the form of serial doses of 3BP in a fresh medium (DMEM/F12 containing 1% FBS) for 24 h. Medium was aspirated, centrifuged and cell pellets were added to pellets of corresponding dishes which were collected by scraping. Pellets were kept on ice and lysed using a radioimmunoprecipitation assay (RIPA) lysis buffer (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, 1 mM PMSF and 2 mM EDTA) containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by sonication, extracted at 4 °C for 30 min and then centrifuged at 14,000 g for 20 min. SDS-PAGE and western analyses blot analyses were done under reducing denaturing conditions. Protein samples (40 μg per lane) were used. Protein samples were added to 4 X denaturing sample buffer (200 mM Tris.Cl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 8% (v/v) 2-mercaptoethanol) and then protein samples were loaded in 12.5% polyacrylamide gel containing 0.1% SDS. SDS-PAGE was performed. Proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA). The membranes were incubated with rabbit polyclonal anti-caspase-3 antibody (1:200). Detection of protein was carried out using ECL Advance Western blotting detection systems (Amersham Biosciences, Little Chalfont, UK) according to manufacturer's instructions.

H_2O_2 assay

Using the Amplex® red H_2O_2 assay kit (Molecular probes, invitrogen, CA, USA), estimation of H_2O_2 was carried out according to manufacturer's instructions. H_2O_2 was estimated in cell-free media treated with exogenous H_2O_2 (250 μM) to investigate the effect of pyruvate and lactate in scavenging H_2O_2. H_2O_2 assay was carried out also after treating cultured C6 cells with serial doses of pyruvate and lactate to investigate the effects of pyruvate and lactate on ROS-steady state. 3BP-induced H_2O_2 production was estimated using the same kit to investigate the effect of pyruvate and lactate on scavenging 3BP-induced H_2O_2 production.

ATP assay

Energetics of C6 glioma were determined 24 h after treatment using an ATP determination kit from Molecular probes (Eugene, OR, USA) according to manufacturer's instructions. C6 cells were seeded at a density of 1×10^4 cells/well into 96-well plates followed by incubation for 24 h. Treatment was given using fresh medium (DMEM/F12 containing 1% FBS) for 24 h. Medium was removed followed by addition of the ATP standard reaction solution with protection from light. ATP levels were estimated in C6 cells using

the luminometer function of Infinite™ M200 microplate reader.

Clonogenic assay

Under complete aseptic conditions, C6 cells (1×10^3 cell/plate) were seeded in 6 cm plates in nutrient medium. Plates were shaken gently for even distribution. Plates were incubated in a CO_2 incubator for 10 h. Cells received treatment in the form of 3BP, citrate and a combination of both. Cells were incubated in CO_2 incubator. Daily follow up using Nikon phase contrast light microscopy was done until colonies in control plates form 50 cells or more. Aspiration of medium was carried out followed by careful washing of plates gently using 1X PBS with avoidance of disturbing attached colonies to the base of plates. Cells were fixed with 100% methanol for 15 min. Methanol was aspirated and plates were stained with 0.5% crystal violet (in 2% ethanol) for 30 min at room temperature. Crystal violet was removed carefully and plates were rinsed with tap water carefully and air dried at room temperature. Colonies per plate were counted. Plates were photographed by digital camera.

In vitro glioma tumor model

In vitro tumor models (C6 glioma spheroids) in 96-well plates were established as previously reported (Friedrich et al. 2009). Briefly, 1.5% agar in autoclaved distilled water was prepared and heated in an oven. Agar (100 μl) was poured per plate using a multichannel pipette under complete aseptic conditions. C6 glioma cells (1×10^4) were suspended in 200 μl nutrient medium. Glioma cells were seeded in 96-well plates previously coated with an agar layer followed by incubation for 96 h. Half of the medium per well was replaced by the same amount of fresh nutrient medium containing treatment in the form of citrate (3 and 5 mM) and/or 3BP (15 and 30 μM). Medium containing treatment was incubated for 72 h. For assaying spheroid viability after treatment, MTT solution (1 mg/ml) was added and followed by incubation for an additional 3–4 h. After centrifugation, the supernatant was aspirated slowly and carefully from each well (without touching spheroids) and 150 μl of DMSO/plate was added to dissolve the insoluble formazan crystals. Viability of cells in tumor spheroids was estimated be measuring absorbance at 550 nm using an Infinite™ M200 microplate reader.

Migration assay (wound healing assay)

Effect of pyruvate and lactate on metastatic migratory power of the C6 glioma was investigated in an in vitro model. Using the wound healing migration assay (Shankardas et al. 2010), the effect of 3BP on inhibiting migratory power was investigated. Effect of 3BP on inhibiting migratory power against the effect of pyruvate and lactate was also investigated. Combinatory effect of both 3BP and citrate on inhibiting the migratory power of the C6 glioma was also investigated. Briefly, C6 cells were seeded at a density of 1 $X10^6$ cells per well in 6 well plates and cells were incubated in a CO_2 incubator for about 24 h until cells reached 90–95% confluency. Marker lines were drawn along the bottom of the plates. Using a yellow P200 pipette tip, three parallel scratch wounds were made perpendicular to the marker lines. Washing with 1 X PBS was done once carefully and gently to remove scratched cells without disturbing both sides of the wound. Immediate photographs were taken using Nikon phase contrast microscopy (Nikon, Tokyo, Japan) attached to a screen. This procedure made it possible to image the entire width of the wound using a 4× objective. The wounds were observed using a phase contrast inverted microscope (Nikon, Tokyo, Japan). Images were taken at 12 h and 24 h of both areas flanking the intersections of the wound and the marker lines (at least 8 images per experimental condition). Migratory distances and images were analyzed by digitally drawing imaginary lines (using Image J software) averaging the position of the migrating cells at the wound edges. The cell migration distance was determined by measuring the area of the wound under different experimental conditions in comparison with the control.

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. † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ indicate significance within the same treatment condition.

Results

Pyruvate and lactate are structural analogs of 3BP (Fig. 1a). Based on structural similarity between 3BP and both pyruvate and lactate, we hypothesized that pyruvate and lactate may influence 3BP-induced anticancer effects. As lactate is a key metabolite produced through the glycolytic phenotype of cancer cells even in the presence of oxygen (Warburg effect), lactate may carry important beneficial advantages for cancer cells. Lactate is reported to play a major role in cancer progression and metastasis (Fischer et al. 2007; Brizel et al. 2001; Yabu et al. 2011; Walenta et al. 2004). We hypothesized also that 3BP may antagonize the Warburg effect through antagonizing the effects of lactate and depriving cancer cells of lactate-based effects.

Pyruvate scavenged exogenous and endogenously produced H_2O_2 while lactate did not

First, we investigated effects of pyruvate and lactate on scavenging H_2O_2 in a cell- free system through using the sensitive Amplex® red hydrogen peroxide assay kit. Our data revealed that pyruvate, but not lactate, significantly scavenged H_2O_2 in a cell-free system ($p<0.001$) (Fig. 1b). Cancer cells are undergoing increased steady-state ROS conditions in comparison with normal cells (Aykin-Burns et al. 2009). In cancer cells lactate is produced intracellularly from glucose catabolism even in the presence of oxygen (Warburg effect) and then extruded to the extracellular space. As a simulation to the high lactate content in tumors (Walenta et al. 2004), we studied the effect of serial doses of exogenous lactate and its analog, pyruvate on the ROS-steady state condition of the C6 glioma cells cultured in 96-well plates. ROS steady-state was estimated using the same kit. Our data revealed that pyruvate reduced significantly the steady-state ROS levels of the C6 glioma ($p<0.001$) while lactate did not (Fig. 1c). The metabolism of cancer cells in the direction of production of lactate may help cancer cells in maintaining the increased steady-state ROS levels. Conversion of most of the pyruvate as an end product to lactate -according to the Warburg effect- seems to deprive cancer cells from the antioxidant effect of pyruvate.

As 3BP can induce significant generation of H_2O_2 (El Sayed et al. 2012), we hypothesized that this may be related to the property of 3BP to decrease production of pyruvate in the glycolysis pathway (by acting as an upstream inhibitor to pyruvate formation step in the glycolytic pathway) which deprives cancer cells from its antioxidant function. In addition to that, we hypothesized that 3BP may exert an antagonistic function to pyruvate and lactate (based on structural similarity) (Fig. 1a) which may further explain the oxidative stress effect induced by 3BP. We investigated the effect of pyruvate and lactate on 3BP-induced generation of H_2O_2. We did the experiments both as a co-treatment of 3BP with pyruvate and lactate and as a pretreatment of pyruvate and lactate followed 30 min later by treatment with 3BP. Our data revealed that the amount of H_2O_2 generated by 3BP was significantly decreased ($p<0.001$) by the presence of exogenous pyruvate. Similar results were obtained in the co-treatment condition as in the pretreatment condition. The pyruvate-mediated scavenging effect on 3BP-induced H_2O_2 generation was significant ($p<0.001$) while lactate did not have any scavenging effect on 3BP-induced H_2O_2 generation (Fig. 1d) even though lactate is a structural analog of pyruvate

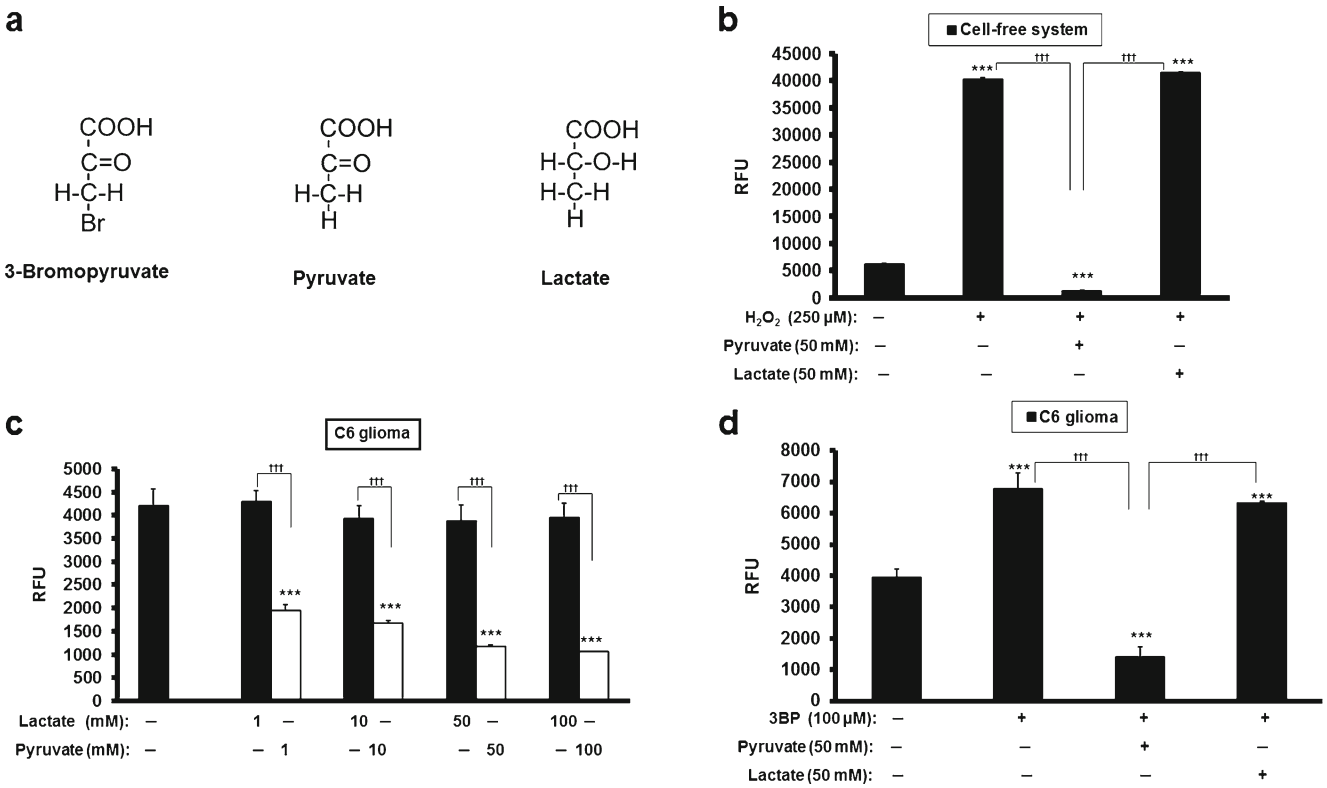


Fig. 1 Effect of pyruvate and lactate on exogenous and endogenously produced H_2O_2. **a** Pyruvate and some important structurally-related compounds. **b** Effect of pyruvate and lactate on scavenging H_2O_2 in cell-free system **c** Effect of pyruvate and lactate on steady-state ROS levels in C6 glioma. **d** Effect of pyruvate and lactate on 3BP-induced H_2O_2 production. Data are mean ± SEM of 3 independent experiments. ***$p<0.001$ indicate significance between control and treatment conditions. ††† $p<0.001$ indicates significance between treatment conditions within the same group

and a glycolysis downstream product. Pyruvate deficiency (due to conversion to lactate according to Warburg effect) may lower the antioxidant power of cancer cells. Previously, we reported also that antioxidants e.g. NAC and GSH protected significantly against 3BP-induced C6 glioma cell death (El Sayed et al. 2012).

Pyruvate protected against C6 glioma cell death induced by exogenous H_2O_2 but with no protection against DAO/D-serine-induced C6/DAO cell death

Then, we investigated the effect of pyruvate as a scavenger of H_2O_2 in protecting C6 glioma cells treated with exogenous H_2O_2. C6 glioma cells were treated with serial doses of pyruvate, lactate, acetate and GSH (2 mM) followed 30 min later by treatment with exogenous H_2O_2 (500 μM). Our data revealed that pyruvate and GSH significantly protected C6 glioma cells against H_2O_2-induced glioma cell death ($p<0.001$). Acetate and lactate had no protecting effects against H_2O_2-induced C6 glioma cell death. Similar results were obtained when cells received the above mentioned treatment as a co-treatment (Fig. 2a).

Unexpectedly, although DAO/D-serine can induce significant H_2O_2 production in C6/DAO glioma cells (Park et al. 2006) causing significant cell death that is protected by antioxidants as edaravone (Chung et al. 2010), pyruvate did not protect C6/DAO cells against DAO/D-serine-induced glioma cell death (Fig. 2b). Instead, pyruvate increased significantly the DAO/D-serine-induced cell death ($p<0.001$). Lactate had no effect on DAO/D-serine-induced C6/DAO cell death (Fig. 2b).

Pyruvate and lactate exert antagonistic effects with 3BP as it regards an effect on C6 viability

To elucidate how important is pyruvate for cellular metabolism especially energy generating pathways in cancer cells, sources and fate of pyruvate were listed (Fig. 3a and b). Pyruvate occupies a central position in metabolism and seems vital in energy generating pathways.

As mentioned above, 3BP is structurally related to pyruvate. At the same time, 3BP exerts an oxidant effect which is antagonistic to the antioxidant effect exerted by pyruvate. We hypothesized that 3BP may exert an antagonistic effect to

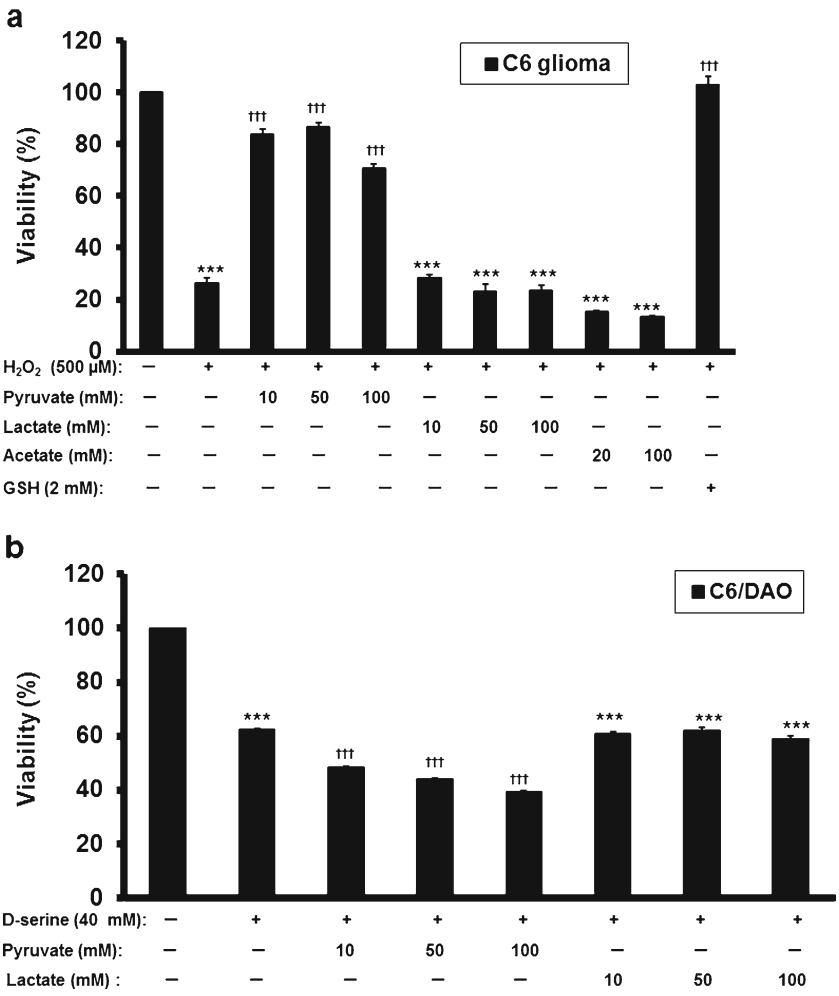


Fig. 2 Effect of pyruvate and lactate on viability of C6 glioma treated with exogenous H_2O_2 and DAO/D-serine. **a** Effect of pyruvate, lactate, acetate and GSH on C6 glioma cell death induced by exogenous H_2O_2. **b** Effect of pyruvate and lactate on viability of C6/DAO glioma treated with D-serine. Data are mean ± SEM of 3 independent experiments. *** $p<0.001$indicate significance between control and treatment conditions. ††† $p<0.001$ indicates significance between 500 μM H_2O_2 and other treatment groups. ††† $p<0.001$ also indicates significance between 40 mM D-serine and other treatment groups

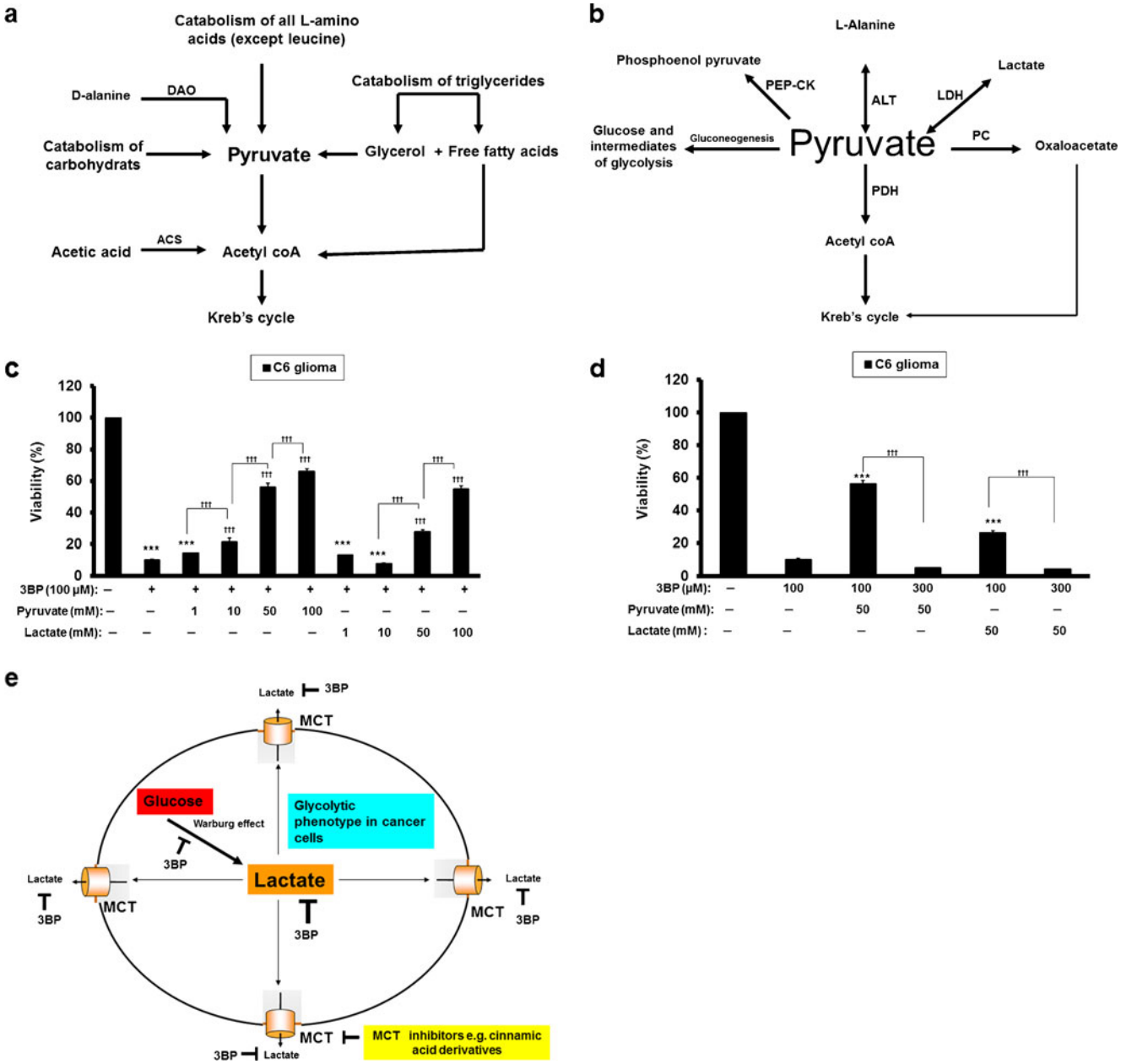


Fig. 3 Pyruvate and lactate have antagonistic effects to 3BP as regard effect on viability of C6 glioma. **a** Pyruvate occupies an important central position in metabolism. **b** Metabolic fate of pyruvate. **c** Pyruvate and lactate protected significantly C6 cells against effect of 3BP. **d** Pyruvate and lactate were antagonized by higher doses of 3BP. **e** 3BP targets formation and effects of lactate. ACS, acetyl CoA synthase, ALT, alanine transaminase, LDH, lactate dehydrogenase, PEP-CK, phosphoenolpyruvate carboxykinase, PC, pyruvate carboxylase, PDH, pyruvate dehydrogenase, MCT, Monocarboxylate transporters. Data are mean ± SEM of 3 independent experiments. *** $p<0.001$ indicates significance between control and treatment conditions. ††† $p<0.001$ indicates significance between 100 µM 3BP and other treatment conditions. ††† $p<0.001$ also indicates significance between treatment conditions within same group

pyruvate and its analog lactate as it regards effect on cancer cell viability. First, we investigated the effect of serial doses of pyruvate (as a pretreatment) on protecting glioma cells treated with the 3BP (100 µM). Our data revealed that the 3BP effect was significantly antagonized by pyruvate in a dose-dependent manner ($p<0.001$) (Fig. 3c). On increasing 3BP concentration to a higher dose (300 µM) against a fixed pyruvate concentration (50 mM), 3BP could overcome significantly the protective effect of pyruvate ($p<0.001$) (Fig. 3d). This may suggest an antagonistic mechanism of action of 3BP versus pyruvate.

We later investigated the effect of 3BP on glioma cell death in the presence of exogenous lactate (to mimic the tumor microenvironment in which glycolysis-derived lactate is abundant in the glycolytic phenotype, a characteristic of tumors as reported in the pioneering work of Warburg) (Fig. 3e). We did the experiments under 2 different

conditions: in the first condition 3BP was added as a co-treatment with lactate at the same time. In the second condition, 3BP was added after pre-incubation of C6 glioma cells for 30 min with serial doses of lactate. Our data revealed that lactate exerted a dose-dependent significant protection of C6 glioma viability against 3BP-induced cell death ($p<0.001$) (Fig. 3c) with similar results using both treatment conditions. The first condition (co-treatment) mimics treatment of glycolytic tumors using 3BP when lactate is present extracellularly in which both 3BP and lactate co-exist. The lactate-mediated protection against 3BP-induced C6 glioma cell death was overcome significantly ($p<0.001$) by treatment with higher doses of 3BP (300 μM) (Fig. 3d).

Pyruvate and lactate antagonized the ATP-depleting effect of 3BP

Pyruvate is an important intermediate in energy generating pathways. Pyruvate can be converted to acetyl CoA via mitochondrial oxidative decarboxylation (the action of pyruvate dehydrogenase complex) which is the step immediately before start of the citric acid cycle. Pyruvate can synthesize oxaloacetate by the action of pyruvate carboxylase. Moreover, Pyruvate can be converted anaerobically to lactate. Pyruvic acid and lactate can restore all intermediates of glycolysis via gluconeogenesis (Cori cycle) (Fig. 3a and b) (Robert et al. 2003). Moreover, 3BP is an analog of both pyruvate and lactate.

As 3BP is a potent antiglycolytic agent which significantly depleted ATP in C6 glioma cells (El Sayed et al. 2012), we also investigated whether pyruvate and lactate can exert a protecting effect against 3BP-induced depletion of ATP in C6 glioma cells. Our data revealed that both pyruvate and lactate significantly protected the energetics of C6 glioma cells treated with 3BP ($p<0.001$). On increasing the dose of 3BP to 300 μM, protection induced by pyruvate and lactate was significantly overcome ($p<0.001$) (Fig. 4a). This may suggest that 3BP induced an antagonistic effect with both pyruvate and lactate (Fig. 4a).

Pyruvate and lactate increased significantly the migratory power of C6 glioma cells which was antagonized by 3BP

The anticancer effects of 3BP were reported to be due to inhibition of HK II (Ko et al. 2001), inhibition of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Ganapathy-Kanniappan et al. 2009), oxidative stress effect and free radical generation (El Sayed et al. 2012). 3BP can decrease the level of glycolysis end products e.g. lactate (Nakano et al. 2011) and exert antagonistic effects to both pyruvate and lactate which may be critical for cancer cell survival and migration. As metastatic potential is an important feature of aggressive malignant tumors which represents an important cause of death due to cancer disease, we investigated the effects of exogenous pyruvate and lactate on the migratory power of C6 glioma cells. We also investigated the effect of 3BP as a general suggested anticancer agent in decreasing the migratory power of C6 glioma cells. A wound healing migration assay was done according to the previous report by Shankardas et al. (Shankardas et al. 2010). The gap induced by scratching the monolayer of C6 glioma cells was gradually filled by migrating cells from both sides of the gap in a time-dependent manner. 3BP (100 μM) was enough to induce massive C6 glioma cell death at both sides of the wound leading to enlargement of the diameter of the wound (Fig. 4b and d) within the first 12 h after treatment. 3BP (100 μM) was very powerful in inhibiting the migration power of C6 glioma cells, stopping and detaching them from both sides of the gap leading to disappearance of the gap and full detachment of all C6 glioma cells (almost 90–100% confluent C6 cells which were tightly adherent to each other and to the six well plate then became detached as a result of treatment with 3BP) (Fig. 4b, c and d). Lactate and pyruvate increased filling the gap more significantly than the untreated control ($p<0.001$) at 12 h after wound initiation (Fig. 4c). 3BP was powerful in antagonizing significantly the migration enhancing effect of pyruvate and lactate ($p<0.001$) (Fig. 4c and d).

By the end of 24 h, C6 glioma cells in control wells had almost completely filled the gap. Pyruvate and lactate enhanced filling the gap more significantly than untreated control wells ($p<0.05$), while 3BP significantly antagonized the effects of lactate and pyruvate ($p<0.001$) and kept the wide gap between both sides even in the presence of lactate and pyruvate (Fig. 4b, c and d).

Effect of pyruvate and lactate on glioma cell death induced by NaF and citrate

Targeting the glycolysis pathway at many points may enhance cancer cell killing (Fig. 5a). Prior investigations are a must to decide which antiglycolytics act synergistically to each other and which antiglycolytics do not act synergistically to allow selection of the best combinations. The glycolytic inhibitor, NaF (enolase inhibitor) exerted a significant potent decrease in viability of C6 glioma cells in the millimolar range in a dose-dependent manner in an MTT assay (Fig. 5b). For NaF, the viability of the C6 glioma started to decrease at a dose of 1 mM and progressively decreased in a dose-dependent manner with a maximal decrease at dose of 15 mM NaF. Pruvate and lactate did not protect C6 glioma cells against NaF effect (Fig. 5c). Citrate (PFK inhibitor) had a strong killing effect on C6 glioma at a dose of 9 mM. (Fig. 5d). Our data revealed that pruvate and lactate did not protect C6 glioma cells against citrate effect (Fig. 5c and d).

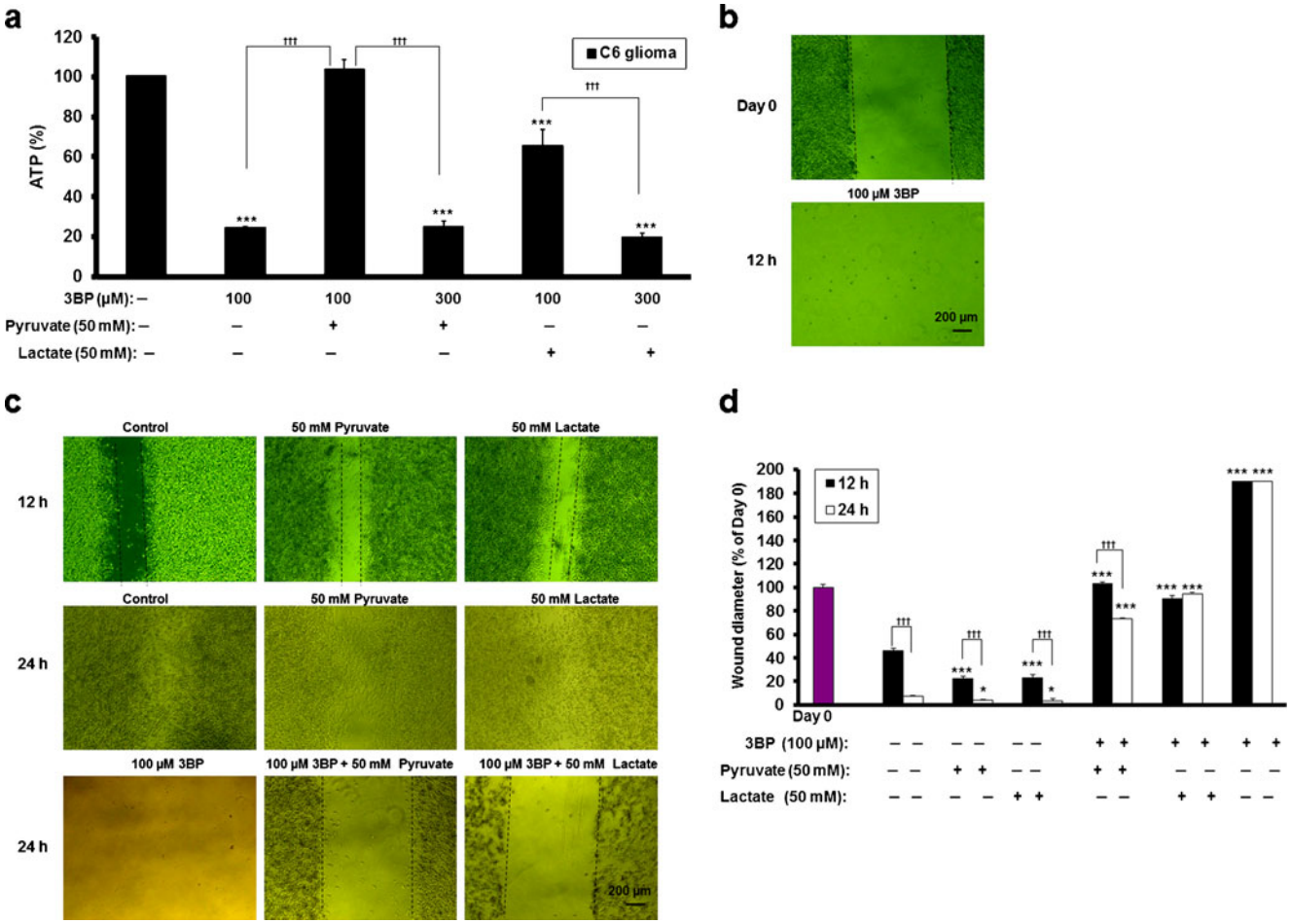


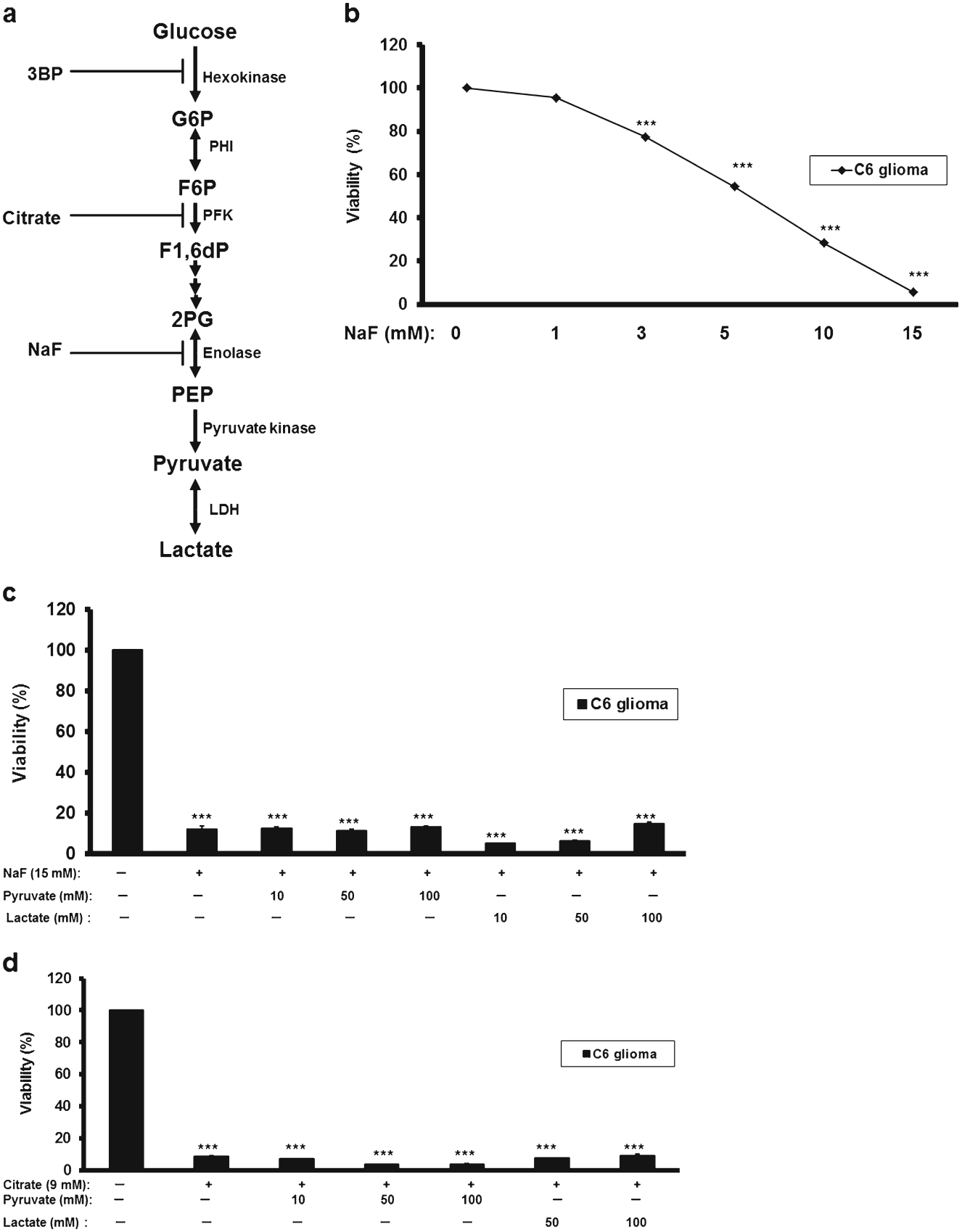
Fig. 4 3BP antagonized effects of pyruvate and lactate as regard ATP levels and migratory power. **a** Pyruvate and lactate had antagonistic effects to 3BP as regard effect on ATP levels of C6 glioma. **b** 3BP depleted migratory power of C6 glioma 12 h after treatment. (**c** and **d**) Pyruvate and lactate enhanced significantly the migratory power of C6 glioma which was depleted by 3BP. Data are mean ± SEM of 3 independent experiments. *$p<0.05$ and *** $p<0.001$ indicate significance between control and treatment conditions. ††† $p<0.001$ indicates significance between treatment conditions within same group

Glycolysis double and triple inhibition

We hypothesized that cancer therapy using a combination of antiglycolytics may carry the benefit of combined therapy that includes a strong anticancer effect at lower concentrations with lower toxicity of antiglycolytic agents that work by different mechanisms of action and target different enzymes in the glycolysis pathway. For citrate (PFK inhibitor), cytotoxicity to C6 glioma was mild but significant ($p<0.01$) at dose of 3 mM (Fig. 6a) We investigated the effect of a small dose of citrate (3 mM) and serial doses of 3BP (in the micromolar range). Also, we investigated a possible combinatory effect between combination of NaF and citrate and a triple combination of the three glycolytic inhibitors 3BP, citrate and NaF. Our data revealed that serial 3BP had a strong significant synergistic effect when combined with citrate (Fig. 6a). Combinatory effect between 3BP (15 μM) and NaF (3 mM) was slightly stronger than either of them alone (Fig. 6b). With serial increase in the dose of 3BP (30 and 45 μM) with a fixed dose of NaF (3 mM), antagonistic effects were observed (Fig. 6b). NaF (3 mM) had a significant combinatory effect with citrate (3 mM) ($p<0.001$) (Fig. 6c). A triple combination of low toxic doses of 3BP (15 μM), citrate (3 mM) and NaF (3 mM) decreased significantly the viability of C6 glioma cells (Fig. 6c).

3BP in combination with citrate depleted significantly the energetics of C6 glioma cells

As both 3BP and citrate are inhibitors of the first 2 key enzymes of glycolysis (HK and PFK, respectively), doses of 15 μM 3BP and 3 mM citrate exerted a mild but significant decrease ($p<0.001$) in ATP levels in glioma cells. We investigated the effect of combination of both 3BP and citrate on depleting the energetics of 3BP. There was a significant


a
Glucose
3BP
Hexokinase
G6P
PHI
F6P
Citrate
PFK
F1,6dP
2PG
NaF
Enolase
PEP
Pyruvate kinase
Pyruvate
LDH
Lactate
b
Viability (%)
120
100
80
60
40
20
0
***
C6 glioma
NaF (mM): 0 1 3 5 10 15
c
Viability (%)
C6 glioma
NaF (15 mM): – + + + + + + +
Pyruvate (mM): – – 10 50 100 – – –
Lactate (mM) : – – – – – 10 50 100
d
Viability (%)
C6 glioma
Citrate (9 mM): – + + + + + +
Pyruvate (mM): – – 10 50 100 – –
Lactate (mM) : – – – – – 50 100

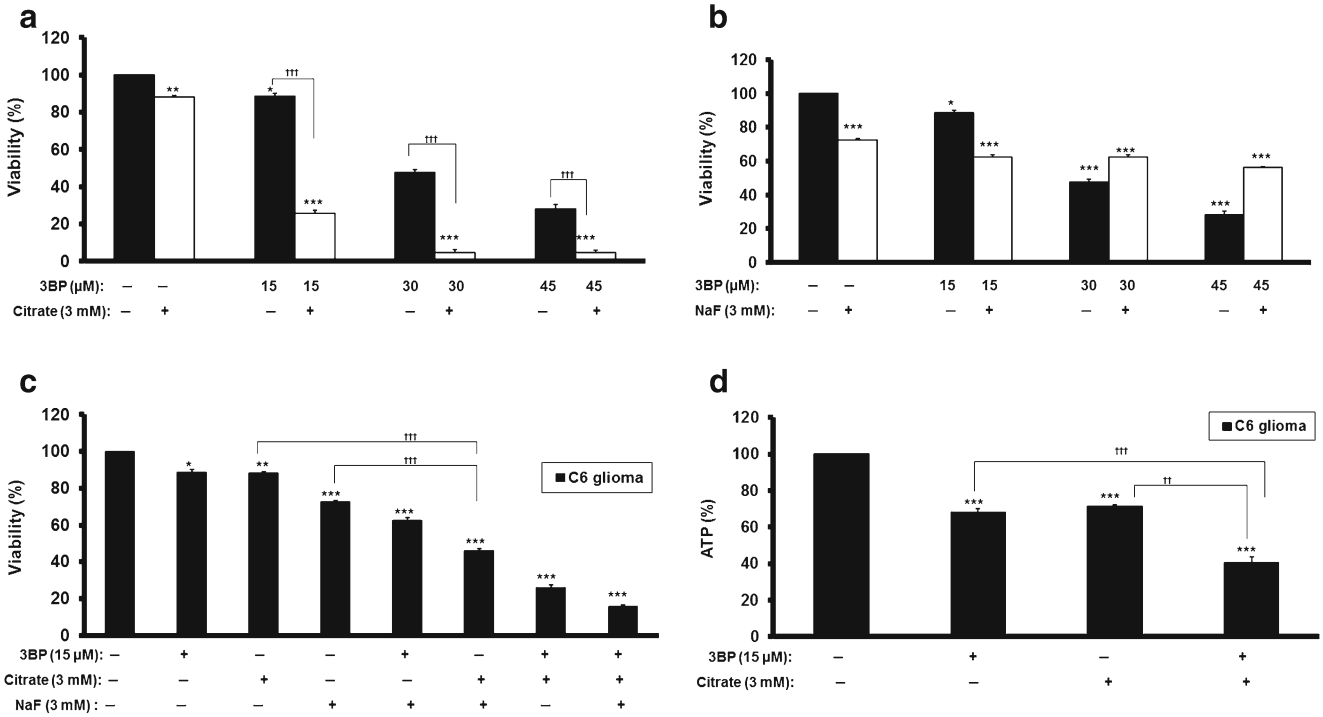


Fig. 6 Effect of antiglycolytic combinations on viability and energetics of C6 glioma cells. **a** Citrate had a synergistic effect with serial doses of 3BP. **b** NaF has no combinatory effect with serial doses of 3BP. **c** Effect of glycolysis double and triple inhibition on viability of C6 glioma cells. **d** Effect of glycolysis double inhibition on ATP levels of C6 glioma cells. Data are mean ± SEM of 3 independent experiments. *$p<0.05$, ** $p<0.01$ and *** $p<0.001$ indicate significance between control and treatment conditions. †† $p<0.01$ and ††† $p<0.001$ indicate significance between treatment conditions within same group

synergistic depletion of energetics of C6 glioma cells ($p<0.001$) (Fig. 6d).

3BP in combination with citrate decreased significantly the viability of C6 glioma spheroids

As tumor spheroids represent an in vitro simulation of three dimensional growth of tumors, we established that spheroid model to investigate the effect of combination of inhibitors of glycolysis (3BP and citrate). Multicellular tumor spheroids (MCTSs) were reported to be an in vitro tumor model which simulates micrometastasis and gives an expected idea about sensitivity of in vivo xenograft models to anticancer therapy (Friedrich et al. 2009). Our data revealed that the synergistic combination of 3BP with citrate significantly decreased viability of C6 glioma spheroids ($p<0.001$) in a dose-dependent manner (Fig. 7a). This reflects an expected potent effect of citrate and 3BP on future treatment of glioma tumors.

◀ **Fig. 5** Effect of pyruvate and lactate on cell death induced by citrate and NaF. **a** Targeting glycolytic enzymes using 3BP, citrate and NaF. **b** NaF induced a significant dose-dependent cell death of C6 glioma. **c** Pyruvate and lactate did not protect C6 glioma against effect of NaF. **d** Pyruvate and lactate did not protect C6 glioma against effect of citrate. Data are mean ± SEM of 3 independent experiments. *** $p<0.001$ indicates significance between control and treatment conditions

Glycolysis double inhibition depleted totally the clonogenic power of the C6 glioma

Combination of low effective doses of 3BP (15 μM) and citrate (3 mM) depleted maximally and significantly ($p<0.001$) the clonogenic power of the C6 glioma. No colonies were detected in six well plates which received both 3BP and citrate treatment. Either of the tested doses of the above mentioned agents alone was not effective in inhibiting totally the clonogenic power of the C6 glioma (Fig. 7b and c).

Glycolysis double inhibition inhibited the migratory power of the C6 glioma

Moreover, the migratory power of the C6 glioma which determines the metastatic potential of C6 glioma cells was powerful and decreased the gap diameter in a time-dependent manner (Fig. 7d and e). By 24 h, the gap was almost filled with migratory cells from both sides of the scratched area as an indication of the strong migratory power of the C6 glioma. A low dose of 3BP (20 μM) was not powerful enough to maintain the initial gap diameter 12 h after scratching as day 0 diameter. The same was true for low dose citrate (2 mM). Effects of 3BP and citrate individually were not significant when compared with the untreated control. The synergistic combination

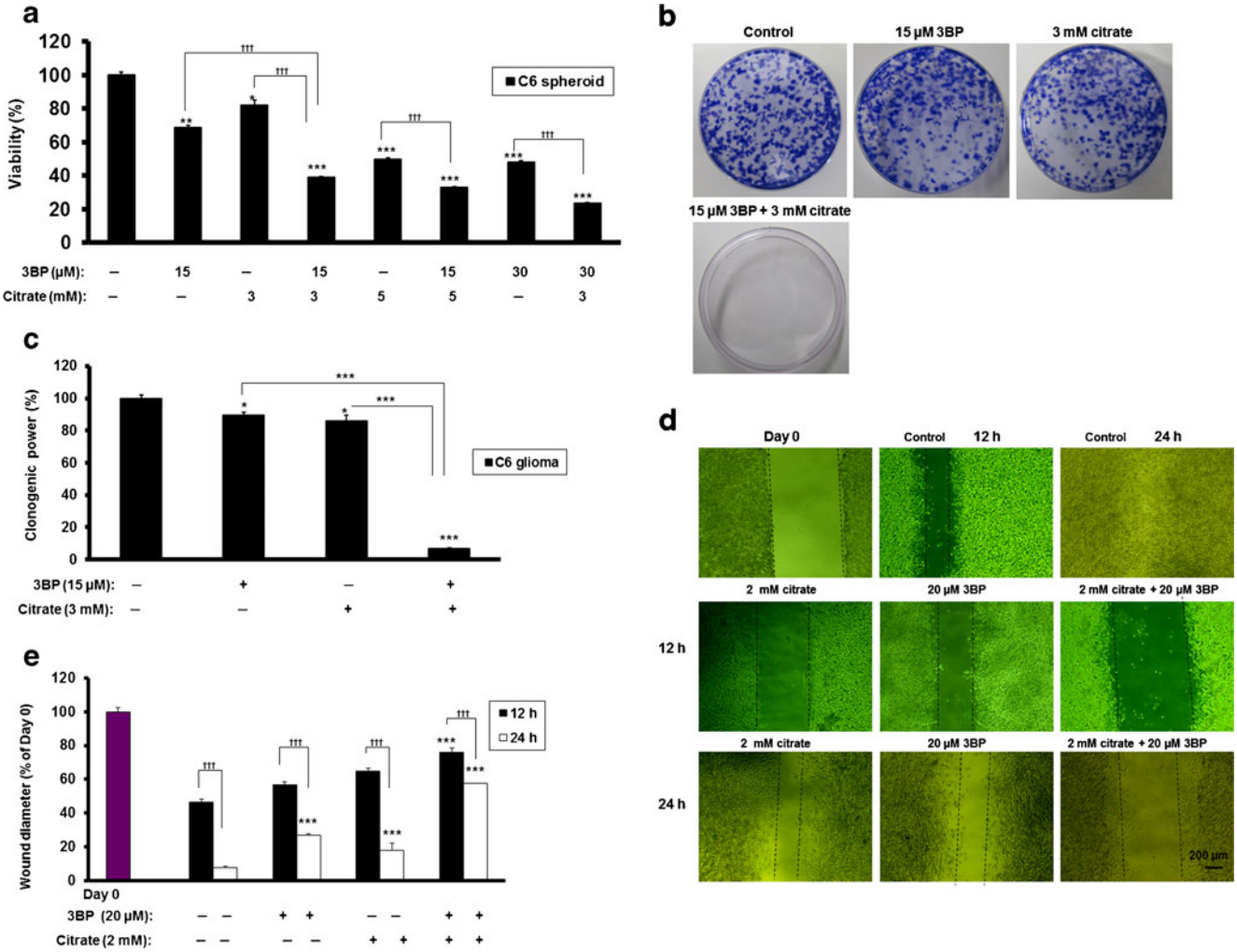


Fig. 7 Effect of glycolysis double inhibition on viability of C6 glioma spheroids, clonogenic power and migratory power of C6 glioma. **a** Glycolysis double inhibition using citrate with 3BP decreased significantly the viability of C6 glioma spheroids. (**b** and **c**) Glycolysis double inhibition depleted significantly the clonogenic power of C6 glioma. (**d** and **e**) Glycolysis double inhibition depleted significantly the migratory power of C6 glioma. Data are mean ± SEM of 3 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ indicate significance between control and treatment conditions. ††† $p<0.001$ also indicate significance between treatment conditions within same group

between 3BP and citrate maintained significantly ($p<0.001$) the diameter of the gap at about 80% of the gap diameter at day 0. After 24 h, the migratory power of C6 glioma cells was stronger than the inhibitory power of the low dose of 3BP and citrate individually and a significant drop in inhibition of migratory power occurred ($p<0.001$). More than half the gap was filled during the next 12 h (the period starting from 13 h to 24 h after treatment) in the control, 3BP (20 μM) and citrate (2 mM) but not in the combination group. Although the migratory power of C6 was strong, it could not progress against combined low doses of 3BP and citrate (Fig. 7d and e).

3BP induced a caspase-dependent C6 glioma cell death

As 3BP was reported to induce apoptotic and necro-apoptotic glioma cell death (El Sayed et al. 2012), we investigated if 3BP-induced apoptosis is caspase-dependent or caspase-independent using activation of cleaved caspase-3 in cell lysates of C6 glioma treated with serial doses of 3BP in an immunoblot experiment. Our data revealed that 3BP induced cleavage of pro-caspase-3 into its cleaved bands (p20, p17 and p11) (Fig. 8a).

3BP decreased significantly the viability of the C6 glioma and U373MG human glioblastoma cells in a dose- and time-dependent manner

U373MG is an aggressive human glioblastoma cell line that is chemoresistant and radioresistant (Lau et al. 2008). U373MG expresses the multidrug resistance-associated protein (MRP), which is a membrane transporter related to multidrug resistance that does not occur through P-glycoprotein (Mohri et al.

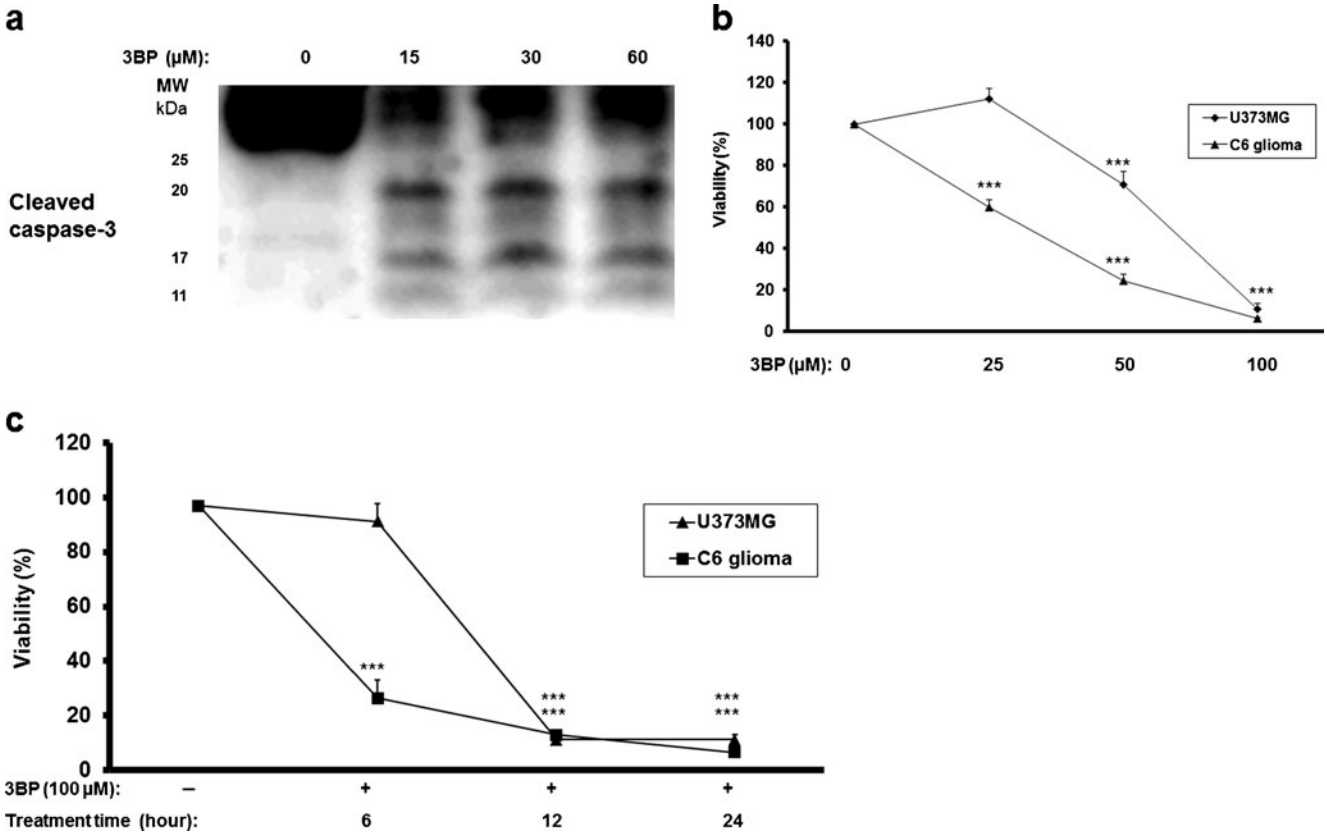


Fig. 8 3BP-induced death in glioma cells is caspase-dependent and occurred in a dose- and time-dependent manner. **a** 3BP induced a caspase-dependent cell death in glioma cells. **b** 3BP induced a significant cell death in U373MG and C6 glioma cells in a dose-dependent manner. **c** 3BP induced a significant cell death in U373MG and C6 glioma cells in a time-dependent manner. Data are mean ± SEM of 3 independent experiments. *** $p<0.001$ indicate significance between control and treatment conditions

2000). 3BP was very powerful in decreasing significantly the viability of U373MG in a dose-dependent manner. 3BP at doses (50 and 100 μM) was significant ($p<0.001$) in decreasing the viability of U373MG human glioblastoma cells (Fig. 8b). A dose 100 μM 3BP decreased totally the viability of U373MG within 24 h. As for the C6 glioma, all tested doses of 3BP (25, 50 and 100 μM) were all significant ($p<0.001$) in decreasing viability of the C6 glioma. 3BP (100 μM) was powerful in decreasing the viability of the U373MG and C6 glioma in a time-dependent manner ($p<0.001$). The C6 glioma was more sensitive than U373MG 6 h after treatment. Viability of almost all U373MG and C6 glioma cells was lost within the first 12 h after treatment ($p<0.001$). The same death values were continuous during the next 12 h (Fig. 8c).

Discussion

Normal cells depend on oxidative phosphorylation to get necessary energy. Oxidative phosphorylation provides cells with 36 ATP molecules (Xu et al. 2005) and requires intact mitochondria and the presence of oxygen. When oxygen is lacking, normal cells use the less efficient glycolysis pathway which gives only 2 ATP molecules. Glycolysis is inhibited in the presence of oxygen (Pasteur effect) (Baierlein and Foster 1968). In cancer cells, glycolysis proceeds to produce ATP and lactate even in the presence of oxygen (Warburg effect) (Warburg 1956).

Pyruvate and lactate are structurally related to 3BP (Fig. 1a) and are transported through the MCT (Zorzano et al. 2000). Structural similarity between pyruvate and lactate (glycolysis end products) and 3BP may suggest that 3BP may act as an antagonist for both pyruvate and lactate as a novel mechanism of action of 3BP.

Pyruvate exerted a significant antioxidant function both in a cell-free system (Fig. 1b) and in cell-based system through decreasing the steady-state ROS condition in the C6 glioma. Lactate did not affect the steady-state ROS condition in the C6 glioma (Fig. 1c). 3BP induced generation of H_2O_2 (Fig. 1d). Pyruvate is a potent scavenger of exogenous H_2O_2 (Fig. 1b) and endogenously produced H_2O_2 due to the effect of 3BP (Fig. 1d). Lactate had no antioxidant function (Fig. 1 b-d). Pyruvate protected glioma cells significantly against H_2O_2-induced glioma cell death while lactate did not protect C6 glioma cells against the effect of H_2O_2. Reduced glutathione had a protecting effect

while acetate had no protecting effect against H_2O_2-induced glioma cell death (Fig. 2a). No protection effect was found when pyruvate treatment was applied to C6/DAO cells receiving D-serine treatment which is known to induce H_2O_2 production due to a DAO/D-serine reaction. Unexpectedly, a more significant decrease in C6/DAO viability was the result with serial doses of pyruvate (Fig. 2b). This may be explained because DAO/D-serine induces GSH depletion (Krug et al. 2007; Orozco-Ibarra et al. 2007). Pyruvate becomes toxic to cells when glutathione is depleted (Marí et al. 2002). This may be the reason why pyruvate unexpectedly did not protect against D-serine-induced death in C6/DAO cells. Interestingly, catabolism of D-serine upon DAO reaction results in the formation of β-hydroxylpyruvic acid (HPA). HPA is a structural analog to pyruvate, lactate and 3BP. HPA was toxic to glial cells in the millimolar range. HPA induced caspase-dependent C6 glioma cell death. Apoptosis induced by HPA was dose- and time-dependent (Chung et al. 2010). Further investigation is necessary to elucidate the possible effects of the structural similarity between 3BP and the DAO/D-serine metabolite, HPA on cellular metabolism.

Also, decreased production of lactate is a common advantage of glycolytic inhibitors acting upstream of the lactate formation step (Nakano et al. 2011; Pereira da silva et al. 2009). Deprivation of pyruvate may be achieved by glycolytic inhibitors acting upstream of the pyruvate formation step in the glycolysis pathway as does 2-deoxyglucose (Stanbrook and McMurtry 1983).

Pyruvate occupies a central position in the metabolism of carbohydrates, proteins and lipids in the human body (Robert et al. 2003). However, pyruvate can be formed from other sources e.g. from dehydrogenation of lactate by activity of LDH, transamination of L-alanine, catabolism of all L-amino acids except L-leucine and DAO activity on D-alanine etc. (Fig. 3a). Antiglycolytic drugs e.g. 2-deoxyglucose and 3BP can decrease pyruvate and lactate production by targeting glycolytic enzymes acting upstream to the pyruvate and lactate formation steps in the glycolysis pathway. Moreover, pyruvate can form oxaloacetate. Both acetyl CoA and oxaloacetate initiate the first step of the Krebs cycle (Fig. 3b). This confirms the previous report by Pedersen (Pedersen 2007) who reported that 3BP can inhibit both power plants for energy production in cancer cells i.e. glycolytic and mitochondrial pathways for energy production. This is confirmed by previous reports that 3BP was reported to be an inhibitor of pyruvate dehydrogenase (PDH) which forms acetyl CoA from pyruvate (Nemeria et al. 1998; Korotchkina et al. 1999). Consistent with this is the report that 3BP decreased the content of acetyl CoA in the tissues (Rícný and Tucek 1981).

3BP-induced glioma cell death was protected by serial doses of pyruvate and serial doses of lactate (Fig. 3c) and this protection was overcome by higher doses of 3BP (Fig. 3d). Based on that, the close structural similarity between 3BP and lactate may confer many advantages to 3BP over other antiglycolytics regarding its effect on lactate. 3BP may act as a functional antagonist to lactate as regard its effect on viability of C6 glioma cells in which both 3BP and lactate exerted antagonistic effects i.e. lactate protected against 3BP-induced glioma cell death and that was overcome on increasing the dose of 3BP (Fig. 3c-d). Lactate was reported to be a competitive inhibitor to 3BP (Mulet and Lederer 1977). Moreover, lactate and 3BP are transported through the same subtype of MCT transporters, MCT1 (SLC5A8) (Végran et al. 2011; Thangaraju et al. 2009) Competing with pyruvate suggests that 3BP may partially affect the mitochondrial pathway for energy production as conversion of pyruvate to acetyl CoA is the introductory step into Krebs cycle. MCT1 is a protein which participates in the bidirectional transmembrane transport of lactate and pyruvate (Halestrap and Price 1999). The direction of exchange depends on the pH gradient between intracellular and extracellular environment (Halestrap and Price 1999; De Bruijne et al. 1983; Garcia et al. 1994; McCullagh et al. 1996). Moreover, within the same tumor, oxygenated and hypoxic regions coexist. The Warburg effect is manifest in hypoxic regions in which lactate is formed from glucose metabolism and extruded from intracellular compartment to the extracellular space. Lactate in the extracellular space acidifies the tumor microenvironment and can be transported via MCT1 to the aerobic oxygenated regions of the tumor to be used to fuel oxidative phosphorylation for energy production (Semenza 2008).

Targeting lactate as a treatment for cancer can be done at 3 levels: The first level is to target its formation e.g. using antiglycolytics. The second level is to target its transport using MCT inhibitors which prevent extrusion of lactate e.g. cinnamic acid derivatives (α-cyano-4-hydroxy-cinnamic acid) (Colen et al. 2011) or siRNA directed against MCT (Mathupala et al. 2007). The third level is to target the effect of lactate itself using lactate analogs excluding pyruvate which may fuel aerobic regions of tumors. As 3BP exerts an antagonistic effect to lactate, 3BP may induce potent anticancer effects with no need for MCT inhibitors. Antiglycolytic agents decrease formation of lactate. 3BP has superiority over other antiglycolytic agents as it regards an effect on lactate as 3BP inhibits formation of lactate and antagonizes its effects (Fig. 3e).

Massive depletion of the energetics of C6 glioma cells was induced by 3BP. Pyruvate and lactate significantly protected against 3BP-induced ATP depletion in C6 glioma cells (Fig. 4a). Accumulation of lactate in the extracellular space in highly glycolytic cancer cells plays an important role in maintaining low pH (acidosis) in the extracellular space which facilitates invasiveness of cancer cells. Acidity

of the extracellular compartment in tumors adds a survival advantage to cancer cells over normal cells. Extracellular acidity establishes an unfavorable environment for normal cells and facilitates cancer invasion (Stubbs et al. 2000). As tumor cell acidity exerted by exported acids e.g. lactate from the intracellular compartment to extracellular compartment is a major cause for cancer cell resistance to chemotherapy, 3BP may help in treatment of such resistance by competing with lactate either as a monotherapy or when combined with currently used chemotherapeutics.

LDH is a prognostic marker for survival time in patients suffering from terminal malignancy (Suh and Ahn 2007). 3BP causes a time-dependent inactivation of LDH in addition to the ability of 3BP to compete with lactate (Mulet and Lederer 1977). 3BP is a substrate for LDH in which the affinity of LDH towards 3BP is 9–11 times less than its affinity towards pyruvate. 3BP can compete with pyruvate for lactate dehydrogenase activity (Dell'Antone 2009).

3BP was reported to compete with pyruvate for its uptake (Lang et al. 1987). Lactate and pyruvate are transported via MCT1 (SLC5A8) which transports acetate, lactate, pyruvate and 3BP (Babu et al. 2011). Competing with pyruvate may be a novel anticancer mechanism of action added to the well known anticancer mechanisms of action of 3BP. 3BP was reported to block catabolism of glucose to pyruvate and lactate at more than one point in the glycolysis pathway i.e. HK II inhibitor (Ko et al. 2001), GAPDH inhibitor (Ganapathy-Kanniappan et al. 2009) in addition to the ability of 3BP to induce generation of H_2O_2 (El Sayed et al. 2012). 3BP-mediated inhibition of glycolysis at many points allows 3BP to decrease the production of ATP, pyruvate and lactate through inhibition of the high glycolysis rates in cancer cells.

For induction of the Krebs cycle while glycolytic output of pyruvate and lactate is blocked by 3BP, other sources of pyruvate may come from protein breakdown (all L-amino acids except L-leucine) and breakdown of triglycerides into glycerol that synthesizes pyruvate through gluconeogenesis. 3BP may affect the metabolic reactions of pyruvate e.g. oxidative decarboxylation of pyruvate to produce acetyl CoA, which is the critical step to start the Krebs cycle (Fig. 3a) (Robert et al. 2003).

3BP interferes with Warburg effect through breaking the catabolism of glucose to lactate. Being a structural analog to pyruvate and lactate, 3BP may be competitive with pyruvate and lactate (Figs. 3 and 4), which adds more interference to the Warburg effect (Fig. 7e). This may be supported by the report of Mulet et al. (Mulet and Lederer 1977) who reported that 3BP inhibited the LDH enzyme which converts pyruvate to lactate and vice versa leading to a decrease in the levels of lactate and pyruvate. Moreover, acidic extracellular pH of tumors enhances invasiveness of cancer cells (Stubbs et al. 2000). Hyperglycolytic tumors are characterized by hypoxia, tumor acidity and excessive production of lactate (end product of glycolysis). All that may be correlated with metastasis and may lead to chemoresistance to currently used chemotherapeutics especially chemotherapeutics that are weak bases e.g. doxorubicin (Raghunand et al. 2003).

As 3BP could overcome lactate effects and induced C6 glioma cell death (Fig. 3c), 3BP may help in decreasing tumor acidity and enhancing chemosensitivity. Nakano et al. reported that 3BP inhibited ATP-binding cassette transporters (ABC transporters) in cancer cells that play an important role in chemoresistance (Nakano et al. 2011).

As 3BP induced massive significant inhibition of C6 glioma cell migration and antagonized the pyruvate and lactate stimulatory effect on migration of glioma cells (Fig. 4b-d), 3BP can be regarded as a treatment for malignant metastasis either as a monotherapy or a combination therapy with other chemotherapeutics. The earliest report was by Ko et al. (Ko et al. 2004) who reported that hepatocellular carcinoma tumors (AS-30D cell line) were completely eradicated in 19 out of 19 SD rats bearing the tumors in which animals lived for a long time with no distant metastasis after 3BP monotherapy. The AS-30D cell line used is highly metastatic and chemoresistant (Kobryn and Fiskum 1992). We reported recently that direct metastatic spread of the C6 glioma xenograft tumors in SD rats occurred in untreated control SD rats and did not occur in rats receiving 3BP as a monotherapy or as a combination with the DAO gene therapy (El Sayed et al. 2012). Antimetastatic properties of 3BP can be attributed to many therapeutic effects induced by 3BP. Metastatic spread necessitates enormous amounts of ATP that is depleted by 3BP. Metastasis is enhanced in the presence of lactate in the cancer microenvironment (Goetze et al. 2011). Lactate can be regarded as the facilitator and gatekeeper of metastasis. Lactate can be regarded as a prognostic marker for cancer progression, metastasis and recurrence after treatment. Lactate predicts the decrease in survival of cancer patients (Walenta et al. 2000). 3BP decreased lactate formation and antagonized its effects. This may explain why the Warburg effect seems vital for cancer cells and the value of 3BP to antagonize the Warburg effect.

Metastasis requires pyruvate to fuel the mitochondrial power plant for energy production. 3BP inhibited the upstream steps before pyruvate formation and antagonized effects of pyruvate (Figs. 1, 3 and 4). 3BP deprived cancer cells from the antioxidant effect of pyruvate (Fig. 1d). Metastasis requires massive glucose consumption to feed glycolysis to attain the glycolytic phenotype. 3BP significantly decreased glucose consumption in prednisolone-resistant pediatric acute leukemia cells (Hulleman et al. 2009). Metastasis requires reducing equivalents (NADH) to be oxidized to NAD+(coenzyme of the GAPDH step in glycolysis pathway) to feed glycolysis or to provide ATP and lactate.

3BP depleted reducing equivalents (El Sayed et al. 2012; Ihrlund et al. 2008) and lactate (Nakano et al. 2011). Lactate was recently reported to impede migration of monocytes and release of cytokines as an immune suppression effect. Lactate induced also a significant enhancement in migratory power of tumor cells in Boyden chamber assays (Goetze et al. 2011) which agree with our data using a wound healing assay (Fig. 4c and d). As for other glycolytic inhibitors e.g. sodium fluoride (NaF) and citrate, both target different enzymes in the glycolysis pathway (Fig. 5a). NaF exerted a significant dose-dependent decrease in C6 glioma viability (Fig. 5b). The effect of NaF was not inhibited by serial doses of pyruvate and lactate (Fig. 5c). Citrate (PFK inhibitor) induced potent death of C6 glioma cells which was not protected by pyruvate or lactate (Fig. 5d). Pyruvate and lactate, end products of glycolysis, did not protect glioma cells against NaF or citrate-induced glioma cell death but protected only against 3BP-induced glioma cell death.

Combination between serial doses of 3BP (HK II inhibitor) and citrate was strongly synergistic in which massive glioma cell death occurred while each drug separately exerted a minor degree of glioma cell death (Fig. 6a-c). Combination of NaF with a small dose of 3BP (15 μM) was more effective in inducing C6 glioma cell death than using 3BP or NaF alone. Antagonism between 3BP and NaF was evident at higher doses of 3BP (Fig. 6b). NaF induced a significant cell death of glioma cells when NaF was combined with citrate (Fig. 6c). Combination of antiglycolytics which target different points in the glycolysis pathway (glycolysis double inhibition and triple inhibition) (Fig. 5a) exerted a significant effect in decreasing glioma cell viability (Fig. 6c). Our data revealed that the best combination was between 3BP and citrate and when both were combined with NaF (glycolysis triple inhibition), a further decrease in glioma viability occurred.

Combination of low effective doses of 3BP and citrate depleted significantly C6 glioma cell energetics (Fig. 6d) which may be explained in light of synergistic combination due to inhibition of 2 key glycolytic enzymes.

In an in vitro tumor model, viability of C6 glioma spheroid decreased significantly on receiving a combined glycolysis double inhibition treatment using 3BP and citrate in a dose-dependent manner (Fig. 7a). All combinations between 3BP and citrate were synergistic in decreasing viability of C6 spheroids which predicts an expected potent effect in an in vivo tumor model (Friedrich et al. 2009). Glycolysis double inhibition using 3BP and citrate decreased significantly the clonogenic power of the C6 glioma (Fig. 7b and c).

Based on our data and previous reports, 3BP can be looked at as a potent general anticancer drug with a broad antiglycolytic range i.e. 3BP targets the glycolysis pathway at its beginning (HK step), its middle (GAPDH step), and at its end (LDH step) and at pyruvate and lactate effects, which obviously interfere with Warburg effect and disrupt the microenvironment suitable for cancer cells.

Drug resistance of cancer cells still constitutes a major obstacle to obtain a cure from cancer disease. Chemoresistance and radioresistance increase the possibility of cancer proliferation and metastasis. The emergence of resistance can be attributed to the presence of cancer stem cells that are not sensitive to cancer treatment (Dean 2009). Cancer chemoresistance may be due to factors related to the cancer microenvironment e.g. extracellular acidosis may cause resistance to chemotherapeutics that are weak bases in nature e.g. activities of mitoxantrone, doxorubicin and daunorubicin are all inhibited by low extracellular pH (Stubbs et al. 2000). Inhibition of glycolysis was reported to overcome drug resistance induced by cancer cells (Xu et al. 2005). Lactate produced through glycolysis participates in establishing a cancer microenvironment which resists chemotherapy. 3BP decreased lactate production (Nakano et al. 2011) and is antagonistic to its effects. Cancer cells may become chemoresistant via the aberrant expression of the drug-expelling transporters, ABC transporters (Dean 2009). Glycolysis inhibition using 3BP inactivates ABC transporters to restore drug sensitivity in malignant cells. Inhibition of glycolysis by 3BP enhances the tumoricidal effects of anticancer agents on ABC transporter-expressing multiple myeloma cells (Nakano et al. 2011). Expression of genes associated with glucose metabolism is different in prednisolone-sensitive and prednisolone-resistant precursor B-cell lineage leukemic patients. Inhibition of glycolysis by 3BP and other antiglycolytics decreased prednisolone resistance in childhood acute lymphoblastic leukemia cells (Hulleman et al. 2009). Chemoresistance in cancer cells is determined by intracellular ATP levels and delivery of liposomal ATP delivery renders chemosensitive cancer cells chemoresistant (Zhou et al. 2011).

3BP had been reported to have a significant combinatory effect with cancer chemotherapeutics e.g. with cisplatin and oxaliplatin (Ihrlund et al. 2008). 3BP had a strong synergistic effect with citrate. 3BP had a synergistic effect with prednisolone in treatment of prednisolone-resistant pediatric acute leukemia cells (Hulleman et al. 2009). Moreover, the precursor of 3BP, (3-bromo-2-oxopropionate-1-propyl ester, 3-BrOP) had a potent antileukemia effect in all tested acute leukemia cell lines including the patient samples and the chemoresistant cell lines. 3-BrOP had a significant combinatory effect with antimycin A and rapamycin (Akers et al. 2011).

3BP induced caspase-dependent cell death in C6 glioma (Fig. 8a) and decreased significantly the viability of C6 glioma and the chemoresistant and radioresistant U373MG human glioblastoma cells in a dose- and time-dependent manner (Fig. 8b and c).

In conclusion, 3BP is a promising potent general anticancer agent that exerts potential anticancer effects through multiple mechanisms of action. 3BP interferes with the Warburg effect and glycolytic phenotype. 3BP is simple as it regards its structure and powerful as regards its anticancer effects. 3BP inhibits the beginning, middle and end of glycolysis pathway and antagonizes lactate and pyruvate effects. 3BP exerts potent synergistic effects with citrate (PFK inhibitor). Citrate was potent in decreasing glioma viability even in the presence of lactate and pyruvate. 3BP exerted strong anti-metastatic effects and chemosensitizing effects. As a promising suggested anticancer agent, 3BP carries a lot of hope in the management of cancer disease.

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Conflict of interest Authors declare that there is no conflict of interest.

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