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Glioma Spheroid Cells (3D Tumor Models) Are Less Responsive to 3-Bromopyruvate than Cultured Cells: Lack of Tumors Eradication

Salah Mohamed El Sayed[1,2,*], Hussam Baghdadi[1], Faten M. Omran[3], Ahmed M. Okashah[4], Samer A. El-Sawy[2], Hytham Mahmoud Abd elatif[3], Wafaa A. Abdellah[3], Azza Mahmoud Ahmed Abouelela[3], Hassan El-Alaf[5], Elussainy MA Elussainy[6], Sayed Mostafa El Sayed[7,8], Hesham I. Abdallah[7,8], Osama Al-hadramy[9], Tamer M. Soliman[10], Amr El-Dardear[11], Mohamed Abdel-haleem[12], Manal M.H. Nabo[13]

[1]Department of Clinical Biochemistry and Molecular Medicine, Taibah Faculty of Medicine, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia
[2]Department of Medical Biochemistry, Sohag Faculty of Medicine, Sohag University, Egypt
[3]Department of Medical Pharmacology, Sohag Faculty of Medicine, Sohag University, Egypt
[4]Academic Affairs Director And Consultant Clinical Immunologist, Al-Madinah Directorate of Health, Al-Madinah Al-Munawwarah, Ministry of Health, Saudi Arabia
[5]Department of Medical Physiology, Sohag Faculty of Medicine, Sohag University, Egypt
[6]Department of Medical Physiology, Kafr Elshekh Faculty of Medicine, Kafr Elshekh University, Egypt
[7]Anatomy and Embryology Department, Faculty of Medicine, Ain Shams University, Egypt
[8]Anatomy and Embryology Department, Faculty of Medicine, Taibah University, Saudi Arabia
[9]Division of Cardiology, Department of Medicine, Taibah Faculty of Medicine, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia
[10]Department of Clinical Pathology, Sohag Faculty of Medicine, Sohag University, Egypt
[11]Department of Pediatrics, Taibah Faculty of Medicine, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia
[12]Department of Ear, Nose and Throat, Taibah Faculty of Medicine, Taibah University, Al-Madinah Al-Munawwarah, Saudi Arabia
[13]Department of Pediatrics, Al-Rayyan Medical Colleges, Al-Madinah Al-Munawwarah, Saudi Arabia
*Corresponding author: salahfazara@yahoo.com

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Abstract Gliomas and glioblastomas are space-occupying brain tumors with variable aggressive behaviour. Glioblastomas are the most lethal brain tumors and are not responsive to current chemotherapy and radiotherapy. To date, no satisfactory curative treatment exists. 3-bromopyruvate (3BP) is a promising chemotherapeutic that proved effective in treating gliomas and other malignancies in so many reported studies. 3BP is both an alkylating agent and antimetabolite (pharmacological antagonist of lactate, the Warburg effect). Our previous publications proved effectiveness of 3BP in treating glioma cell lines (2D models) and early glioma spheroids (3D models) where 3BP was added early after culturing glioma cells. No report is there regarding treating well-established glioma spheroids (well-established avascular 3D tumor models) using 3BP that we report here. In this study, our data revealed that 3BP effectively and maximally killed cultured glioma cells causing cellular fragmentation that correlated with maximal glioma cell death. However, the picture was less promising when treating well-established 3D glioma spheroids with 3BP where glioma viability and size decreased significantly (in a dose-dependent manner) but not maximally. There was no significant difference in spheroids cell killing at high versus very high doses of 3BP. 3BP may face many problems regarding delivery and targeting to glioma cells inside spheroid body. A similar effect may be faced when treating clinical tumors with 3BP. 3BP formulations inside lipid nanocarriers (liposomes), PEGylated liposomes or targeted liposomes may improve 3BP-induced tumor cells killing. More future research is needed to explore the reasons why 3BP effects in cell lines were not effectively translated into 3D models and how to overcome the obstacles.

Keywords: *3-bromopyruvate, spheroids, glioma, viability*

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1. Introduction

Since the most exciting report that 3-bromopyruvate (3BP) eradicated all primary and metastatic hepatoma tumors in tumor-bearing animals, [1] hundreds of research studies investigated 3BP effects in almost all cancer types with promising variable degrees of success. 3BP is a promising alkylating agent and antimetabolite (pharmacological antagonist of lactate, the Warburg effect) that kills cancer cells by many different mechanisms e.g. blocking energy production, antagonizing the Warburg effect and inhibiting basic requirements for tumour growth and spread. [2,3] That gives 3BP superiority over many other cancer chemotherapeutics that are limited in their pharmacological spectrum. In previous reports, we explored many anticancer effects and benefits of 3BP. [2,3] In subsequent research studies, we reported novel anti-angiogenic effects of 3BP [4] in addition to its safety that was added to the promising clinical effectiveness in treating xenograft animal models bearing aggressive malignancies. [5]

Anticancer power of 3BP benefits so much from the structural similarity-based pharmacological and chemical antagonism with lactate effects (The Warburg effect) and pyruvate (a key metabolic intermediate). [6] That deprives cancer cells from many vital requirements for tumour survival, growth, metastasis and angiogenesis. [6] However, biochemical nature of 3BP subjects this tiny biomolecule to a lot of biochemical reactions *in vivo* that may not translate its excellent power in cancer cell killing *in vitro*. In our previous report, treatment of a patient with metastatic melanoma proved safety of 3BP but the efficacy was less than expected. [7]

Many biological differences may exist between animal and human tissues when administering 3BP. Many obstacles may hinder achieving cancer cure using crude unformulated 3BP. So, 3BP was recommended to be formulated in different ways to improve its therapeutic efficacy. [3] In the majority of reported animal studies, 3BP was administered intraperitoneally while in human studies, intravenous administration of 3BP was the suitable root. That certainly subjects delivered 3BP dose to thiol groups of serum glutathione, proteins and tissue proteins. 3BP was previously reported to be inactivated and metabolized when being conjugated with glutathione. [8] In the most promising tumour eradication reported by Ko et al., [1] 3BP was given to experimental animals in small doses (less than 2 mg/kg) in a relatively reasonable amount of solvents (less than 2 ml PBS/animal) which is different from the dose reported in treating a patient with metastatic melanoma (1 mg/kg/ml given through intravenous drip infusion). When a larger dose of 3BP was given (5 mg/kg) intraperitoneally to laboratory animals, mild peritonitis was reported that is mostly chemical peritonitis [9] which confirms 3BP-induced toxicity at relatively large doses. In this study, we aimed at investigating the effects of 3BP in inducing cancer cells killing in 3D tumour microenvironment to simulate a tumour model. We aimed also at comparing 3BP effects in 2D versus 3D models.

2. Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), 3-bromopyruvate (3BP), agarose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma (St. louis, MO, USA). Cell culture C6 rat glioma cells (Dainippon Pharmaceutical, Osaka, Japan) were maintained in DMEM/F12 containing 15% (v/v) horse serum, 2.5% (v/v) FBS and 1% penicillin– streptomycin at 37°C under a humidified atmosphere containing 5% CO_2.

2.1. MTT Cellular Viability Assay

Investigated doses of 3BP were in micromolar range at the scheduled final concentrations (15, 30, 60 and 100 µM). Doses of 3BP were prepared for investigating their effects on the viability and morphology of C6 glioma cells using the MTT assay. Images were captured under a phase contrast microscope.

2.2. *In vitro* 3D Glioma Spheroid Tumor Models

Using 96-well plates, regularly cultured C6 glioma cells were plated as described before. [10] Briefly, 1.5% agar solution dissolved in distilled water was put in suitable heat conditions inside an oven. To the 96-well plates, dissolved gar solution (100 µl) was poured in each plate using a multichannel pipette under complete sterile laboratory circumstances. Trypsinized C6 glioma cells (1×10^4) were washed with nutrient medium then put in 200 µl of the same nutrient medium. Glioma cells were seeded in each well of the 96-well plates in which agar layer was added. All plates were incubated for 96 h to allow for the growth of glioma spheroids during which regular change of nutrient medium was done every 24 hour for 3 days. Decided treatment doses of 3BP were added in time every 24 hours starting from day 4 for 4 consecutive days. For assaying spheroid cells viability after treatment, MTT solution (1 mg/ml) was added and followed by incubation for an additional 3-4 h inside CO_2 incubator. Centrifugation of the whole 96 well plates was done to settle the spheroids down and allow for aspirating the supernatant. Culture medium supernatant was aspirated slowly and carefully from each well (without touching glioma spheroids). 150 µl of DMSO were added by multichannel pipettor to each well of the plate. Insoluble formazan crystals were then dissolved using dimethyl sulfoxide. Viability of cells in tumor spheroids was estimated be measuring absorbance at 550 nm using a microplate reader. The total length of spheroids diameters in 10 microscopic fields was measured and averaged using Image J software. The total number of closed vascular polygons in 10 microscopic fields was counted and averaged.

2.3. Statistical Analysis

Statistical analyses and graphical presentations were done using Microsoft excel and SPSS version 16. Paired two tailed Student's t-test. Values were expressed as Mean ± SEM. All data shown represent results from three or more independent observations. ***indicates $p < 0.001$.

3. Results

3.1. Effects of 3BP on Viability of C6 Glioma Cells

3BP markedly disturbed both morphology and viability of cultured C6 glioma cells. Glioma cells lost their characteristic spindle-shaped morphology and became distorted. Majority of glioma cells detached from the culture plate leaving distorted fragments (Figure 1A). MTT viability assay confirmed that where 3BP significantly and maximally decreased C6 glioma cellular viability by more than 97% ($p<0.001$) (Figure 1B).

3.2. Effects of 3BP on Viability of 3D C6 Glioma Spheroids

C6 glioma spheroids started to attain a globular morphology from the 1st day of seeding (day 0) in culture medium over the agar layer. Spheroids started to acquire a globular shape rapidly (day 1) (Figure 2A). In a previous publication, we demonstrated that 3BP given in the 1st seeding day markedly impaired C6 spheroids growth in a dose-dependent manner. [5] Herein, 3BP induced a dose-dependent marked (but not maximal) decrease in glioma spheroids sizes (Figure 2B). 3BP given starting from day 4 for 4 consecutive days did not maximally decrease glioma spheroids cellular viability. C6 glioma spheroids significantly lost the majority of cellular viability in a dose-dependent manner (Figure 2C) ($p<0.001$). However, significantly viable portions of C6 glioma cells in the core of spheroids bodies still exist. At dose 60 µM, 3BP-induced C6 glioma cell death was significant ($p<0.001$) but not maximal. At dose 100 µM, 3BP-induced C6 glioma cell death was not significantly different from that induced by 60 µM (Figure 2C). A very high dose 3BP (100 µM) significantly ($p<0.001$) but not maximally decreased glioma spheroids cellular viability (Figure 2D).

4. Discussion

3BP is a promising anticancer drug that proved effective for treating almost all types of malignant cells significantly and maximally. [1,2] Our data confirmed that 3BP distorted the morphology and significantly reduced the viability of C6 glioma cells converting them into separate fragments (Figure 1A), which was associated with a maximal highly significant decrease in glioma viability (Figure 1B). That is quite consistent with our previously published data.

Herein, we cultured glioma spheroids cells over an agar layer to allow for the development of C6 glioma spheroids. The globular configuration started rapidly from the 1st day of seeding and becomes more obvious in the 2nd day and so on (Figure 2A). Herein, we did not treat glioma spheroids with 3BP immediately after seeding. Instead, we allowed the development of well-established 3D spheroids growths that were quite reasonable at day 4 post-seeding to mimic 3D tumours inside the human body (apart from developing a vasculature). We started 3BP treatment at this stage to simulate adding 3BP to well-formed tumours inside human body. Our data were promising that 3BP significantly decreased viability of C6 glioma cells inside the spheroids bodies. However, our data were less powerful than expected where glioma cells killing at a relatively high dose of 3BP (60 µM) was not so different from that at very high dose of 3BP (100 µM) (Figure 2C). Moreover, 3BP did a relatively powerful effect in significantly decreasing the sizes of glioma spheroids but still not maximally enough to eradicate such tumours (Figure 2D) as was previously reported by Ko et al. [1] In the report by Ko et al. animals were treated after 5–6 days with an intraperitoneal. injection of 1 ml freshly prepared phosphate buffered solution, PBS (2.0 mM 3-BrPA in 1 ml PBS, pH 7.5), and then for 4 days with the same dosage. That given dose in grams = 2/1000 (molar concentration in moles) X 167 (molecular weight of 3BP) X 1/1000 (given volume in litres) = 0.334 mg/rat that is almost 1 mg/kg (weight of each rat is about 330 grams).

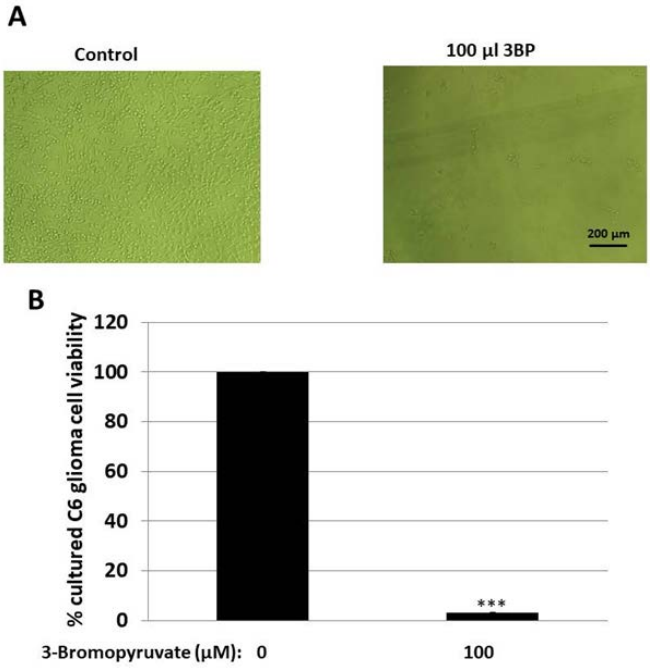

Figure 1.

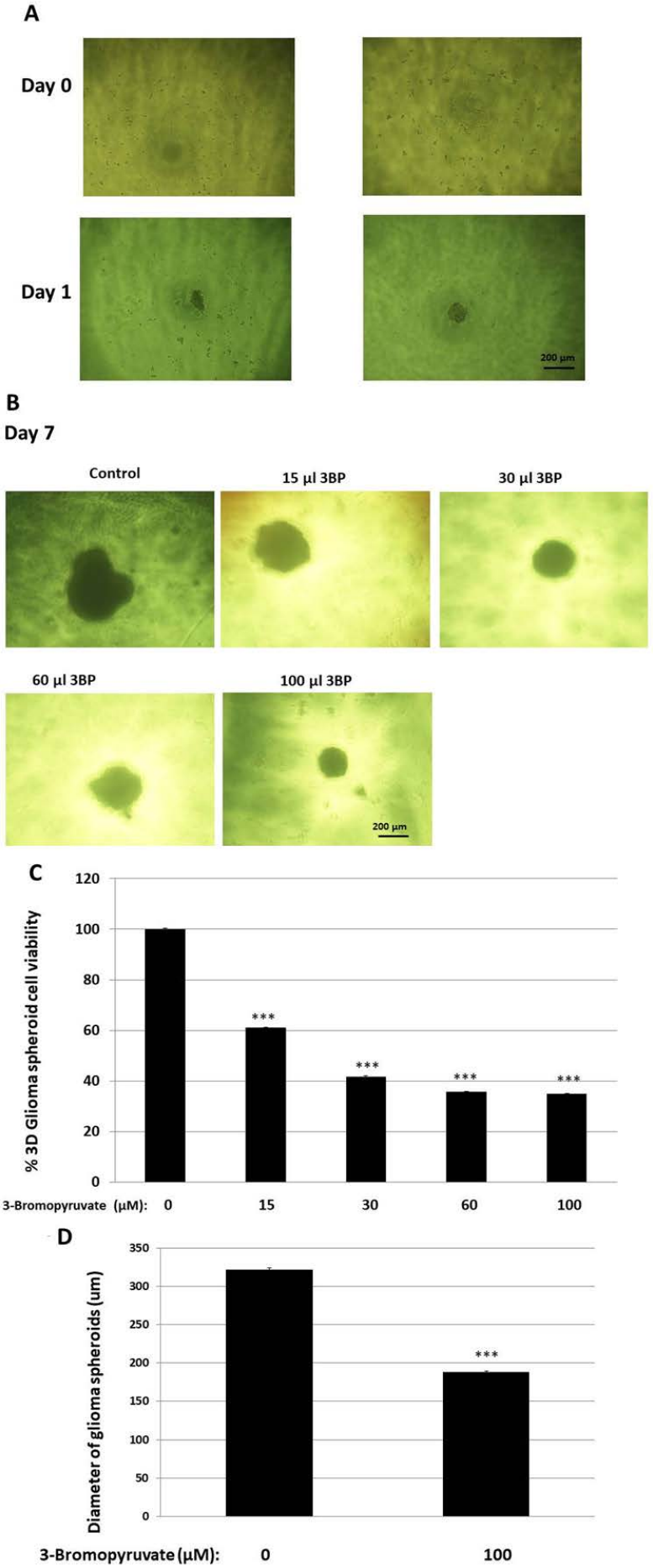
A
Day 0
Day 1
200 µm
B
Day 7
Control
15 µl 3BP
30 µl 3BP
60 µl 3BP
100 µl 3BP
200 µm
C
% 3D Glioma spheroid cell viability
120
100
80
60
40
20
0
***
***
***
***
3-Bromopyruvate (µM): 0 15 30 60 100
D
Diameter of glioma spheroids (um)
350
300
250
200
150
100
50
0
***
3-Bromopyruvate (µM): 0 100

Figure 2.

In conclusion, further research studies are highly needed to investigate the causes beyond the discrepancy between 3BP effects in 2D cell cultures versus 3D glioma spheroids. Pharmacodynamics and pharmacokinetics of 3BP can be maximally modified for better potentiation of 3BP-induced anticancer effects and minimizing the unwanted side effects of 3BP. Formulating 3BP inside lipid nanocarriers (liposomes), PEGylated liposomes or targeted liposomes may improve 3BP-induced tumor cells killing, hide 3BP from thiol groups in the circulation and minimize the unwanted side effects. [3]

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Conflict of Interest

The author declares that there is no financial or non-financial competing interests with any other partner. There is no financial benefit. The article is fully supported by the author.

Disclosure

The authors report no conflicts of interest in this work.

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