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D-Amino acid oxidase-induced oxidative stress, 3-bromopyruvate and citrate inhibit angiogenesis, exhibiting potent anticancer effects

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Abstract Angiogenesis is critical for cancer growth and metastasis. Steps of angiogenesis are energy consuming, while vascular endothelial cells are highly glycolytic. Glioblastoma multiforme (GBM) is a highly vascular tumor and this enhances its aggressiveness. D-amino acid oxidase (DAO) is a promising therapeutic protein that induces oxidative stress upon acting on its substrates. Oxidative stress-energy depletion (OSED) therapy was recently reported (El Sayed et al., Cancer Gene Ther, 19, 1-18, 2012). OSED combines DAO-induced oxidative stress with energy depletion caused by glycolytic inhibitors such as 3-bromopyruvate (3BP), a hexokinase II inhibitor that depleted ATP in cancer cells and induced production of hydrogen peroxide. 3BP disturbs the Warburg effect and antagonizes effects of lactate and pyruvate (El Sayed et al., J Bioenerg Biomembr, 44, 61-79, 2012). Citrate is a natural organic acid capable of inhibiting glycolysis by targeting phosphofructokinase. Here, we report that DAO, 3BP and citrate significantly inhibited angiogenesis, decreased the number of vascular branching points and shortened the length of vascular tubules. OSED delayed the growth of C6/DAO glioma cells. 3BP combined with citrate delayed the growth of C6 glioma cells and decreased significantly the number and size of C6 glioma colonies in soft agar. Human GBM cells (U373MG) were resistant to chemotherapy e.g. cisplatin and cytosine arabinoside, while 3BP was effective in decreasing the viability and disturbing the morphology of U373MG cells.

Keywords D-amino acid oxidase · 3-bromopyruvate · Citrate · Angiogenesis · Glycolysis double inhibition · Glioblastoma

Abbreviations

3BP	3-Bromopyruvate
CHCA	α-cyano-4-hydroxycinnamic acid
DAO	D-amino acid oxidase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GBM	Glioblastoma multiforme
hEGF	Human epidermal growth factor
hFGF-B	Human fibroblast growth factor-B
HPA	β-hydroxypyruvate
HUVECs	Human umbilical vein endothelial cells
MCT	Monocarboxylate transporters
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OSED	Oxidative stress-energy depletion
PFK	Phosphofructokinase
pHe	Extracellular pH
VEGF	Vascular endothelial growth factor

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Introduction

Glioblastomas are the most aggressive primary brain tumor with a dismal prognosis and low median survival (less than 12 months) due to resistance to radiation and chemotherapy (Bao et al. 2006). High-grade gliomas e.g. glioblastoma multiforme (GBM) is characterized by extensive microvascular proliferation and a higher degree of vascularity than low-grade gliomas and normal brain (Lopes 2003).

D-amino acid oxidase (DAO) plays important roles in the pathophysiology of schizophrenia (Abou El-Magd et al. 2010a; Ono et al. 2009). DAO may be regarded as a promising anticancer therapeutic whether used in protein therapy (Fang et al. 2002) or gene therapy (Stegman et al. 1998; El Sayed et al. 2012a). DAO induces production of hydrogen peroxide by acting on D-amino acids e.g. D-serine (Park et al. 2006) or D-proline (Fang et al. 2002). DAO for anticancer therapy may be obtained from red yeast (*rhodotorula gracilis*) (Stegman et al. 1998), pig (Fang et al. 2002) or mouse (El Sayed et al. 2012a). Differences among human, porcine and yeast DAO were reported (Kawazoe et al. 2007a). Recently, we reported oxidative stress-energy depletion (OSED) therapy using DAO combined with glycolytic inhibition (El Sayed et al. 2012a).

Angiogenesis in tumors is critical for cancer cell survival, proliferation, invasion and metastasis (Folkman 1971). There is a positive correlation between the histological grade of tumors and increased vascular density (Brem et al. 1972; Aronen et al. 1994). Also, histological grading of astrocytomas correlates with glucose consumption (Di Chiro et al. 1982). Antiangiogenic agents are gaining popularity in cancer treatment. For example Bevacizumab, a humanized monoclonal antibody that binds to and inhibits the activity of vascular endothelial growth factor (VEGF), was successful in the treatment of pediatric GBM especially in combination with current chemotherapeutics (Vredenburgh et al. 2007). Targeting angiogenesis seems promising for cancer therapy.

3-bromopyruvate (3BP) is a hexokinase II (HK II) inhibitor that exerts potent anticancer effects through inhibition of glycolysis (Ko et al. 2001). Interestingly, gliomas are driven by glycolysis (Oudard et al. 1997) which correlates with tumor angiogenesis (microvascular density) and tumor microvascular blood volume (Aronen et al. 2000). 3BP antagonizes the actions of lactate (El Sayed et al. 2012b) which may be produced through the Warburg effect (production of lactate as an end product of glycolysis in cancer cells even in the presence of oxygen) (Warburg 1956). 3BP is transported into cancer cells by the same transporters as for lactate and pyruvate i.e. monocarboxylate transporters (MCT) (Zorzano et al. 2000) which can be blocked by α-cyano-4-hydroxycinnamic acid (CHCA) (Colen et al. 2011). The influx of lactate through endothelial cell MCT1 was reported to enhance tumor angiogenesis through an NF-κB/IL-8 pathway (Végran et al. 2011). MCT1 expression was reported in C6 glioma (Mac and Nałecz 2003), an experimental model for studying the growth and invasion of GBM (Grobben et al. 2002). Another glycolytic inhibitor, citrate, was reported to act synergistically with 3BP (El Sayed et al. 2012b). Citrate is a potent inhibitor of glycolysis through inhibition of phosphofructokinase (Marín-Hernández et al. 2006), and a safe natural substance available in citrus fruits.

In this study, we investigated the effects of DAO-induced oxidative stress, 3BP and citrate on the angiogenic power of vascular endothelial cells in vivo. We investigated also novel anticancer effects of these treatments.

Materials and methods

Chemicals and reagents

D-Serine, D-proline, agar and citrate were purchased from Wako (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), 3-bromopyruvate (3BP), agarose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cis-platinum(II)-diammine-dichloride (Cisplatin), cytosine β-D-arabinofuranoside (cytosine arabinoside) and porcine kidney D-amino acid oxidase (DAO) were purchased from Sigma (St. louis, MO, USA). One DAO unit was defined as that which can oxidatively deaminate one micromole of D-alanine to pyruvate per min at pH 8.3 at 25 °C in the presence of catalase (Lot 18 H0370, 0.15 units/mg solid). DMEM/F12, Geniticin (G418) and the Penicillin-Streptomycin-Glutamine antibiotic mixture were from Invitrogen Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). α-cyano-4-hydroxycinnamic acid (CHCA) was obtained from Nacalai Tesque (Kyoto, Japan). HuMedia-EG-2, human epidermal growth factor (hEGF), hydrocortisone hemisuccinate, human fibroblast growth factor-B (hFGF-B), heparin and the gentamicin-amphotericin antibiotic mixture were purchased from Kurabo (Osaka, Japan).

Maintenance of vascular endothelial cells

Human umbilical vein endothelial cells (HUVECs) (Kurabo, Osaka, Japan) were maintained in HuMedia-EG-2 supplemented with FBS 2 % (v/v), hEGF (10 ng/ml), hydrocortisone hemisuccinate (1.34 μg/ml), hFGF-B (5 ng/ml), heparin (10 μg/ml) and antibiotics (0.1 %v/v, gentamycin 50 mg/ml and amphotericin-B 50 μg/ml).

In vitro angiogenesis assay

Using an in vitro angiogenesis assay kit (Millipore, CA, USA), which contains a matrix mixture (ECMatrix ™) and diluent

10X buffer, the inhibitory effects of DAO, 3BP, citrate and CHCA against angiogenesis were investigated. Briefly, diluent 10X buffer (100 μl) was added to 900 μl of ECMatrix™ in a sterile microfuge tube. After slow mixing, the solution was kept on ice to avoid solidification. Then, 50 μl of this mixture was added per well of a pre-cooled 96-well tissue culture plate. Incubation at 37 ^{0}C for 1 h was done to allow the matrix solution to solidify. Endothelial cells (1×10^4 HUVECs) were seeded on the surface of the polymerized ECMatrix™. Incubation in a CO_2 incubator was conducted for 30 min to help cellular adhesion. HUVECs received treatment in the form of 3BP (60 μM), citrate (9 mM), and DAO protein (50 mU/ml) together with D-proline (20 mM), D-serine (20 mM) or CHCA (10 mM). Cells were incubated for 16 h to allow for the development of cellular network structures. Tube formation was inspected under a light microscope (40 X magnification) after staining with a crystal violet solution (0.5 % crystal violet in a solution of 50 % ethanol and PBS containing 5 % formaldehyde). Images were captured using a camera attached to a phase contrast microscope (Nikon, Tokyo, Japan). The degree to which angiogenesis has progressed was estimated according to the pattern recognition values provided by the manufacturer (Table 1). Tubule branch points were counted; the total length of capillary tubes 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.

Growth delay assay

C6 glioma cells were maintained in DMEM/F12 supplemented with 2.5 % FBS, 15 % horse serum and antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin and 292 μg/ml L-glutamine). The stable transformant, C6/DAO, was derived from C6 glioma cells transfected with pEF-BOS-Neo mDAO as reported previously (Park et al. 2006). C6/DAO cells overexpressing mouse DAO were maintained in DMEM/F12 supplemented with 2.5 % FBS, antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin and 292 μg/ml L-glutamine), 15 % horse serum and 250 μg/ml G418. C6 and C6/DAO glioma cells (1×10^4) were seeded in 6-cm well plates in triplicate. Cells were allowed to grow in a humidified CO_2 incubator for 3 days. After that, C6 and C6/DAO cells were counted and divided into 7 groups: the first group was the control, the second group received D-serine (5 mM), the third group 3BP (15 μM), the fourth group 3BP (15 μM) with D-serine, the fifth group 3BP (30 μM), the sixth group 3BP (30 μM) and D-serine and the seventh group 3BP (60 μM). All the treatments lasted for 48 h. On day 5, the medium was aspirated and the cells were trypsinized and counted. Cells were plated again in new plates and allowed to grow for another 72 h. On day 8, cells were counted in all treatment groups. The effect of serial doses of 3BP and/or 3 mM citrate on the growth delay of C6 glioma cells was also investigated.

Table 1 Steps of angiogenesis

Pattern of angiogenesis	Value
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible, no sprouting.	2
Visible sprouting of new capillary tubes	3
Closed polygons begin to form	4
Complex mesh-like structures develop	5

Anchorage-independent growth assay

C6 glioma cells have the power to form colonies in soft agar without being attached to substratum. The anchorage-independent growth assay was conducted as reported previously (El Sayed et al. 2012a). Effects of serial doses of 3BP (15 and 30 μM) and citrate (3 mM) were investigated on the formation and size of colonies using Image J software.

MTT viability assay

U373MG human glioblastoma cells were maintained in DMEM supplemented with 10 % FBS and 1 % antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin and 292 μg/ml L-glutamine). Equimolar serial doses of 3BP, cisplatin and cytosine arabinoside in the micromolar range (25, 50 and 100 μM) were tested for effects on the viability and morphology of U373MG cells using the MTT assay. Images were captured under a phase contrast microscope.

Results

DAO, 3BP, citrate and CHCA inhibited angiogenesis

Since DAO, citrate and 3BP exert potent anticancer effects in OSED therapy (El Sayed et al. 2012a, b), we investigated if they exhibit an anti-angiogenic effect. Potential anti-angiogenic activity would widen the spectra of those reagents as anticancer agents. As shown in Fig. 1, DAO-induced oxidative stress significantly decreased vascular tube formation (tubulogenesis) in an in vitro angiogenesis assay using both D-serine ($p<0.01$) and D-proline ($p<0.001$). With D-proline as a substrate of DAO, no vascular tubes were formed (Fig. 1a and b). 3BP strongly inhibited angiogenesis ($p<0.001$) (Fig. 1a and b). Citrate also significantly inhibited vascular tube formation ($p<0.001$) (Fig. 1a and b). DAO/D-proline, DAO/D-serine, 3BP and citrate inhibited the formation of branching points of angiogenesis tubules ($p<0.001$) (Fig. 1c). With D-serine as a substrate of DAO, vascular tube

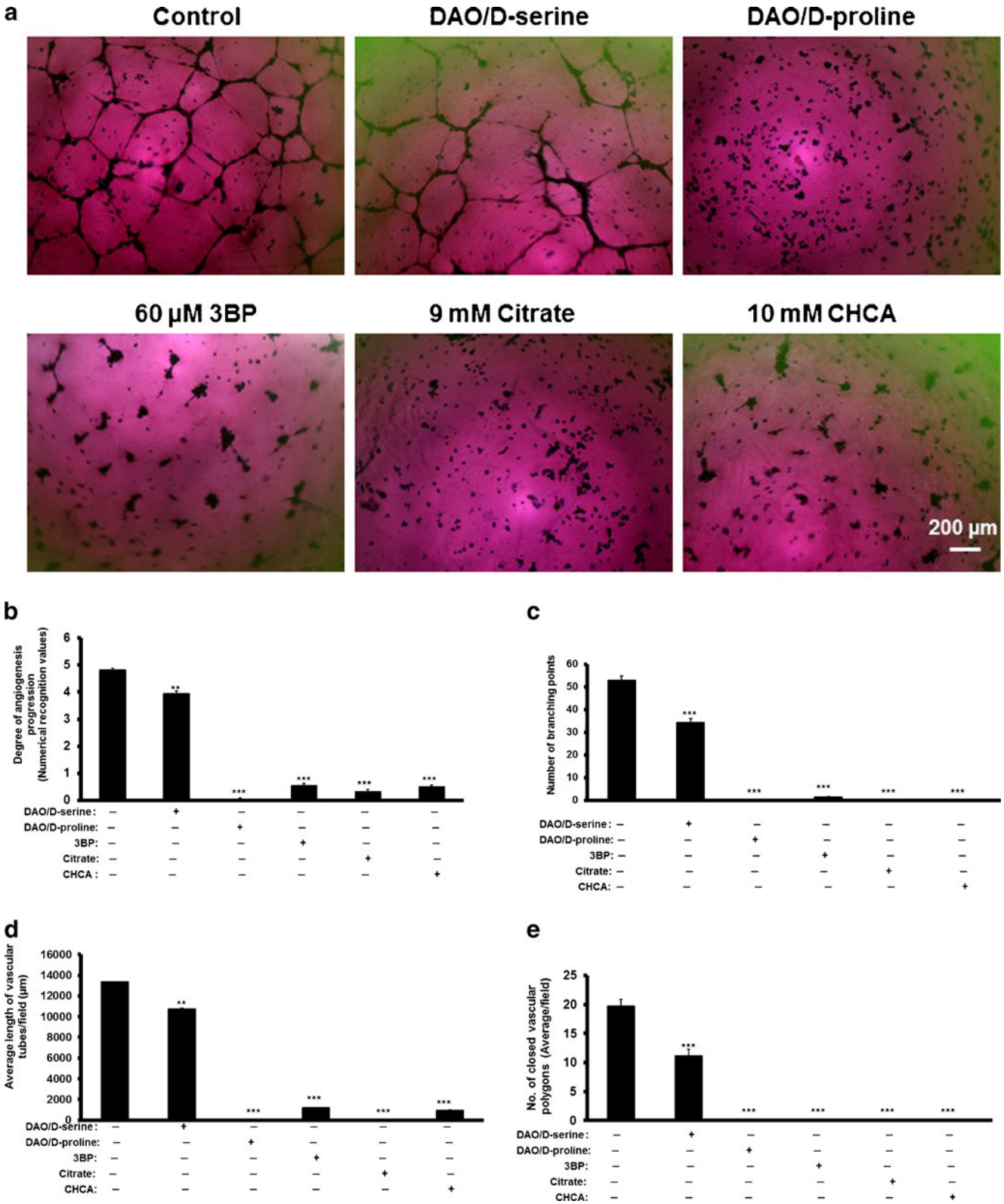

Fig. 1 DAO, 3BP, citrate and CHCA significantly inhibited angiogenesis in an in vitro assay. (**a**) Effect of DAO (50 mU/ml), 3BP (60 µM), citrate (9 mM) and CHCA (10 mM) on tubulogenesis and formation of closed polygons in HUVECs (stained with crystal violet and visualized at 40 X magnification). DAO substrates used were D-proline (20 mM) and D-serine (20 mM). (**b**) Effect on the progression of angiogenesis. (**c**) Effect of treatment on the number of vascular branching points (average no. per microscopic field). (**d**) Average length (µm) of vascular tubes per microscopic field. (**e**) Average number of closed vascular polygons per microscopic field. Data are the mean ± SEM for 3 independent experiments. ** $p<0.01$ and *** $p<0.001$ indicate a significant difference between the control and treatment conditions

formation and the average total length of vascular tubes per microscopic field decreased significantly ($p<0.01$). D-proline was effective as a substrate of DAO as DAO/D-proline inhibited the formation of vascular tubes significantly ($p<0.001$) (Fig. 1d). DAO/D-proline, DAO/D-serine, 3BP and citrate decreased the number of closed vascular polygons significantly ($p<0.001$) (Fig. 1e). Interestingly, CHCA (inhibitor of MCT) significantly inhibited the tubulogenesis of HUVECs ($p<0.001$) (Fig. 1a and b). CHCA inhibited the formation of vascular tubules, vascular branching points and vascular polygons (Fig. 1a–e).

OSED delayed growth of C6 glioma cells

Tumors recur after treatment mainly because not all the tumor cells are killed. To investigate the effect of OSED on the killing of cancer cells, we performed a growth delay assay which allows tumor cells already treated with OSED to recover, so as to investigate the ability of cancer cells to regrow after withdrawal of treatment (Genç et al. 2004). As illustrated in Fig. 2, the proliferation phase allows C6 glioma cells to reach confluency, while the treatment phase represents the effect of treatment on the viability of confluent cells. Whenever tumor cells fail to grow or their growth becomes delayed (in the growth delay phase), this indicates how powerful the treatment was. C6 glioma cells were treated with D-serine, 3BP and citrate as indicated in Fig. 2a.

Previously we reported the significant effect of a combination of DAO/D-serine and 3BP on the viability of C6 glioma (El Sayed et al. 2012a). We investigated here if this OSED (DAO/D-serine combined with 3BP) treatment still delayed the growth of C6 glioma cells after the withdrawal of treatment. Our data revealed that treatment of C6 glioma cells with D-serine did not result in a significant delay in growth and did not have any significant combinatory effect with serial doses of 3BP (Fig. 2b). D-serine delayed significantly the growth of C6/DAO cells ($p<0.001$) (Fig. 2c). Serial doses of 3BP delayed the growth of C6 and C6/DAO cells ($p<0.001$) (Fig. 2b and c). Citrate (3 mM) delayed the growth of C6 glioma ($p<0.001$) although the growth of C6 glioma cells continued (Fig. 2d). Serial doses of 3BP (15 and 30 μM) and a small dose of citrate (3 mM) were effective in delaying the growth of C6 glioma ($p<0.001$). Glycolysis double inhibition using serial doses of 3BP combined with citrate resulted in a significant growth delay effect on C6 glioma ($p<0.001$) which was maintained even after withdrawal of treatment and was stronger than that using a high dose of 3BP (60 μM) (Fig. 1b and c) or a high dose of citrate alone (Fig. 2d).

Anchorage-independent growth assay

To study the anticancer effect of glycolysis double inhibition further, we investigated the effect of serial doses of 3BP

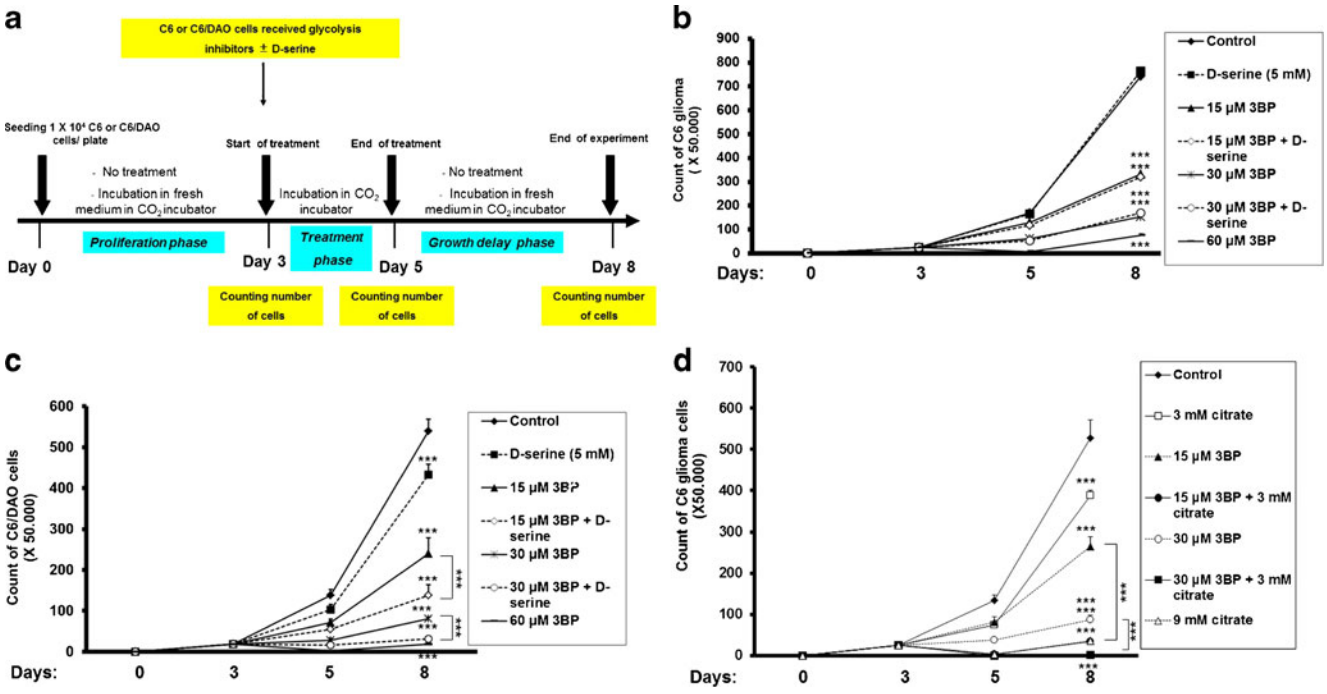

Fig. 2 Growth delay assay. (**a**) Experimental study design. (**b**) Effect of D-serine combined with serial doses of 3BP on the growth of C6 glioma cells. (**c**) Effect of D-serine combined with serial doses of 3BP on the growth of C6/DAO glioma cells. (**d**) Effect of citrate (3 mM) combined with serial doses of 3BP on the growth of C6 glioma cells. Data are the mean ± SEM for 3 independent experiments. *** $p<0.001$ indicates a significant difference between the control and treatment conditions and between the different treatment conditions

combined with a low effective dose of citrate (3 mM) on the number and size of colonies of C6 glioma in an anchorage-independent growth assay. Our data revealed that the effect of 3BP on the number of colonies was dose-dependent i.e. 3BP at 15 and 30 μM significantly decreased the number of glioma colonies ($p<0.01$ and 0.001, respectively). Citrate (3 mM) significantly decreased the number and size of C6 glioma colonies ($p<0.01$) (Fig. 3a and b). Significant effects of 3BP and citrate on the size of glioma colonies were observed ($p<0.001$) (Fig. 3a and c). The combination of 3BP (15 μM) and citrate significantly decreased the number and size of glioma colonies ($p<0.001$). 3BP (15 μM) induced a significant decrease in the size of C6 glioma colonies ($p<0.001$). A greater decrease in the size of C6 glioma colonies occurred with a larger dose of 3BP (30 μM) ($p<0.001$). The combination of serial doses of 3BP and a small dose of citrate (3 mM) was significant in inducing a further decrease in the size of C6 glioma colonies ($p<0.001$). Colony size was minimal when 30 μM 3BP was combined with citrate (Fig. 3a and c).

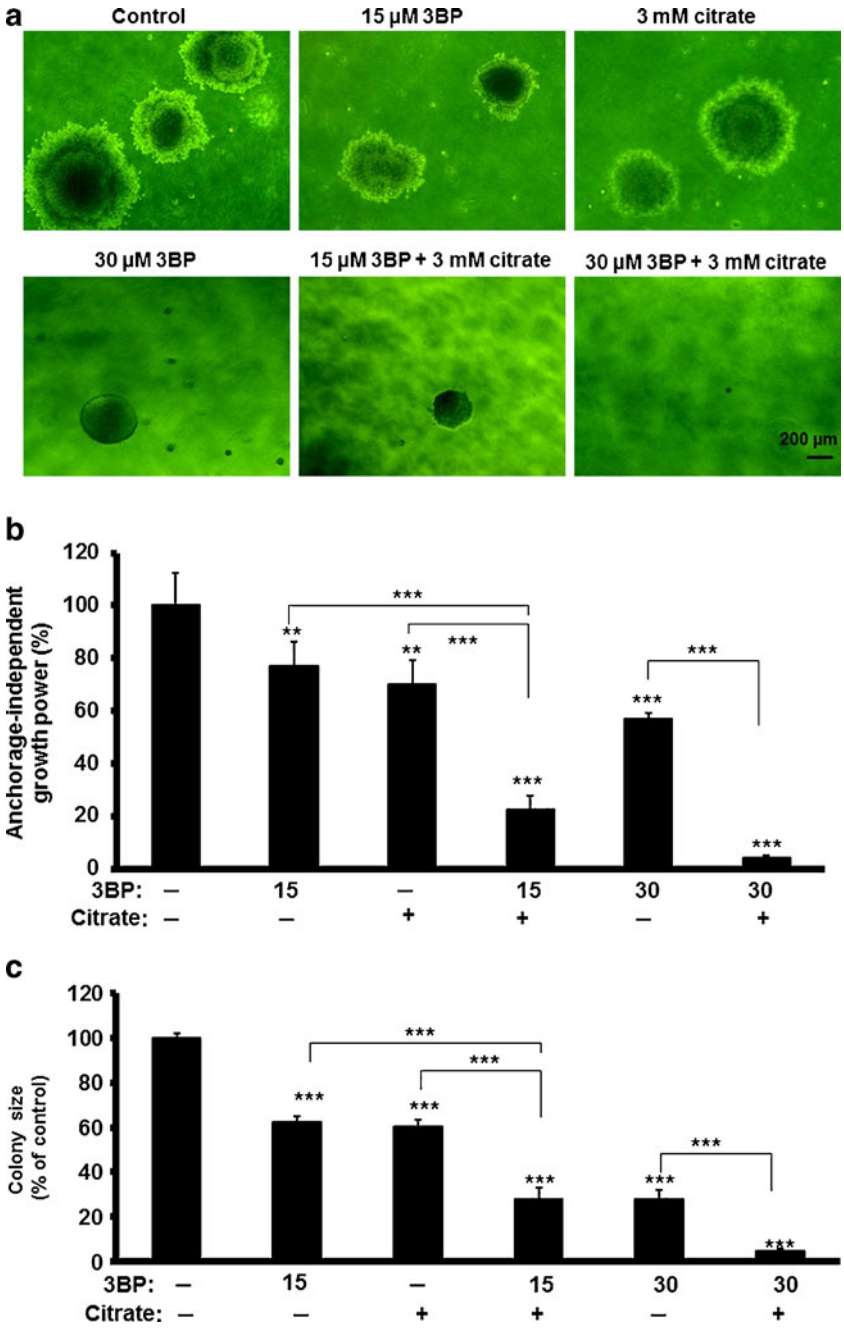

Fig. 3 Anchorage-independent growth assay. (**a**) Effect of combining citrate with serial doses of 3BP on C6 glioma colonies. (**b**) Effect of citrate (3 mM) plus serial doses of 3BP on the number of C6 glioma colonies. (**c**) Effect of citrate (3 mM) plus serial doses of 3BP on the size of C6 glioma colonies. Data are the mean ± SEM for 3 independent experiments. ** $p<0.01$ and *** $p<0.001$ indicate a significant difference between the control and treatment conditions and between the different treatment conditions

3BP is stronger than cisplatin and cytosine arabinoside as an anticancer drug

Cisplatin and cytosine arabinoside are among the most popular chemotherapeutics used in the treatment of gliomas and other types of cancer. They exert anti-glioma effects in the micromolar range (1–100 μM) (Walker and Allen 1988; Wolff et al. 2002; Kelsen et al. 1991) which is similar to the range reported for 3BP (El Sayed et al. 2012a; Qin et al. 2010). We investigated the comparative effects of 3BP versus cisplatin and cytosine arabinoside and their combination on the morphology and viability of the human GBM cell line U373MG. Our data revealed that U373MG cells were less sensitive to cisplatin and cytosine arabinoside in that neither agent altered the fibroblastic morphology (Fig. 4a). 3BP dramatically distorted the morphology and caused massive cell damage in U373MG. Both cisplatin and cytosine arabinoside decreased viability significantly but

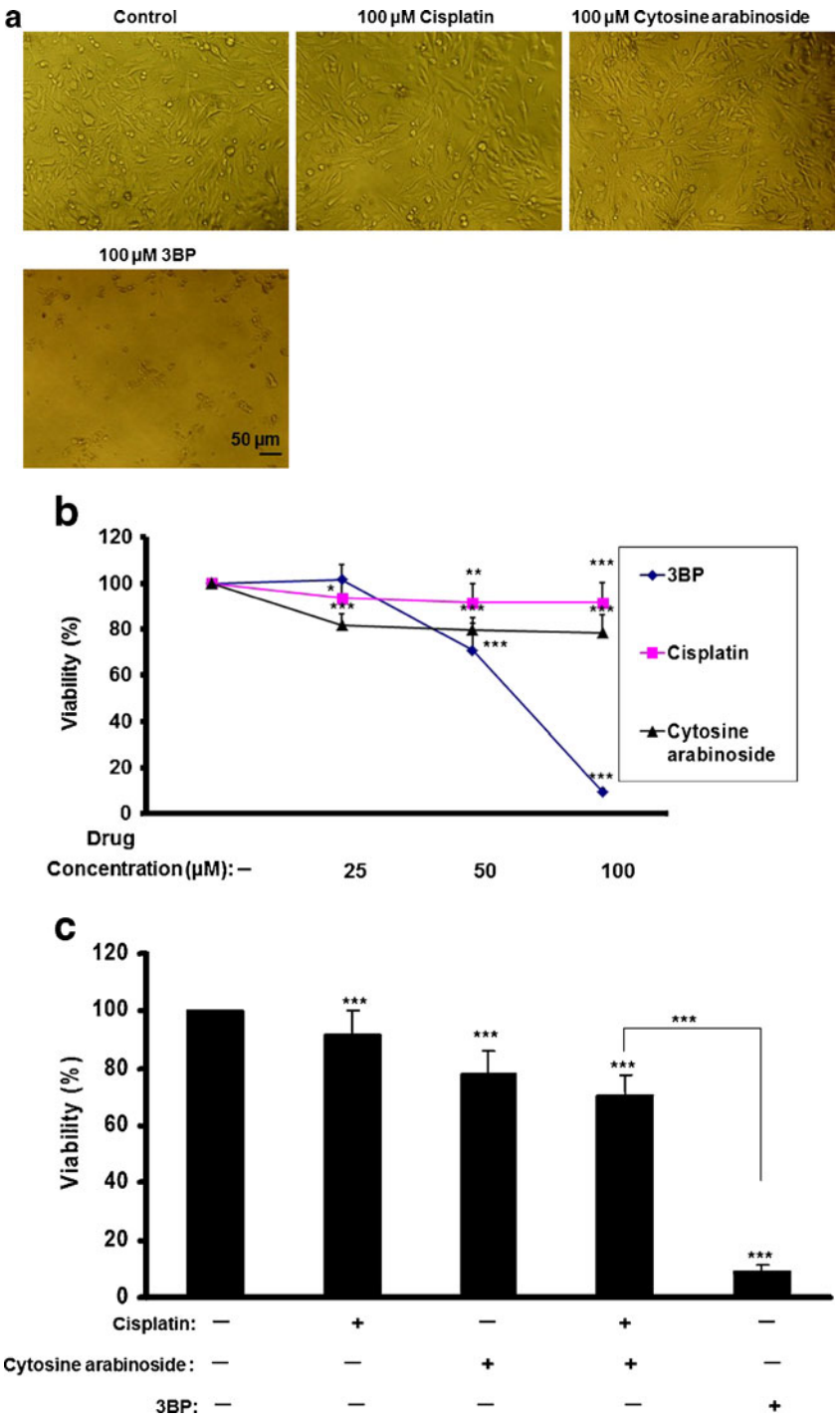

Fig. 4 3BP is stronger than ciaplatin and cytosine arabinoside in decreasing the viability of U373MG cells . (**a**) 3BP distorted the morphology of U373MG cells, while cisplatin and cytosine arabinoside did not. (**b**) 3BP decreased the viability of U373MG more strongly than cisplatin and cytosine arabinoside. (**c**) 3BP decreased the viability of U373MG cells more strongly than a combination of cisplatin and cytosine arabinoside. Data are the mean ± SEM for 3 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ indicate a significant difference between the control and treatment conditions and between the different treatment conditions

with a mild degree of GBM cell death (< 20 % decrease in viability at 100 μM) in a dose-independent manner (Fig. 4b). This lack of dose-dependent cytotoxic effects of cisplatin and cytosine arabinoside meant that the viability of U373MG cells was maintained even at large doses (Fig. 4a–c) and after the combination of cisplatin and cytosine arabinoside (Fig. 4c). 3BP caused a dose-dependent decrease in the viability of U373MG cells (Fig. 4b). 3BP was stronger than both cisplatin and cytosine arabinoside (alone and in combination) in reducing the viability of U373MG cells (Fig. 4a–c). 3BP (100 μM) caused the greatest decrease in viability (>90 % at 100 μM). 3BP was stronger than both cisplatin and cytosine arabinoside in disturbing the morphology of U373MG cells (data not shown).

Discussion

Tumor angiogenesis is a critical process by which new blood vessels are formed from pre-existing ones. Angiogenesis is vital for tumor growth and precedes malignant tumor formation (Plate 1999). It is also necessary for cancer progression and metastasis (Folkman 1971). GBM, the most aggressive type of glioma has a poor prognosis despite aggressive multimodal therapeutic approaches i.e. surgery, radiotherapy and chemotherapy (Stupp et al. 2005). GBM is very aggressive due to a massive and diffuse infiltration of the surrounding normal brain tissue (Maher et al. 2001), and is among the most highly angiogenic tumors in which the degree of neovascularization predicts the degree of malignancy and prognosis (Lopes 2003). Prolonged survival was reported in patients suffering from GBM who responded well to anti-angiogenic treatment (Sorensen et al. 2011). The development of more effective novel therapeutic strategies for glioma seems essential.

Targeting angiogenesis seems to be a promising treatment for cancer. Vascular endothelial cells are highly glycolytic and can withstand hypoxia (Quintero et al. 2006). Critical steps of angiogenesis include endothelial cell proliferation, migration, adhesion and reorganization, which are energy-dependent (Rahman et al. 2010). Anticancer agents targeting steps of angiogenesis are promising, especially if they have additional mechanisms for their anticancer effects.

The activity of DAO toward its substrates including D-serine results in the formation of β-hydroxypyruvate (HPA, a structural analog of 3BP) which is cytotoxic to glial cells (Chung et al. 2010), while DAO activity toward D-DOPA represents an alternative pathway for the production of dopamine (Kawazoe et al. 2007b). Activity of DAO can be inhibited by antipsychotics e.g. chlorpromazine (Iwana et al. 2008) and risperidone (Abou El-Magd et al. 2010a). To determine the structural basis for its catalytic activity, we revealed the crystal structure of wild-type human DAO (Kawazoe et al. 2006) and reported the production of mutant P-219-L human DAO through engineering of the wild-type enzyme (Abou El-Magd et al. 2010b).

Our data revealed that DAO/D-proline, DAO/D-serine, 3BP and citrate induced significant anti-angiogenic effects. Using porcine DAO as an anticancer agent, D-proline was more powerful than D-serine as a substrate for inhibition of angiogenesis (Fig. 1a–e). DAO/D-proline, DAO/D-serine, 3BP and citrate significantly decreased the progression of angiogenesis, inhibited the formation of vascular branching points, inhibited vascular tubulogenesis, decreased the length of vascular tubes and inhibited the formation of vascular polygons (Fig. 1a–e). Interestingly, DAO coupled with its substrates, 3BP and citrate, have other anticancer effects i.e. DAO/D-proline and DAO/D-serine induced oxidative stress and decreased significantly the survival of cancer cells (Fang et al. 2002; El Sayed et al. 2012a). 3BP has many mechanisms of action as an anticancer agent e.g. depletion of energy and reducing equivalents in cancer cells, oxidative stress (El Sayed et al. 2012a) and antagonistic effects on lactate and pyruvate (El Sayed et al. 2012b). 3BP was reported to exert synergistic effects with many anticancer agents including citrate (El Sayed et al. 2012b).

Interestingly, CHCA (MCT inhibitor) was a powerful inhibitor of angiogenesis with a novel mechanism of action through inhibition of vascular tubulogenesis, prevention of the development of branching points, reduction in the length of vascular tubes and inhibition of the formation of vascular polygons (Fig. 1a–e). Our results agree with previous reports that lactate transported through the endothelial cell MCT1 enhances tumor angiogenesis (Végran et al. 2011) i.e. inhibition of MCT using CHCA had an antiangiogenic effect as CHCA prevents the transport of lactate.

In addition to its anti-angiogenic effect (Fig. 1a–e), citrate exerted a synergistic effect with cisplatin in the treatment of mesothelioma (Zhang et al. 2009). Citrate (4–6 g/day) has been used in the treatment of thyroid carcinoma in a child (Halabe Bucay 2009), the treatment of stomach cancer (Lu et al. 2011) and the treatment of antibiotic-resistant postoperative wounds in cancer patients (Nagoba et al. 2011). Citrate induced dose-dependent lympholytic activity in leukemia and lymphoma cell lines with minimal effects on normal cells e.g. peripheral blood mesenchymal cells (Yousefi et al. 2004).

Extracellular pH (pHe) in tumors is acidic, ranging from 6.2 to 6.9 (Cardone et al. 2005), which adds a chemoresistant advantage to tumor cells against chemotherapeutics that are basic in nature e.g. mitoxantrone and doxorubicin. The activity of chemotherapeutics that are acid in nature e.g. 5-fluorouracil and chlorambucil is enhanced by low pHe (Mahoney et al. 2003). Citrate (citric acid) is acidic in solution and its activity may be enhanced by low pHe in tumors, which may support the use of citric acid as a future cancer chemotherapeutic. In vitro, citrate caused a decrease

in pH of the culture medium (DMEM) in a dose-dependent manner i.e. 0.1 % citrate (4.76 mM) maintained an alkaline DMEM (pH: 7.2), while 0.25 % citrate (10.71 mM) reduced the pH to 6.22 (Chan et al. 1999). Amaral et al. reported that a high dose of citrate, e.g. 15 % citrate (0.7 M), for 7 days moderately decreased the viability of macrophages (Amaral et al. 2007). Also, Malheiros et al. reported progressive and continuous cell growth of NIH3T3 fibroblasts treated with 0.1 % citrate for 7 days, while moderate cytotoxic effects occurred at 0.5 % (23.8 mM) (Malheiros et al. 2005). The viability of human dental pulp cells was mildly affected by 0.1 % citrate and moderately affected by 2.25 % citrate after treatment for 5 days (Chan et al. 1999). Based on that, the in vitro cytotoxicity of high doses of citrate e.g. more than 0.25 %, may be partially due to citrate-induced acidosis.

Importantly, there is a close relationship between glycolysis and angiogenesis in cancer cells: e.g. in ovarian cancer cells, the Akt oncogene and VEGF are activated (Schmidt et al. 2008). Ovarian cancer (Krockenberger et al. 2007) and colorectal cancer cells exhibit active angiogenesis and high levels of glucose metabolism (Mazurek et al. 2000). Akt activation was reported to cause the high glycolytic phenotype of cancer cells (Elstrom et al. 2004) and angiogenesis, i.e. PI3/Akt signalling mediates expression of VEGF (Xia et al. 2006). VEGF stimulates tumor angiogenesis. VEGF expression correlates with a poor prognosis for breast (Linderholm et al. 2003) and ovarian (Hefler et al. 2006) cancer. VEGF has functional autocrine activity and significance for both endothelial cells and tumor cells (Bachelder et al. 2001).

As tumor cells may begin to proliferate immediately after chemotherapy, the growth delay assay evaluates the proliferative potential of tumor cells (Genç et al. 2004). The growth delay phase in the assay reflected the ability of C6 glioma cells to resume growth after the removal of D-serine and 3BP treatment (Fig. 2a). D-Serine did not delay the growth of C6 glioma cells and did not have a significant combined effect with serial doses of 3BP on growth (Fig. 2b). DAO/D-serine-induced oxidative stress delayed the proliferation of C6/DAO cells significantly (Fig. 2c). A significant combinatory effect was found for 3BP and DAO/D-serine in delaying the proliferation of C6 glioma cells (Fig. 2c). Significant growth delay occurred when serial doses of 3BP were combined with citrate (Fig. 2d). This can be explained in light of the massive decrease in glioma energetics when 3BP was combined with citrate (glycolysis double inhibition) (El Sayed et al. 2012b).

A significant decrease in anchorage-independent growth of C6 glioma colonies was observed when serial doses of 3BP were combined with citrate as it regards the number and size of glioma colonies (Fig. 3a–c). U373MG cells are resistant to chemotherapy and radiotherapy (Lau et al. 2008). U373MG cells express the multidrug resistance-associated protein (MRP) that causes multidrug resistance not through P-glycoprotein (Mohri et al. 2000). The morphology was totally distorted with a loss of fibroblastic morphology of GBM cells on treatment using 3BP, while cisplatin and cytosine arabinoside did not affect the morphology of U373MG (Fig. 4a). Interestingly, 3BP was more effective than some currently used chemotherapeutics e.g. cisplatin and cytosine arabinoside. The anticancer effect of 3BP was stronger than that of cisplatin and cytosine arabinoside at an equimolar dose, whether cisplatin and cytosine arabinoside were used alone or in combination (Fig. 4b and c).

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Conflict of interest The authors declare that there is no conflict of interest.

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