ORIGINAL RESEARCH

Ultrasound-guided direct delivery of 3-bromopyruvate blocks tumor progression in an orthotopic mouse model of human pancreatic cancer

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Abstract Studies in animal models of cancer have demonstrated that targeting tumor metabolism can be an effective anticancer strategy. Previously, we showed that inhibition of glucose metabolism by the pyruvate analog, 3-bromopyruvate (3-BrPA), induces anticancer effects both in vitro and in vivo. We have also documented that intratumoral delivery of 3-BrPA affects tumor growth in a subcutaneous tumor model of human liver cancer. However, the efficacy of such an approach in a clinically relevant orthotopic tumor model has not been reported. Here, we investigated the feasibility of ultrasound (US) image-guided delivery of 3-BrPA in an orthotopic mouse model of human pancreatic cancer and evaluated its therapeutic efficacy. In vitro, treatment of Panc-1 cells with 3-BrPA resulted in a dose-dependent decrease in cell viability. The loss of viability correlated with a dose-dependent decrease in the intracellular ATP level and lactate production confirming that disruption of energy metabolism underlies these 3-BrPA-mediated effects. In vivo, US-guided delivery of 3-BrPA was feasible and effective as demonstrated by a marked decrease in tumor size on imaging. Further, the antitumor effect was confirmed by (1) a decrease in the proliferative potential by Ki-67 immunohistochemical staining and (2) the induction of apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphospate nick end labeling staining. We therefore demonstrate the technical feasibility of US-guided intratumoral injection of 3-BrPA in a mouse model of human pancreatic cancer as well as its therapeutic efficacy. Our data suggest that this new therapeutic approach consisting of a direct intratumoral injection of antiglycolytic agents may represent an exciting opportunity to treat patients with pancreas cancer.

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Introduction

The incidence of pancreatic cancer expected among men and women in the USA in 2012 is 43,920, with an estimated 37,390 deaths [1]. Only 15–20 % of patients are candidates for surgical resection, with 80 % of the patients treated with curative intent recurring in the first 2 years after surgery [2]. The standard care for locally advanced pancreatic cancer, conventional chemotherapy or

chemoradiation, increases median survival to approximately 10 to 13 months, but rarely results in long-term survival due to the inherent resistance of pancreatic tumors to chemotherapy and radiotherapy [3, 4].

The low perfusion found in pancreatic malignant lesions compared to the normal pancreas is a major contributing factor of resistance to chemotherapy [5–7]. The decreased blood supply narrows the therapeutic window by impeding the adequate delivery of systemic chemotherapy to the tumor cells. Additionally, it reduces the nutrient supply, resulting in hypoxia of the tumor cells, up-regulation of survival pathways, and an increase in glucose metabolism [8]. Each one of these factors can cause resistance to chemotherapy; however, together they result in an extremely resistant tumor phenotype that leads to the morbid prognosis associated with pancreatic cancer treatment [9, 10].

Recently, there has been considerable progress with the use of ultrasound (US) to deliver therapeutics directly into pancreatic tumors to overcome the physiological obstacle of low blood flow [11, 12]. We therefore hypothesized that an approach that involved US-guided delivery of an effective antiglycolytic agent directly into the tumor could circumvent the therapeutic obstacle of low blood flow and target the increased glycolytic, chemoresistant phenotype of pancreatic tumors. To test this hypothesis, we selected a nude mouse model of pancreatic cancer, using the pancreatic adenocarcinoma cell line Panc-1 as it is characterized by mutated *kras* and *p53* genotype, constitutive expression of HIF-1α and has also been shown to be resistant to nutrient deprivation [13].

Recent data show that the tumor metabolism of pancreatic cancer is regulated by oncogenic KRAS and that tumor maintenance primarily depends on glucose metabolism [14]. In addition, data from proteomic analysis demonstrated up-regulation of glycolytic enzymes in pancreatic cancer [15]. These findings support the view that tumor metabolism could be the "Achilles' heel" of cancer [16]. It is therefore not surprising that the glycolytic inhibitors, iodoacetate and 3-bromopyruvate (3-BrPA), have already shown anticancer effects in pancreatic cancer cell lines [17]. Although 3-BrPA is capable of promising effects when given as combination therapy [18] in pancreatic tumor models, until now there is no report on the efficacy of 3-BrPA as monotherapy for pancreatic cancer, especially when administered under image guidance. Here, we investigated the potential efficacy of direct ultrasound-guided intratumoral delivery of 3-BrPA to pancreatic cancer. The rationale was to possibly overcome both the physiological and molecular obstacles to drug therapy associated with treatment failure in pancreatic cancer.

Methods

Reagents, cell culture, cell viability, intracellular ATP level, and lactate production

3-BrPA was purchased from Sigma Chemical (St. Louis, MO, USA). For immunohistochemical studies, the detection kit and the Ki-67 antibody were purchased from Dako Inc. (Carpinteria, CA, USA). For apoptosis analysis, a terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphospate nick end labeling (TUNEL) kit was purchased (Millipore, Bedford, MA, USA). The human pancreatic cancer cell line Panc-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). Panc-1 cells were cultured in MEM supplemented with 10 % FBS (Invitrogen Inc, Carlsbad, CA, USA). 3-BrPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). For viability assay, Panc-1 cells were seeded in 12-well plates at a density of 5×10^4 cells/mL. After 24 h, cells were treated with increasing concentrations of 3-BrPA (10–100 μM), and after 24 h, cell viability was determined using trypan blue exclusion assay. Intracellular ATP levels were measured using Cell Titer-Glo Luminescence Cell Viability Assay kit (Promega, Durham, NC, USA) according to the manufacturer's protocol. In brief, Panc-1 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL. After 24 h, cells were treated with increasing concentrations of 3-BrPA (0–75 μM), and after 24 h, ATP levels were determined. The lactate production was estimated using the Lactate Assay kit (Bio-Vision, Mountain View, CA, USA) according to the manufacturer's instructions. In brief, Panc-1 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL. After 24 h, cells were treated with increasing concentrations of 3-BrPA (0–75 μM), and after 24 h, lactate levels were determined.

Orthotopic tumor model

Animal studies were performed as approved by the Johns Hopkins University Animal Care and Use Committee. For the in vivo experiments, 3–4-week-old female athymic nude mice (body weight, 20–26 g) were used (Crl:NU-*Foxn1*[nu] strain; Charles River Laboratory, Germantown, MD, USA). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Panc-1 tumor cells (1.5×10^6 cells in 50 μL medium/mouse) were orthotopically implanted in anesthetized mice. Briefly, a small left abdominal flank incision was made, and the spleen and pancreas were exteriorized. Panc-1 tumor cells were injected into the tail of the pancreas with a Hamilton syringe. A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage.

US-guided intratumoral injection

Two to 4 weeks after tumor implantation, animals underwent US imaging of the pancreas, to confirm the presence of a pancreatic tumor (VEVO2100, Visual Sonics Inc., Toronto, Ontario, Canada). The ultrasound equipment was used according to the manufacturer's (VEVO2100, Visual Sonics Inc., Toronto, Ontario, Canada) guidelines. A MS-550D MicroScan transducer (probe) with 40 MHz (broadband width 22–55 MHz) suitable for mouse tumor model imaging was used. All intratumoral injections were done under US guidance using a 27-gauge needle. The volume of the solution injected was five times the tumor volume ($1/2 \times \alpha \times \beta \times \gamma$, where α, β, and γ represent the length, breadth, and width in millimeters, respectively, as measured on US imaging).

For US-guided intratumoral treatments, 13 animals were randomized into two groups: group 1 consisted of mice treated with an intratumoral injection of 3-BrPA (1.75 mM) ($n=6$) and group 2 consisted of mice treated with intratumoral injection of saline ($n=7$). Tumor size was measured on a weekly basis using US.

The animals were sacrificed 4 weeks after the treatment. After sacrifice, the organs were surgically removed, fixed in 10 % formaldehyde solution, and embedded in paraffin. Histological sections were obtained along the maximum length of pancreas, and hematoxylin and eosin (H&E) staining, TUNEL staining, and Ki-67 staining were performed as described below. Histopathological examination of H&E-stained sections was performed in consultation with a pathologist.

Ki-67 proliferation index

Immunohistochemistry was performed by the standard biotin–streptavidin–peroxidase method on 4-μm-thick formalin-fixed, paraffin-embedded tissue sections. After deparaffinization in xylene and rehydration in descending concentrations of alcohol, an antigen retrieval step was performed in 10 % target retrieval solution (Dako) for 25 min using a steamer. Endogenous peroxidase was blocked by 3 % hydrogen peroxide. The sections were incubated with the diluted primary antibody overnight. The immunoreactivity was detected using the LSAB+ Kit (Dako) at room temperature according to the manufacturer's instructions. The 3,3′-diaminobenzidine (DAB) (Liquid DAB+; Dako) solution was used as a chromogen. Sections were lightly counterstained with hematoxylin. The section without primary antibody served as negative control. Ki-67-stained tissue sections were evaluated by light microscopy (Nikon SMZ800 microscope). A total of five to ten fields were viewed at ×10, and the number of Ki-67 positive cells and the total cell number were recorded. The Ki-67 labeling index was calculated using the formula: Ki-67 labeling index (%)=(the number of positive cells/the number of total cells)×100.

TUNEL assay

The TUNEL apoptosis detection kit (Millipore, Jaffrey, NH, USA) was used for DNA fragmentation fluorescence staining according to the manufacturer's protocol. In brief, tissue sections were deparaffinized and incubated with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 min. Fluorescein-conjugated avidin was applied to the sample, which was then incubated in the dark for 30 min. Positively stained fluorescein-labeled cells were visualized and photographed by fluorescence microscopy. For negative controls, TdT was omitted.

Statistical analysis

All experiments were performed independently at least three times. Statistical comparisons of data sets were carried out by Student's t tests. Change in tumor volume over time was plotted by intervention group, and the area under the curve was calculated. The area under the curve across the intervention groups was compared using Kruskal–Wallis nonparametric test. Alpha was set at 0.05.

Results

3-BrPA treatment causes cell death and ATP depletion and inhibits lactate production in Panc-1 cells

In order to assess the efficacy of 3-BrPA on human pancreatic cancer, we first analyzed the effect of 3-BrPA in vitro on Panc-1 cells. As shown in Fig. 1a, treatment with 3-BrPA resulted in a dose-dependent increase in Panc-1 cell death, with an IC_{50} of 60 ± 10 μM. Next, quantification of intracellular ATP and lactate production after 24 h of treatment with different concentrations of 3-BrPA (ranging from 0 to 75 μM) showed a dose-dependent decrease in both ATP and lactate levels (Fig. 1b, c). Increased glycolysis is a phenotypic trait almost invariably observed in human cancers that confers a selective growth advantage on transformed cells by creating an acidic environment. Our results indicate that treatment with the glycolytic inhibitor 3-BrPA depletes intracellular ATP and lactate production resulting in pancreatic cancer cell death.

US-guided intratumoral injection of 3-BrPA exhibits anticancer activity in an orthotopic tumor model of pancreatic cancer

The effects of US-guided intratumoral therapy with 3-BrPA on proliferation and apoptosis were studied by comparing the tumor Ki-67 index and TUNEL staining between treated and untreated mice 7 days after treatment. All intratumoral injections were successful, and all animals tolerated the
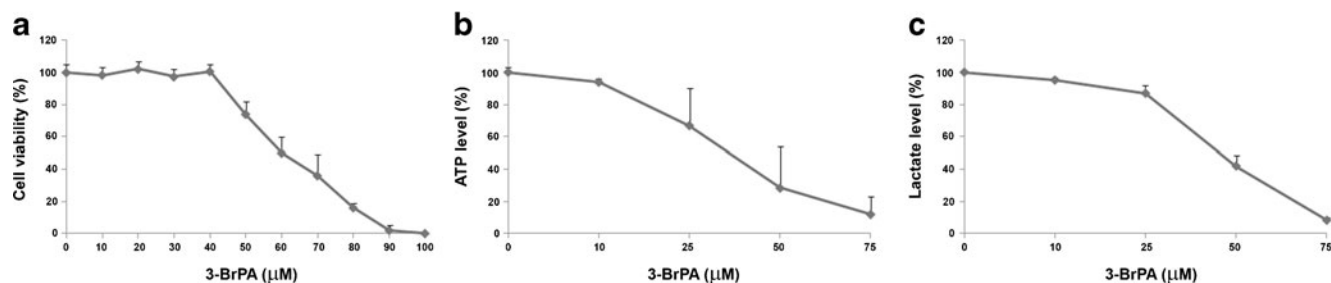
Fig. 1 3-BrPA treatment promotes cell death in Panc-1 cells. Panc-1 cells treated with 3-BrPA show a dose-dependent decrease in **a** cell viability, **b** intracellular ATP levels, and **c** lactate production

procedure well. A characteristic US image of the tumor during treatment is shown in Fig. 2. This is the first report of antiglycolytic–chemotherapeutic being delivered under US guidance in a murine model of human pancreatic cancer.

A significant difference in tumor volume (cubic millimeter) between treated and control animals was first noted 2 weeks after treatment and was maintained throughout the follow-up period, culminating at the end of the study with average tumor volumes about fivefold higher in the control than 3-BrPA-treated animals (Fig. 3a, b). In the 3-BrPA-treated group, in five out of six animals, the tumor volume at the end of the follow-up period was smaller than the initial tumor volume, indicating a profound tumor response. Only one animal in the 3-BrPA-treated group progressed, and this animal had a tumor volume that was comparable to control (saline-treated) animals at the end of the study. It is noteworthy that this animal had unusual activity (motion) possibly related to stress during anesthesia which may have affected our ability to deliver the drug effectively to the tumor as we had been able to in other animals, especially since percutaneous injection in such a small tumor requires complete immobility and is technically challenging. This could have explained the unfavorable outcome. On the other hand, all animals in the control (saline-treated) group showed a consistent increase in tumor volume throughout the study.

Examination of H&E-stained slides of the pancreatic tumor showed that intratumoral treatment with 3-BrPA resulted in a marked decrease in tumor proliferation as measured by the Ki-67 index 7 days after treatment when compared to the control treatment (Fig. 4a, b and Table 1). Analysis of TUNEL-stained slides of the pancreatic tumors demonstrated an increase in apoptosis in treated tumors when compared to control tumors (Fig. 4c, d). Further, histopathology revealed that a significant number of neutrophils were present in 3-BrPA-treated tumors (Fig. 4e). Moreover, the tumor border was well demarcated and the normal pancreatic tissue was intact. In control (saline-treated) tumors on the other hand, only a small number of neutrophils was observed 7 days after treatment, and the tumor was invading the adjacent normal pancreatic tissue (Fig. 4f). All control tumors showed invasion of the tumor into the normal pancreas, whereas 3-BrPA-treated tumors were well demarcated. None of the surrounding organs including the pancreas showed any sign of toxicity on histopathological analysis.

Fig. 2 Typical US image of a pancreatic tumor during treatment. Note the *needle in the center* of the tumor (*arrow*)
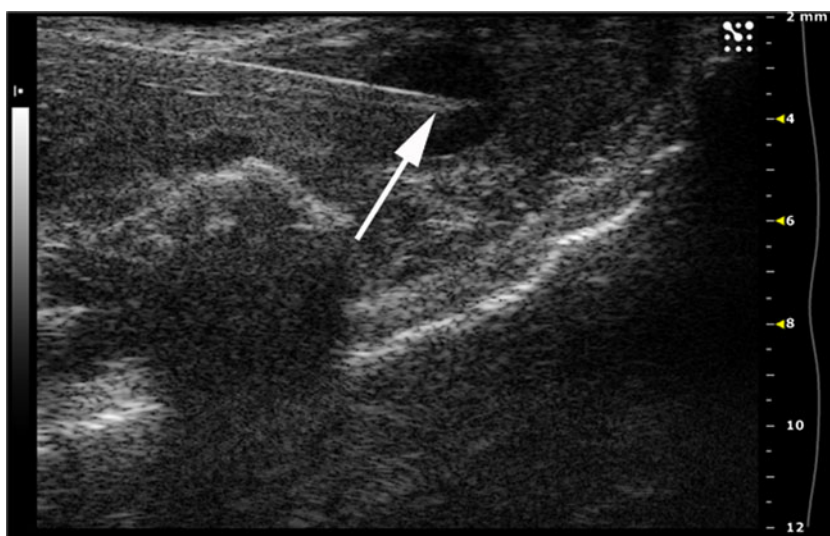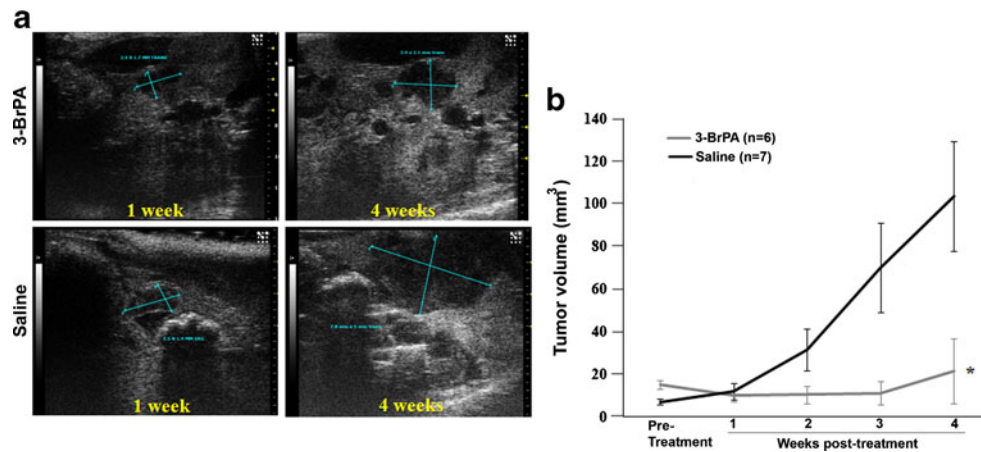
Fig. 3 US images of pancreatic tumors in control (saline-treated) and 3-BrPA-treated mice. Pancreatic tumor growth as measured on weekly US after intratumoral treatment with 3-BrPA or saline. **a** Typical US images of 3-BrPA-treated and control (saline-treated) tumors, 1 week (*left*) and 4 weeks (*right*) after treatment. **b** A graphical representation of the US image-based tumor size in control and 3-BrPA-treated animals. *P* value for comparing the area under the curve across the intervention groups is 0.022 using Kruskal–Wallis nonparametric test

Discussion

Here, we report that US-guided intratumoral treatment with 3-BrPA was a safe and effective therapy for pancreatic cancer in an orthotopic xenograft model and superior to systemic delivery. In vitro, we show that 3-BrPA treatment of Panc-1 cells induced anticancer effects by blocking ATP and lactate production. Although the cytotoxic effect of 3-BrPA on the human pancreatic cancer cell line Panc-1 has been previously reported in two different studies [17, 18], conflicting reports of IC$_{50}$ concentrations and the use of tetrazolium-based assay provided a rationale for a comprehensive examination of this cytotoxic effect. Note, previously, we have shown that 3-BrPA directly reacts with tetrazolium leading to inaccurate estimation of cytotoxic effects [19]. Our data emanating from the standard, conventional Trypan blue staining validate the cytotoxicity of 3-BrPA on Panc-1 cells. Two key contributing factors to the chemoresistance of pancreatic cancer are the decreased tumor blood flow rates coupled with increased rate of tumor cell glycolysis in comparison to normal tissues [9, 10]. This mismatched ratio results in a resistant molecular phenotype associated with poor response to therapy. In this study, we demonstrated that combining an image-guided locoregional approach with the delivery of an antiglycolytic agent could circumvent the extent of treatment resistance seen in pancreatic cancer.

US is ideally suited to guide injection of anticancer therapy directly into focal masses in the pancreas. A variety of agents have been injected into pancreatic malignancies. Chang et al. [20] demonstrated that injection of lymphocytes (cytoimplant) into pancreatic cancer under endoscopic US (EUS) guidance is feasible and safe. However, analysis of the interim results of

Fig. 4 Antitumorigenic effects of intratumoral therapy with 3-BrPA on pancreatic tumor. **a**, **b** Typical Ki-67 staining of 3-BrPA-treated tumor (*bottom*) and saline-treated tumor (*top*). **c**, **d** Typical TUNEL staining of 3-BrPA-treated tumor (*bottom*) and saline-treated tumor (*top*). **e**, **f** H&E-stained slides of 3-BrPA showing a significant number of neutrophils (*arrow*) and control (saline-treated) tumor with only a few neutrophils. (Ki-67 and H&E stain, original magnification ×100; TUNEL stain, original magnification ×40)
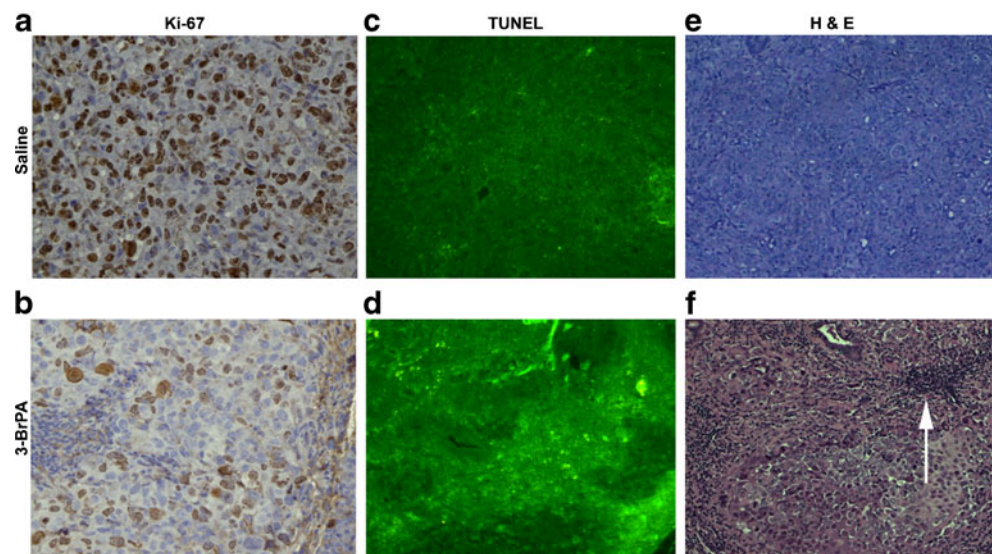
Table 1 Ki-67 proliferation index in Panc-1 tumors after intratumoral treatment

Treatment group	Proliferation index (mean ± SD)	
	7 Days posttreatment	4 Weeks posttreatment
3-BrPA	35.7±0.5	44.9±9.6
Control	50.5±3.0	63.4±6.0

a randomized clinical trial comparing cytoimplant with gemcitabine suggested that the cytoimplant patients did worse than the chemotherapy patients, and the trial was suspended. Another recent study demonstrated that repeated EUS-guided injections of ONYX-015 (an E1B-55K-deleted adenovirus for oncolytic adenoviral therapy) in combination with gemcitabine were safe if ONYX-015 was administered transgastrically [21]. No convincing evidence proving the efficacy of ONYX-015 was found. Finally, a phase III study evaluating the efficacy of EUS-guided injection of an adenovirus vector carrying the transgene for human tumor necrosis factor (TNFerade) is ongoing. These studies clearly demonstrate that endoscopic US-guided intratumoral therapy is feasible in pancreatic cancer patients. In this study, we could not use endoscopic US due to the size of the animal; however, abdominal US imaging of the pancreas and pancreatic tumors was feasible in mice and suitable for guidance of the needle for intratumoral treatment.

In addition to anticancer agents, several ablative agents have been studied for safety and feasibility after direct delivery to the pancreas, including radiofrequency ablation (RFA), photodynamic therapy, and ethanol injection. The major drawback of these techniques is that they are associated with severe toxicity. RFA and photodynamic therapy have been shown to cause cholecystitis, intrahepatic abscess formation, biloma, and pleural effusion [22–24]. Ethanol injection into the pancreas can cause injury to the large pancreatic duct, especially in the head of the pancreas, resulting in acute pancreatitis [25]. Extrapancreatic leakage of injected ethanol may injure the surrounding organs or portal vein and induce various complications such as peritonitis or bleeding. In our study, we found that treatment with 3-BrPA into the pancreatic tumor resulted in no toxicity to the normal pancreatic parenchyma or surrounding tissues, and no deleterious clinical signs or symptoms were observed in the animals after treatment.

Like many solid tumors, pancreatic cancers rely on glycolysis for their increased energy requirements, even in the presence of oxygen [15, 26]. In the current study, we hypothesized that the increased dependency on glycolysis in cancer cells could be exploited for therapeutic benefits and tested this hypothesis in an orthotopic xenograft model of pancreatic cancer, using 3-BrPA as a single pharmacologic tool to inhibit glycolysis. We found that intratumoral treatment resulted in a decrease in tumor size after treatment in five of six animals, which persisted throughout the study, indicating that the tumors were highly sensitive to inhibition of glycolysis. Even though the amount of the drug injected into the tumors was five times that of the tumor volume, no evidence of toxicity was observed within the normal pancreatic parenchyma, indicating that normal pancreatic cells are indeed not sensitive to glycolysis inhibition. This finding is reassuring when contemplating the translation of this approach to the clinical setting.

One of the critical pathologic turning points in cancer is the initiation of local invasion leading to the dissemination of tumor cells. Analysis of H&E-stained slides of the tumors from the 3-BrPA-treated group showed that the tumors were well demarcated with no invasion into the surrounding pancreas and characterized by infiltration of lymphocytes, whereas control (saline-treated) tumors showed invasion into the pancreatic tissue and only a few lymphocytes were present. It has been shown that a decrease in extracellular pH, resulting from the increased acid production as a result of enhanced glycolysis, increases MMP activity and invasion in cancer cells. We showed that treatment with 3-BrPA decreased the acid excretion of Panc-1 cells in vitro by reducing lactate production in a dose-dependent manner. These results may partly explain the decreased activity of MMP9 and decreased invasion seen in this study. Invasion is an active translocation of neoplastic tumor cells across tissue boundaries through host cellular and extracellular matrix barriers, which requires energy. We demonstrated that treatment with 3-BrPA resulted in a dose-dependent decrease of ATP levels in vitro. The decreased invasion of Panc-1 cells seen after treatment with 3-BrPA may therefore also be the direct result of the decreased amount of ATP present to fuel this active process. Finally, the lymphocytic infiltrate observed in the treated tumors may have contributed to the decreased invasiveness of the tumors and warrants further study.

Although our data clearly show that intratumoral administration of a potent antiglycolytic inhibitor caused significant antitumor effects as monotherapy, we recognize that this animal model is homogeneous. Because human pancreatic tumors are more heterogeneous, the optimal dose of 3-BrPA to selectively inhibit glycolysis in patients will therefore require further investigation. We also showed that treatment with 3-BrPA resulted in an infiltration of lymphocytes in the tumor tissue. The animals used in this study were immunocompromised and the effects on the immune system may therefore be different in immunocompetent animals. Finally, the demonstrated down-regulation of glycolysis has been shown to be a potent sensitizer to other chemotherapeutics. As a result, the potential exists for the use of our approach in combination with systemic chemotherapeutic agents. Such a combination approach is exciting but would warrant further investigation.

In conclusion, this study shows that US-guided intratumoral injection of 3-BrPA in an orthotopic xenograft model of pancreatic cancer resulted in a significant antitumor effect, with no observed adverse events. As such, our study provides proof of principle that the antiglycolytic agent 3-BrPA can be used as a targeted therapy for the treatment of pancreatic cancer.

Conflict of interest Dr. Geschwind is the founder of Presciencelabs LLC, a biotech firm currently developing 3-BrPA for clinical use in liver cancer.

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