Synergistic Antipancreatic Tumor Effect by Simultaneously Targeting Hypoxic Cancer Cells With HSP90 Inhibitor and Glycolysis Inhibitor

Xianhua Cao,1,3 Mark Bloomston,2 Tao Zhang,3 Wendy L. Frankel,4 Guang Jia,5 Bing Wang,3 Nathan C. Hall,5 Regina M. Koch,5 Hao Cheng,3 Michael V. Knopp,5 and Duxin Sun3

Abstract

Purpose: We sought to examine the synergistic antipancreatic cancer effect by simultaneously targeting hypoxic cancer cells with heat-shock protein 90 (HSP90) inhibitor and blockade of energy production.

Experimental Design: The anticancer effects of an HSP90 inhibitor (geldanamycin) in pancreatic cells were investigated in hypoxia and normoxia. A hexokinase II inhibitor, 3-bromopyruvate (3BrPA), was evaluated for selective glycolysis inhibition in hypoxia as a sensitizer of HSP90 inhibitor against pancreatic cancer. The HSP90 client protein degradation was monitored by Western blot. The synergistic antitumor effect of geldanamycin and 3BrPA was evaluated in a xenograft pancreatic cancer model and monitored by a noninvasive dynamic contrast-enhanced magnetic resonance imaging.

Results: Hypoxia enhanced HIF-1α expression by 11-fold in pancreatic cancer cells, and HSP90 inhibitor exhibited a seven- to eightfold higher anticancer effect in hypoxia compared with normoxia via HSP90 client protein degradation. 3BrPA selectively inhibited glycolysis and sensitized geldanamycin against pancreatic cancer cells by 17- to 400-fold through HSP90 client protein degradation. The synergistic antitumor effect of reduced doses of geldanamycin and 3BrPA was confirmed in xenograft models in vivo by more than 75% tumor growth inhibition.

Conclusions: The combination of HSP90 inhibitors and glycolysis inhibitors provides preferential inhibition of cancer cells in hypoxia through HSP90 client protein degradation and selective glycolysis inhibition. This may provide a new therapeutic regimen to battle chemotherapy-resistant pancreatic cancers, by enhancing the synergistic therapeutic efficacy and reducing dose-limiting toxicity.

Pancreatic cancer is the fourth leading cause of cancer death in the United States (1). The high mortality rate is mostly due to the high rate of metastasis and severe resistance to both chemotherapy and radiation. Recently, the combination of gemcitabine and monoclonal antibody against VEGF, EGFR, and Her-2 has been evaluated in several clinical trials in pancreatic cancers (2–4). However, the response and survival rates of treated patients have remained at low levels. A number of biochemical and genetic abnormalities contribute to this low response rate of target-specific therapeutic strategies. First, mutations in both oncogenes (such as K-Ras) and tumor-suppressor genes (such as P53) have been identified in pancreatic cancers. Second, overexpression of growth factors (such as VEGF) and growth factor receptors (such as EGFR), have also been linked to the metastasis and disease progression (5). In addition, the hypoxic microenvironment, induced by hypovascular system in pancreatic tumors, further exacerbates the high rate of metastasis and drug resistance in pancreatic cancer therapy (6).

Due to the complexity of pancreatic cancer, a single drug targeting a particular oncogene is unlikely to be entirely effective for pancreatic cancer therapy. The ansamycin antibiotic geldanamycin provides new therapeutic schemes for pancreatic cancer by inhibiting the molecular chaperone function of heat-shock protein 90 (HSP90), which can downregulate multiple oncogenic proteins simultaneously via proteasomal degradation (7). Most of those proteins, such as hypoxia-inducible factor 1-α (HIF-1α). AKT, K-Ras, v-Src, Raf-1, Bcr-Abl, ErbB2, mutant P53, and EGFR (8), are involved in the regulatory pathways of pancreatic tumor development progress. Thus, HSP90 inhibitors might be able to provide more favorable strategies for pancreatic tumor therapy through simultaneously downregulated multiple targets, especially under hypoxia.

Geldanamycin and 17-AAG have been shown to have antitumor activity against breast cell culture and xenograft animal models. Geldanamycin derivative 17-(allylamino)-17-deethoxygeldanamycin (17-AAG) and 17-dimethylaminoethyamine-17-deethoxygeldanamycin (17-DMAG) was
studied in clinical trials for melanoma, breast, and colon cancer (9–12). Currently, both 17-AAG and 17-DMAG are under clinical trials for solid tumors and leukemia in the National Institute of Health and our institution. However, two challenges still remain for these compounds: (1) Can HSP90 inhibitor be used for pancreatic cancer therapy? (2) Can HSP90 inhibitor be used as a stand-alone agent for cancer therapy, in which dose-limiting toxicity may still be a challenge? Combination with other therapeutic agents may provide benefit for its clinical use.

Inhibition of high-rate glycolysis under hypoxic conditions may provide a sensitization effect for cancer chemotherapy (13–15). The hypoxic environment in solid tumors changes the energy metabolism pathway in cancers and correlates with overexpressed glucose transporters and high rates of glycolysis (16). This metabolism alteration is known as the Warburg effect and has been clinically applied to tumor imaging with positron emission tomography (PET; ref. 17). Cancer cells heavily rely on glucose transporters for glucose uptake and subsequent glycolysis through hexokinase and other glycolytic enzymes for their survival and proliferation. These characteristics are also associated with increased metastasis and poor survival rates in cancer patients (18). Thus, selective inhibition of glucose uptake and/or glycolysis rate in cancer cells provides another novel therapeutic target in pancreatic cancer.

In this study, we intend to test the synergistic effect of HSP90 inhibitors and glycolysis inhibitors in pancreatic cancer cells under hypoxia in vitro and xenograft models in vivo. We hypothesize that glycolysis inhibitors may block the energy production to sensitize anticancer efficacy of the HSP90 inhibitor. We first identified the anticancer efficacy of HSP90 inhibitors (geldanamycin) in human pancreatic cells (Panc-1, BxPC3, MiaPaCa-2) under a hypoxic environment. Then, we evaluated 3-bromopyruvate (3BrPA), a hexokinase II inhibitor as a sensitizer of HSP90 inhibition in pancreatic cancer cells in vitro. Finally, we investigated the synergistic effect of the HSP90 inhibitor and glycolysis inhibitor to target hypoxic cells in a xenograft pancreatic cancer model in vivo.

**Materials and Methods**

**Cell cultures and reagents.** Human pancreatic cancer cell lines MiaPaCa-2, BxPC3, and Panc-1 were cultured at 10% FBS RPMI-1640 or 10% FBS DMEM media at 37°C and 5% CO2. All the antibodies for Western blot analysis were from Cell Signaling Technology. The glycolysis inhibitor 3BrPA, proteasome inhibitors, MG132 and lactacystin were from Sigma-Aldrich (St. Louis, MO). The compound 3BrPA was dissolved in normal saline and neutralized to pH 7.4 with NaOH and further filtered through a 0.2-μm filter to prepare injectable solution for in vivo studies. HSP90 inhibitor, geldanamycin, was kindly provided by Dr. George Wang in the Department of Chemistry, Ohio State University. Geldanamycin was dissolved in dimethyl sulfoxide (DMSO) for in vitro experiment, and geldanamycin was freshly dissolved in DMSO (70% cremophor/ethanol (3:1), and 20% phosphate-buffered saline (PBS) for in vivo experiment. The animal study protocol was approved by the Institutional Animal Care and Use Committee at Ohio State University.

**Hypoxic environment.** Hypoxic conditions were maintained with 1% O2, 5% CO2, and 94% N2 in a modular incubator chamber (Billups-Rotterdam, Inc., Del Mar, CA). The hypoxic chamber was flushed with the hypoxic air for 15 minutes according to the protocol and was then refilled every 8 hours. The hypoxia was further confirmed and monitored by the measurement of hypoxia-induced lactate production and intracellular pH-1a protein levels.

**Tetratozolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenoxy)-2-(4-sulfophenyl)-2H-tetrazolium assay.** The cytotoxicity of a single treatment or combination treatment was measured by tetratozolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenoxy)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (19). Briefly, a total of 2,000 to 5,000 cells were cultured in a 96-well plate for 24 hours. Glycolysis inhibitors (3BrPA, pH 7.0, 1-100 μmol/L) and chemotherapeutic drugs (daunorubicin or geldanamycin, 0.001-10 μmol/L) were added to the cell cultures. The cells were maintained at either a normoxia condition (5% CO2 and 95% air) or in a hypoxia chamber (1% O2 and 5% CO2, 94% N2). After 3 days, MTS (2 mg/mL) and phenazine methosulfate (25 μmol/L) were added directly to the cell culture and incubated for 2 hours at 37°C. The absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm to quantify the number of surviving cells. The IC50 values of the different treatments for cytotoxicity were calculated by WinNonlin software from the dose-response curves of percentage of cell growth versus drug concentration.

**Western blot analysis.** Cells were washed twice with PBS, collected in serum-free media, and lysed in cell lysis buffer (50 mmol/L Tris-HCl, 250 mmol/L NaCl, 5 mmol/L EDTA, 2 mmol/L Na2VO4, 50 mmol/L NaF) with a 1% protease inhibitor cocktail (P8340, Sigma-Aldrich) for 30 minutes on ice and sonicated three times for 20 seconds. Human or mouse tissues were first homogenized in lysis buffer and then are subjected to the same process of cell lysis preparation. Supernatants were collected after 10 minutes centrifugation at 4°C. Protein concentration was determined using BCA protein assay methods (Pierce Biotechnology). The cell lysates were incubated with 2x SDS loading buffer and boiled for 5 minutes. Then, 30 μg of total protein was subjected to electrophoresis and separated in 10% SDS-polyacrylamide gels (Bio-Rad), transferred to nitrocellulose filters, probed with the antibodies of interest, and developed with enhanced chemiluminescence system ECL (Amersham Biosciences, Piscataway, NJ). The ECL signal was quantified using a scanner and a densitometry program (Scion Image, Scion, Frederick, MD).

**Real-time PCR.** Total cellular RNA was extracted and purified using the TRIzol reagent (Invitrogen). One μg of total RNA was used to transcribe the first-strand cDNA with SuperScript II reverse transcriptase (Invitrogen). The real-time PCR was completed on the ABI PRISM Sequence Detector 7000 (PerkinElmer, Branchburg, NJ) using Sequence Detector version 1.7 software (Applied Biosystems, Foster City, CA). SYBR Green PCR Master Mix was purchased from Applied Biosystems. Forward and reverse primers for targeted mRNA were designed using Primer Express version 1.0 software (ABI PRISM, PerkinElmer) based on the human HIF-1α, AKT or actin gene sequence. Fold change of mRNA level was calculated as follows: after completion of the PCR, the baselines and thresholds were set for both samples and internal beta-actin controls. Using Ct values (cycle number in which the sample crosses the threshold value) for samples (sam) and controls, the ΔCt was calculated: ΔCt = Ct sam - Ct actin. The values for each sample were then compared with the control sample (ctl): ΔΔCt = ΔCt sam - ΔCt ctl. The fold change of the mRNA level to the control is 2ΔΔCt.

**Measurement of intracellular ATP and lactate generation.** The cellular ATP content was determined using a bioluminescence assay kit (Sigma-Aldrich). Briefly, 106 cells were treated with a different concentration of 3BrPA under either normoxic or hypoxic conditions. After the indicated times, cells were washed with PBS, lysed in 0.5% Triton X-100, 10 mmol/L Tris-HCl, pH 7.5, 1 mM EDTA and incubated for 30 minutes on ice. After removal of cell debris, the total
protein concentration was calculated, and 10 μL whole-cell lysate was subject to the luminescence kit. ATP content was measured with a luciferin/luciferase assay using a luminometer (model ML2200; Dynatech Deutschland GmbH, Denkendorf, Germany). At the same time, the medium was removed and saved for analysis of lactate generation using an Accutrend lactate analyzer according to the manufacturer's instructions (Roche). The ATP content was calculated using an internal standard, and the data were further normalized by total protein as concentration per microgram total proteins.

**Xenograft models.** The cancer cells (BxPC3 or Panc-1, 10⁷ cells) in matrigel/medium (1:1, 200 μL) were injected subcutaneously into female Nu/Nu nude mice. Tumors were allowed to grow for 3 to 4 weeks. The tumor volumes were measured twice per week with calculated tumor volume (W²/2). When tumors reached 100 to 150 mm³, animals were randomized into different groups for single treatment or combination treatment with at least five mice per group. All compounds and placebo were freshly prepared and injected intraperitoneally twice per week for 3 weeks except the gemcitabine control group, which was injected three times per week. Tumor growth was measured once every 3 days after drug administration and normalized to the initial volumes.

**Noninvasive in vivo tumor image by magnetic resonance imaging.** At the end of the in vivo experiments, all mice were subjected to a 3-tesla clinical magnetic resonance imaging (MRI) system (Achieva, Philips Medical Systems) for a noninvasive in vivo tumor image. The subjects were scanned in a prone position, using a dedicated mouse coil (Philips Research laboratory). High-quality T2-weighted axial and coronal images were obtained using a turbo spin echo sequence (TR/TE = 6,752/77 ms; field of view = 60 × 512 with in-plane resolution 0.12 mm²; matrix = 512 × 512 with in-plane resolution 0.12 × 0.12 mm²; number of excitations = 1; 20 slices; 1-mm slice thickness; gap between slices = 0.3 mm). The image postprocessing and three-dimensional visualization were carried out using the MIPAV software package (20). The calculated tumor sizes were correlated to those measured in ex vivo volumes.

**Evaluate the synergistic effect of combination treatment.** The combination effect of HSP90 inhibitor and glycolysis inhibitor was determined by combination index (CIx) according to the literature (21): CIx = D1,comb/D1 + D2,comb/D2 in which D1 and D2 are drug concentrations that produce x% of cell growth inhibition when used alone; D1,comb and D2,comb are drug concentrations that produce x% of effect when used in combination. The synergism, additivity, and antagonism of the combination effect will be shown when CIx is less than, equal to, or greater than 1, respectively.

**Statistics.** All experiments were performed independently at least three times. Means and standard deviations are plotted in curves and values of P < 0.05 were considered statistically significant.

### Results

**Geldanamycin exhibits better anticancer effect in pancreatic cancer cells through induction of more client protein degradation under hypoxia.** To test whether geldanamycin can have a higher antipancreatic cancer effect in hypoxia than in normoxia, we tested cytotoxicity (MTS assay) of geldanamycin against human pancreatic cancer cells (Panc-1, MiaPaCa-2, BxPC3) with different sensitivity to geldanamycin under a hypoxic environment in comparison with the normoxic condition. The cytotoxicity of the drugs was measured after 72 hours of incubation under a hypoxic chamber flushed with 1% O₂, 5% CO₂, and 94% N₂. As shown in Table 1, under hypoxia, the IC₅₀ for geldanamycin against pancreatic cancer cells (BxPC3, MiaPaCa-2, Panc-1) decreased by six- to 11-fold (P < 0.001). A hypoxic condition significantly enhances the sensitivity of pancreatic cancer cells to geldanamycin. The drug resistance index (RI) calculated by IC₅₀ under hypoxia over IC₅₀ under normoxia showed the seven- to eightfold sensitization of pancreatic cancer cells to geldanamycin compared with normoxia.

The better anticancer activity of geldanamycin under hypoxia is very unique compared with other chemotherapeutic compounds. Normally, hypoxia will induce drug resistance to chemotherapy. For instance, anticancer activity of daunorubicin under hypoxia is decreased with a right-shifted cytotoxicity versus drug concentration curve. The IC₅₀ of daunorubicin under hypoxia was increased by two- to fourfold compared with normoxia (5% CO₂, 95% air; Table 1).

Because HIF-1α is a major regulator for cancer cell survival under hypoxia, and HIF-1α is an HSP90 client protein, we intend to test the hypothesis that high sensitivity of geldanamycin in hypoxia is due to the induction of HIF-1α degradation. Under hypoxia, HIF-1α is overexpressed to promote the cancer cell proliferation and survival (i.e., cancer cells are more heavily relied on by HIF-1α for their survival under hypoxia than under normoxia). Through inhibition of HSP90, geldanamycin would enhance the ubiquitination and proteasomal degradation of premature client proteins in cancer cells including HIF-1α and other downstream proteins stimulated by hypoxia. Our data showed that HIF-1α expression is significantly increased by 11-fold to promote cancer cell survival when the cells were in hypoxia for 2 to 24 hours (Fig. 1A). Geldanamycin (1-5 μmol/L) inhibits HSP90 and induces HIF-1α degradation by 60% after 16 hours of incubation in hypoxia to show high anticancer

### Table 1. IC₅₀ of daunorubicin and geldanamycin in pancreatic cancer cells under normoxia and hypoxia

<table>
<thead>
<tr>
<th>Human pancreatic cancer cell lines</th>
<th>Daunorubicin</th>
<th>Geldanamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia IC₅₀ (μmol/L)</td>
<td>Hypoxia IC₅₀ (μmol/L)</td>
</tr>
<tr>
<td></td>
<td>Normoxia IC₅₀ (μmol/L)</td>
<td>Hypoxia IC₅₀ (μmol/L)</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>0.33 ± 0.11</td>
<td>0.95 ± 0.13</td>
</tr>
<tr>
<td>Panc-1</td>
<td>2.52 ± 0.32</td>
<td>7.74 ± 0.4</td>
</tr>
<tr>
<td>MiaPaCa-2</td>
<td>0.016 ± 0.017</td>
<td>0.026 ± 0.016</td>
</tr>
</tbody>
</table>

NOTE: The cytotoxicity of daunorubicin and geldanamycin under both normoxia and hypoxia were measured by MTS assay in three independent experiments with six replicates in each experiment. Relative cell viability was calculated by normalized intensity to nontreatment cell control under different drug concentrations after 72 h of incubation. IC₅₀s were calculated with WinNonlin 5.0. RI induced by hypoxia was calculated as the ratio of IC₅₀ under hypoxia and IC₅₀ under normoxia.
sensitivity (Fig. 1A). To further confirm if geldanamycin inhibits HSP90 to degrade other client protein degradation under hypoxia, we also tested total AKT levels in pancreatic cancer cells (Panc-1 and BxPc-3). Similar to HIF-1α, total AKT levels were also degraded more significantly by geldanamycin under hypoxia (by 40%-70%) than under normoxia (by 20%-30%; Fig. 1B).

To confirm that the anticancer effect of geldanamycin is through client protein degradation rather than decreasing the transcription of mRNA, we have performed real-time PCR to measure the mRNA level of client protein (e.g., HIF-1α and AKT). The data showed that geldanamycin treatment did not decrease mRNA levels of both HIF-1α and AKT. To further validate that the geldanamycin-induced client protein degradation is through proteasomal pathway, we combined geldanamycin with proteasome inhibitor MG 132 or lactacystin in the treatment of cancer cells (22, 23). The data showed that proteasome inhibitors (MG 132 or lactacystin) reversed the geldanamycin-induced degradation of HSP90 client proteins from 50% to 80% to 120% (Fig. 1C). These data suggest that geldanamycin-induced HSP90 client protein degradation is through proteasomal pathway, and geldanamycin offers advantages in the treatment of hypoxic solid pancreatic tumors.

Glycolysis inhibitor inhibits lactate production and intracellular ATP level in pancreatic cancer cells under hypoxia. Human pancreatic tumors exhibit increased glycolysis rates and use this metabolic pathway for generation of ATP as an energy source. The high rate of glycolysis in pancreatic cancer has been clinically used in PET scanning for tumor imaging and detection in patients (date not shown). In order to show whether glycolysis inhibitors can reduce the glycolysis and the intracellular ATP level in pancreatic cancer cells under hypoxic and normoxic conditions. Both lactate production and intracellular ATP level were measured after 24 hours of incubation of different concentrations of 3BrPA treatment. As Fig. 2A shows, lactate production was threefold higher in the hypoxic condition, which is due to the higher glycolysis rate under hypoxia compared with normoxia. Accordingly, in order to adapt to the hypoxic environment, the intracellular ATP level was increased 40% to maintain the cancer cell growth rate under hypoxia (Fig. 2B). Fifty to 70 μmol/L 3BrPA can inhibit the hypoxia-induced lactate production and intracellular ATP level in this in vitro hypoxic system (Fig. 2A and B). Meanwhile, the same concentration of 3BrPA did not change the metabolism and intracellular ATP level under normoxia. These data suggest that 3BrPA showed the preferential inhibition of the high rate of glycolysis under hypoxia compared with normoxia. The IC_{50} of 3BrPA in pancreatic cancer cells were 80 to 90 μmol/L as tested in MTS assay (Table 2) and showed similar cytotoxicity in both hypoxia and normoxia. An optimal concentration (50–70 μm) was selected to inhibit the intracellular ATP level in only hypoxia and exhibit 20% cell cytotoxicity only in hypoxia.

HSP90 inhibitor and glycosis inhibitor simultaneously target hypoxic pancreatic cancer cells for synergistic anticancer effect. In order to confirm the synergistic anticancer effect of the combination treatment of the HSP90 inhibitor and glycolysis inhibitor in pancreatic cancer cells, 3BrPA and geldanamycin were combined to treat Panc-1 and BxPC3 cells under either normoxia or hypoxia conditions. The concentration of 3BrPA was chosen at 50 μmol/L at which showed no cell killing effects in normoxia but selectively inhibited 50% of the lactate production and intracellular ATP level in a hypoxic environment. As shown in Fig. 2C, the combination of geldanamycin and 3BrPA showed a significant synergistic effect under hypoxia compared with normoxia. Under hypoxia, the IC_{50} of geldanamycin in the combination
treatment of 3BrPA and geldanamycin was 17-fold lower than the IC₅₀ of geldanamycin alone in BxPC3 cells and more than 400-fold lower in Panc-1 cells. In contrast, under normoxia, the IC₅₀ of geldanamycin in the combination of geldanamycin and 3BrPA is threefold lower than that of geldanamycin alone (Table 2). In order to evaluate the synergism of combination, the combination index of different treatments to reach both 50% cell killing and

Table 2. Synergy between 3BrPA and geldanamycin in pancreatic cancer cells under normoxia and hypoxia in three independent experiments

<table>
<thead>
<tr>
<th>Cells</th>
<th>Condition</th>
<th>Effect</th>
<th>IC₅₀ or IC₇₀ of 3BrPA alone (μmol/L)</th>
<th>IC₅₀ or IC₇₀ of geldanamycin alone (μmol/L)</th>
<th>IC₅₀ or IC₇₀ of geldanamycin when combined with 50 μmol/L 3BrPA (μmol/L)</th>
<th>Combination index</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC-3</td>
<td>Normoxia</td>
<td>50%</td>
<td>86.3 ± 6.4</td>
<td>0.85 ± 0.12</td>
<td>0.24 ± 0.13</td>
<td>0.86 ± 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70%</td>
<td>190 ± 11</td>
<td>2.12 ± 0.41</td>
<td>1.2 ± 0.3</td>
<td>0.82 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>50%</td>
<td>90.5 ± 6.7</td>
<td>0.34 ± 0.11</td>
<td>0.02 ± 0.02</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70%</td>
<td>131 ± 8.9</td>
<td>0.76 ± 0.12</td>
<td>0.37 ± 0.1</td>
<td>0.86 ± 0.15</td>
</tr>
<tr>
<td>Panc-1</td>
<td>Normoxia</td>
<td>50%</td>
<td>79.2 ± 6.3</td>
<td>&gt;10</td>
<td>0.021 ± 0.04</td>
<td>&lt;0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70%</td>
<td>93.1 ± 5.8</td>
<td>&gt;50</td>
<td>0.76 ± 0.23</td>
<td>&lt;0.55</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td>50%</td>
<td>76.7 ± 6.8</td>
<td>4.43 ± 2.2</td>
<td>0.007 ± 0.001</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70%</td>
<td>109 ± 8.5</td>
<td>&gt;15</td>
<td>0.34 ± 0.12</td>
<td>&lt;0.48</td>
</tr>
</tbody>
</table>

NOTE: The cytotoxicity of a single geldanamycin treatment or combined with 50 μmol/L 3BrPA treatment under normoxia or hypoxic environment was measured by MTS assay. Relative cell viability was calculated by normalized intensity to nontreatment cell control under different drug concentrations after 72 h of incubation. IC₅₀ or IC₇₀ were calculated by WinNonlin 5.0 in each experiment.
To further study the mechanism of synergism in the combination of geldanamycin and 3BrPA, we evaluated the degradation of HSP90 client proteins using Western blots under both hypoxia and normoxia conditions. As illustrated in Fig. 2D, combination of geldanamycin and 3BrPA (30-50 μmol/L) treatment significantly induced AKT degradation in both BxPC3 and Panc-1 cells by 2.5- to threefold higher compared with geldanamycin treatment alone. In contrast, 3BrPA alone did not show a significant effect (<25%) on the degradation of AKT. These data suggest that the synergism of geldanamycin and 3BrPA in the combination treatment is due to increased degradation of HSP90 client proteins.

Combination of geldanamycin and 3BrPA exhibits anticancer activity in a xenograft model of pancreatic cancer. To confirm the synergistic anticancer activity of combination treatment of geldanamycin and 3BrPA in vivo, we tested the combination efficacy in a xenograft model with human pancreatic cancer cells (Panc-1, BxPC3). According to in vitro cytotoxicity of geldanamycin in our MTS assays, Panc-1 and BxPC3 cell lines showed different sensitivity to geldanamycin. It has been reported in the literature that geldanamycin (5-100 mg/kg) and 3BrPA (1 mL × 2 mmol/L intratumor injection) showed anticancer activity against various cancers with a high dose (13, 24–31). In order to reduce their toxicity and examine the synergistic effect of geldanamycin and 3BrPA, we selected a very small dose of both geldanamycin and 3-BrPA to have minimal effects when they are used alone. We used 2 mg/kg geldanamycin and 2 mg/kg 3BrPA in a BxPC3 xenograft model, twice per week for 3 weeks. In addition, we used 5 mg/kg geldanamycin and 5 mg/kg 3BrPA in Panc-1 xenograft models with the same dose regimen. In both models, we used gemcitabine as the control because it is currently used as first-line therapy for human pancreatic cancer, which was given three times per week for 3 weeks with a dose of 125 mg/kg. The tumor growth rates (tumor size after treatment vs before treatment) were compared among different treatment groups. When tumor sizes reached 800 mm³, the mice were removed from the treatment groups, and the time was recorded and plotted in a survival rate curve to further confirm the antitumor effect.

As shown in Fig. 3A and B, average tumor sizes in the control group were about fivefold bigger than the initial sizes in the BxPC3 tumors at 58 days after the implant of cancer cells and approximately threefold larger in Panc-1 tumors at 75 days after implant. Interestingly, neither single treatment with the gemcitabine, geldanamycin, or 3BrPA had minimal effects, if any, on tumor growth compared with the control groups. However, combination treatments with geldanamycin and 3BrPA in both models showed 75% inhibition of the tumor growth.

The time for tumor sizes to reach 800 mm³ in different groups in the BxPC3 model was plotted in survival rate curves. As shown in Fig. 3C, the median survival rate in the combination treatment group was significantly increased to more than 70 days (40% increase vs control; P < 0.001; Fig. 3C), whereas the median survival rate in either control group or single treatment group was approximately 50 days.

In addition, we also used noninvasive MRI to monitor tumor growth in BxPC3 xenograft tumors after drug treatment (Fig. 4) at the end of the experiments. As seen in Fig. 4, the tumor sizes increased from 765 ± 211 mm³ to 1560 ± 310 mm³ in either single treatment group or control groups, while the combination treatment with geldanamycin and 3-BrPA significantly inhibited the tumor growth with tumor sizes of 301 ± 228 mm³ (P < 0.05). These results suggest that simultaneous
inhibition of HSP90 and glycolysis achieves synergistic antitumor effect in a xenograft model in vivo.

Discussion

Pancreatic cancer is a malignancy with a worldwide incidence of more than 200,000 cases and almost as many deaths every year. Currently, no effective treatment has been developed for this disease. Recent clinical trials have compared gemcitabine as a single agent to combination chemotherapy with one or several other cytotoxic or targeted agents for pancreatic cancers (2, 3, 32–37). However, no statistically significant difference in median survival has been achieved in these combination therapies. In this article, we reported a new strategy for pancreatic cancer therapy by simultaneously targeting the hypoxic cancer cells with HSP90 inhibitor and glycolysis inhibitors in both in vitro and preclinical in vivo animal models.

The antitumor potential of geldanamycin has long been recognized, however, clinical evaluation of geldanamycin has not been pursued due to its severe dose-related toxicity and poor water solubility. For this reason, efforts have been made to modify geldanamycin, generating a number of analogues in attempts to increase clinical efficacy and water solubility and decrease toxicity. Among the successful analogues is 17-allylamino geldanamycin (17-AAG), which is currently in phase II clinical trials for breast cancer therapy at the National Cancer Institute (10–12). However, 17-AAG still has low water solubility. To overcome this problem, another geldanamycin derivative, 17-DMAG, with high water solubility was also developed (38). Although 17-AAG showed improved efficacy and relatively low toxicity, it appears that the dose-limiting toxicity may be still limiting factors for its clinical application.

HSP90 inhibitors have not been tested in pancreatic cancer, and it is not known if they can be used as a stand-alone agent for pancreatic cancer therapy. In this study, we first showed that geldanamycin has a more potent effect against pancreatic cancer under hypoxia by inhibiting the molecular chaperone HSP90 and simultaneously downregulating multiple oncogenic protein targets. Cancer cells highly depend on the function of HSP90 to modulate the folding and assembly of oncogenic proteins including AKT, v-Src, Raf-1, Bcr-Abl, ErbB2, mutant P53, and HIF-1α (39, 40). Geldanamycin binds to the conserved ATP binding pocket at the N-terminus of HSP90 (41), inhibits ATP-dependent chaperone activity (42), enhances ubiquitination and proteasomal degradation of premature client proteins, and therefore exhibits potent anticancer activity (23, 43). Elevated AKT activation has been detected in cancer cells to promote cancer cell survival (44). Geldanamycin induces AKT degradation (45) and shows promising antitumor effects on AKT overexpressing cancer cells. Furthermore, under hypoxic conditions, HIF-1α is dramatically overexpressed 13-fold in pancreatic tumors compared with normal pancreas and correlates with metastasis of pancreatic cancer (46). HIF-1α–regulated pathways further exacerbate the resistance to chemotherapy (5, 6, 46–48). In pancreatic cancer cells, geldanamycin shows seven- to eightfold higher cytotoxicity in a hypoxic environment (Table 1) through HIF-1α and AKT degradation (Fig. 1A and B) compared with normoxic conditions.
conditions. All three pancreatic cell lines show the higher sensitivity to geldanamycin under hypoxia than in normoxia, which is different from other chemotherapeutic agents. These data suggest that HSP90 inhibition would have advantages against pancreatic cancer especially under hypoxia through multiple targets (Fig. 1).

To explore the sensitization strategy for HSP90 inhibitor against pancreatic cancer, we used a combination of HSP90 inhibitor and glycolysis inhibitor in comparison with a single agent alone. An elevated glycolysis rate and the expression of the glycolytic enzymes such as hexokinases in pancreatic tumors has been observed (49), which is consistently confirmed by PET imaging in pancreatic cancer patients (50, 51). These characteristics are also associated with increased metastasis and poor survival rates in cancer patients (18). Our data together with other reports suggest that pancreatic cancer relies on high-rate glycolysis for its survival. It has been reported that HKII inhibitor 3BrPA can reverse the drug resistance induced by high-rate glycolysis in mitochondrial defect cells C6G via the dephosphorylation of BAD and translocation of BAX to mitochondria (13). Other clinical trials have shown the feasibility and safety of combination effects of a glycolysis inhibitor (2-deoxyglucose) and dextocelation or radiation therapy in patients with solid tumors (29–31). Our previous studies also showed that blocking the glucose transporter by phloretine synergistically increased daunorubicin-induced cytotoxicity and apoptosis in drug-resistant cancer cells (K562/Dox) under hypoxia (19). In the current study, the glycolysis inhibitor 3BrPA inhibited both the elevated glycolysis and intracellular ATP level induced by a hypoxic environment (Fig. 2A and B) without affecting the glucose metabolism and intracellular ATP level under normoxia. These data suggest that 3BrPA provides the preferential selectivity for the inhibition of the high rate of glycolysis in pancreatic cancer cells under hypoxia.

The dependency on high rates of glycolysis in cancer can be attributed to either injury of mitochondrial respiration associated with mitochondrial DNA mutations and oncogenic transformation and/or hypoxic stimuli in the tumor tissues (53). In contrast, normal cells with competent mitochondria can generate ATP efficiently through oxidative phosphorylation and can also use alternate energy sources such as amino acids and fatty acids when glycolysis is inhibited. This metabolic adaptation and the different energy metabolism render the cancers dependent on glycolysis to meet their energy requirements, which provide a biochemical basis to preferentially kill cancer cells or sensitize cancer cells to chemotherapeutic drugs under hypoxic conditions.

Therefore, we hypothesized that a combination treatment of pancreatic cancer cells with HSP90 inhibitors and glycolysis inhibitors will provide a synergistic antipancreatic effect, which will benefit to enhance the in vivo therapeutic efficacy and reduce dose-limiting geldanamycin toxicity in pancreatic tumors. In order to show the optimal actions of combined treatment of 3BrPA and geldanamycin, the concentration of 3BrPA in the in vitro studies was chosen through the selective inhibition of the glycolysis rate in cancer cells under hypoxia but not under normoxia (Fig. 2A and B). In vitro treatment of different pancreatic cancer cell lines with both 3BrPA and geldanamycin under hypoxia showed greater efficacy than selective inhibition of high glycolysis rate by 3BrPA alone or geldanamycin alone in pancreatic cancer cells. The antitumor activity of the combination of geldanamycin and 3-BrPA was enhanced by 17-fold in BxPC3 and more than 400-fold in Panc-1 cells compared with single treatment alone (Fig. 2C, Table 2).

Although it is challenging to determine the optimal dose of 3BrPA to selectively inhibit the glycolysis rate in in vivo xenograft tumors, it is predictable that there is more severe hypoxic environment in the solid xenograft tumors. Previous study has been reported to successfully use 3BrPA as an ATP depletion agent to eradicat advanced solid tumors in an in vivo animal model of hepatocellular cancers via a high dose of local injection (24). In our in vivo studies, we selected the drug administration by IP injection and reduced the doses to safety windows with both geldanamycin and 3BrPA. The dose selection was aimed to maximize the synergistic antitumor effect as well as to control the dose in safety windows in vivo for the combination treatment. Two pancreatic cancer cell lines with different in vitro sensitivity to geldanamycin were used in the xenograft models in this dose regimen. A synergistic inhibition on solid tumor growth in both pancreatic cancer xenograft models was observed in the combination treatment groups, while no effect was observed in the single treatment groups with reduced doses (Fig. 3). The synergistic antitumor effect of geldanamycin and 3-BrPA was further confirmed in vivo by the noninvasive MRI tumor images (Fig. 4). These data suggest that the combination treatment of HSP90 inhibitor and glycolysis inhibitor provides a novel therapeutic regimen to treat pancreatic cancers.

In summary, combination of HSP90 inhibitors and glycolysis inhibitors provides preferential inhibition of high glycolysis rate and HSP90 client protein degradation under hypoxia. The synergistic effects of HSP90 inhibitors and glycolysis inhibitors will be beneficial to increase anticancer activity and to overcome drug resistance in pancreatic cancer therapy. Combination of HSP90 inhibitors and glycolysis inhibitors may also decrease the effective dose for both drugs and reduce dose-limiting toxicity. However, it is important to note that extensive pharmacokinetic and pharmacodynamic testing and simulation are warranted to optimize the dose regimen before the application of this strategy in patients with clinical pancreatic cancer.

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Xianhua Cao, Mark Bloomston, Tao Zhang, et al.