Molecular Cancer Therapeutics

The Role of Pyruvate Dehydrogenase Kinase-4 (PDK4) in Bladder Cancer and Chemoresistance

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Abstract

Advanced bladder cancer remains a major source of mortality, with poor treatment options. Cisplatin-based chemotherapy is the standard treatment, however many patients are or become resistant. One potential cause of chemoresistance is the Warburg effect, a metabolic switch to aerobic glycolysis that occurs in many cancers. Upregulation of the pyruvate dehydrogenase kinase family (PDK1–PDK4) is associated with aerobic glycolysis and chemoresistance through inhibition of the pyruvate dehydrogenase complex (PDH). We have previously observed upregulation of PDK4 in high-grade compared with low-grade bladder cancers. We initiated this study to determine if inhibition of PDK4 could reduce tumor growth rates or sensitize bladder cancer cells to cisplatin. Upregulation of PDK4 in malignant bladder cancer cell lines as compared with benign transformed urothelial cells was confirmed using qPCR. Inhibition of PDK4 with dichloroacetate (DCA) resulted in increased PDH activity, reduced cell growth, and G_0–G_1 phase arrest in bladder cancer cells. Similarly, siRNA knockdown of PDK4 inhibited bladder cancer cell proliferation. Cotreatment of bladder cancer cells with cisplatin and DCA did not increase caspase-3 activity but did enhance overall cell death *in vitro*. Although daily treatment with 200 mg/kg DCA alone did not reduce tumor volumes in a xenograft model, combination treatment with cisplatin resulted in dramatically reduced tumor volumes as compared with either DCA or cisplatin alone. This was attributed to substantial intratumoral necrosis. These findings indicate inhibition of PDK4 may potentiate cisplatin-induced cell death and warrant further studies investigating the mechanism through which this occurs. *Mol Cancer Ther; 17(9); 2004–12. ©2018 AACR.*

Introduction

Bladder cancer is the fifth most common solid tumor in the United States with an estimated 79,030 new cases and 16,870 deaths in 2017 (1). Little progress has been made in the treatment of bladder cancer over several decades. As such, outcomes remain poor for advanced stages. The FDA first approved cisplatin-based chemotherapy for the treatment of bladder cancer in 1978 and the most effective regimen was identified in 1985 (2). Because of lack of significant therapeutic advances, it remains the cornerstone of chemotherapy in spite of the fact that up to 50% of patients do not respond and/or develop chemoresistance to treatment. As such, agents that can help overcome cisplatin resistance, or sensitize bladder cancer to cisplatin as a combination therapy are sorely needed.

Cancer cells have the ability to alter their genotypic and/or phenotypic state in order to provide a survival advantage in the hostile tumor microenvironment. The well-described Warburg effect, a metabolic shift in cancers where energy production is diverted from mitochondrial oxidative phosphorylation to aerobic glycolysis in the cytoplasm, is a primary example and is protective in the hypoxic/acidic tumor microenvironment (3). This shift towards aerobic glycolysis results in both dependence on increased glycolysis and has been shown to facilitate chemoresistance (3, 4). Inhibition of cancer-specific alterations in metabolism has been suggested as a mechanism for overcoming chemoresistance, yet the mechanisms controlling this have not been well explained, nor have they been examined extensively in bladder cancer (4, 5). Pyruvate dehydrogenase kinase-4 (PDK4) is a member of a family of isozymes (PDK1–PDK4) that partially mediate the switch to aerobic glycolysis by shunting pyruvate metabolism from the mitochondria to the cytoplasm for glycolysis. Inhibition of PDKs in other tumors slows tumor growth both *in vitro* and *in vivo*, presumably through inhibition of glycolysis, and inhibition of PDK2 sensitizes head and neck squamous cell carcinomas to cisplatin induced cell death (6, 7). Similarly, inhibition of pyruvate kinase M2, a mediator of glycolysis upstream of PDK, reduces bladder cancer tumor growth and sensitizes cells to cisplatin (8, 9)

In a laser capture, microarray pilot study to enrich tumor versus other nontumor elements we found PDK4 expression to be increased 33-fold in high-grade invasive versus low-grade bladder cancers with no overexpression of PDK1 to PDK3 (10). Given this dramatic increase in PDK4, we sought to validate the expression of PDK4 in bladder cancer and explore the impact of inhibition on

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tumor growth and chemoresistance. We hypothesized that PDK4 would be upregulated in bladder cancer cell lines, and that inhibition of PDK4 would result in reductions in proliferation in cell lines and in an animal model.

Materials and Methods

Materials and cell culture

Thoroughly tested and authenticated human high-grade bladder cancer cell lines HTB-9, HT-1376, HTB-5, and HTB-4 were obtained from ATCC. The UROtsa (benign) urothelial cell line was a gift from Dr. Brian Philips, University of Pittsburgh, Pittsburgh, Pennsylvania. Malignant cells were cultured in Eagle's MEM (103700-021; Invitrogen), and UROtsa cells were cultured in DMEM media, supplemented with 10% heat-inactivated FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 5% CO_2 in air atmosphere. All cells were used below 20 passages to reduce cellular drift. Early aliquots from each cell line were tested for Mycoplasma using the MycoAlert Test Kit (Lonza) and found to be mycoplasma free. Dichloroacetate (DCA) was acquired as the sodium salt (sodium dichloroacetate) from Sigma-Aldrich. Cisplatin was acquired at United States Pharmacopeia grade via Sigma-Aldrich. All chemicals were acquired from Sigma-Aldrich unless otherwise noted.

Real-time (quantitative) PCR

Total RNA was extracted using Trizol (Invitrogen). RNA (5 μg) was DNase treated (Ambion) and converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative PCR was performed in 96-well plates using Assays-on-Demand Gene Expression system on a 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters (Applied Biosystems). GAPDH served as the endogenous control. Data analyses were done using comparative Ct (ΔΔCt) or relative standard curve.

Cell counts

HTB-5 and HTB-9 cells (10,000 /well) were plated in 12-well dishes and grown for up to 4 days. Cells were rinsed with PBS, suspended with 0.25% trypsin-EDTA, centrifuged, and resuspended in 1 mL of culture medium. An aliquot of 100 μL of cell suspension was counted with a Coulter Counter (Beckman Coulter Inc.).

Caspase activity assay

Cells were plated at 3.5×10^5 cells per well and allowed to adhere overnight. Cisplatin or DCA was dissolved in media at the indicated concentration. Caspase activity was assessed by measuring the amount of relative fluorescence units generated over 30 minutes per mg of protein using a fluorescent substrate (Ac-DEVD-AMC) as described previously (11).

Pyruvate dehydrogenase activity assay

Pyruvate dehydrogenase (PDH) activity was performed using the Sigma PDH Activity Assay (Sigma Aldrich). In brief, HTB-9 or HTB-5 cells were grown to confluency and treated with 10 mmol/L DCA, then lysed in protein buffer. The assay was performed according to the manufacturer's suggested protocol and the use of a positive control provided by the manufacturer was included to confirm the assay was working as intended.

Synergy analysis

Cells were treated with eight different doses of DCA (312.5 μmol/L to 50 mmol/L) or cisplatin (625 nmol/L to 10 μmol/L) on previous cell death curves and cell death was assessed by hexosaminidase assay as previously described (12). Synergy was assessed by combination index analysis using Compusyn software (13).

Lactate dehydrogenase activity assay

Lactate dehydrogenase activity was assessed as described previously (11). Briefly, media was collected after treatment along with cellular lysate, which was sonicated to ensure complete lysis and samples were centrifuged. LDH activity was assessed by chance in sample absorbance in LDH assay solution using a Bio-Tek (Winooska, VT) Epoch2 spectrophotometer.

Animals

Nu/Nu male mice were purchased from Charles River and maintained at the University of Connecticut Health Center for Laboratory Animal Care under NIH guidelines. All procedures were approved by an institutional animal care committee. Animals were housed in a controlled environment with a 12-hour light–12-hour dark cycle and provided food and water ad libitum.

Xenograft flank model of tumor growth

Male nude mice (Crl nu/nu) were inoculated on right flank with 2×10^6 HTB9 cells in a matrigel (5 mg/mL) suspension (1:1; BD Biosciences). Treatment with DCA (200 mg/kg/day via oral gavage) and/or cisplatin (6 mg/kg i.p. weekly) and/or vehicle controls started when tumor volumes reached an average of 150 mm^3. Tumor volume was determined two to three times/week for 7 weeks. Volume was calculated using the rational eclipse formula ($V = m_1 \times m_2 \times 0.5236$), where m_1 is the length of the short axis and m_2 is the length of long axis as measured with calipers. Animals were euthanized by CO_2 inhalation and death verified by cervical dislocation. Tumors were harvested, weighed, and placed in 10% buffered formalin in PBS for 24 hours and then transferred to PBS.

Histologic analysis

Formalin-fixed tumors were bisected and embedded along the mid-sagittal plane, and serially sectioned for pathologic analysis. TUNEL staining was performed using the Roche In Situ Cell Death Detection Kit according to manufacturer's instructions (Roche) and counterstained with nuclear fast red. Evaluation was performed by a single pathologist in a blinded manner.

Statistical analysis

Data are mean ± SEM. Analysis was performed using Sigma Stat, version 2.03. Differences between multiple groups were examined by one-way ANOVA, followed by *post hoc* Bonferroni comparison or *post hoc* Dunnett's comparison to multiple groups to a control sample. Differences between two groups were assessed by Student *t* test.

Results

In vitro: screening of cell lines for endogenous PDK expression

Our initial data indicated PDK4 was upregulated in high-grade bladder cancer samples in the absence of upregulation of PDK1 to PDK3 (10). To assess whether PDK4 upregulation also

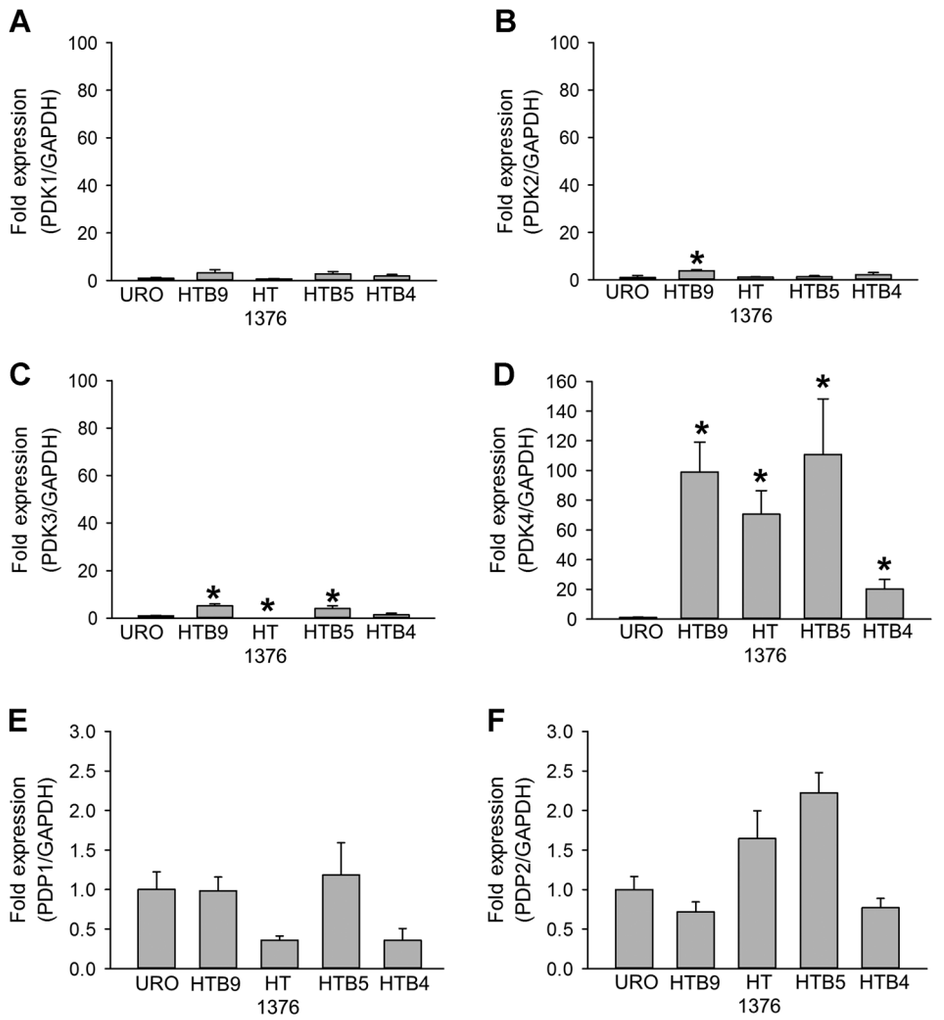

Figure 1.
PDK4 is dramatically upregulated in bladder cancer. PDK1–4 **(A–D)** mRNA expression in benign urothelial cells (URO) and malignant bladder cancer cell lines. PDP **(E, F)** mRNA expression in benign urothelial cells (URO) and malignant bladder cancer cell lines. *, $P < 0.05$ vs. UROtsa.

occurred in cell culture models, UROtsa, HTB-9, HT-1376, HTB-5, and HTB-4 were evaluated for basal PDK1 to PDK4 mRNA expression (Fig. 1). UROtsa cells are a benign urothelial cell line that has been immortalized for cell culture purposes, but retains many characteristics of normal urothelium (14). PDK4 mRNA expression was increased in all malignant cell lines as compared with benign cells (UROtsa), in some cases more than 100-fold above UROtsa (Fig. 1). Pyruvate dehydrogenase phosphatase (PDP) catalyzes the dephosphorylation and activation the PDC, thus reversing the effects of PDKs. PDP levels were also evaluated and no significant difference was noted across benign to malignant cell lines (Fig. 1). Conversely, hypoxia-inducible factor 1α (HIF1α) and peroxisome proliferator-activated receptor alpha (PPARα) are upstream activators of PDKs (15). Expression levels of HIF1α were elevated in two cell lines (HTB-5, HTB-4) and PPARα levels elevated in all malignant cell lines as compared with benign UROtsa cell lines (Supplementary Fig. S1). As such, bladder cancer cell lines HTB-5 and HTB-9 cells were used for further experiments to test the effects of genetic knockdown with siRNA and *in vivo* studies given their high level of PDK4 expression and the tumorigenic property of HTB9 in mice.

In vitro: effect of PDK inhibition on cell growth

DCA is a competitive inhibitor of all PDKs with antitumorigenic effects in multiple solid cancers (16, 17). To validate DCA-mediated inhibition of PDK activity, DCA was given to HTB-9 and HTB-5 cells and PDH activity was assessed. DCA treatment resulted in significant increases in PDH activity after 6 hours in both HTB-9 and HTB-5 cell lines, indicative of inhibition of PDK as expected (Fig. 2). Malignant cell lines HTB-5 and HTB-9 were treated with DCA (10–50 mmol/L) and cell counts measured at 24, 48, and 72 hours. This resulted in significant ($P < 0.05$) decreases in cell counts at 48 and 72 hours in the 25 and 50 mmol/L treatment arms (Fig. 2). Cell-cycle analysis by flow cytometry indicated G_0–G_1 phase arrest in HTB-9 cells consistent with reports in other tumor types (Fig. 2; refs. 17, 18).

To specifically confirm a role for PDK4, HTB-5 cells were transfected with PDK4 siRNA and assessed for cellular proliferation over 48 hours. The siRNA treatment resulted in ∼60% knockdown of PDK4 as assessed by qPCR (manufacturer's suggested method of knockdown validation), which coincided with ∼40% reduction in cellular proliferation (Fig. 3). These data indicate PDK4 inhibition can potently reduce cell proliferation in the absence of inhibition of PDK1 to PDK3.

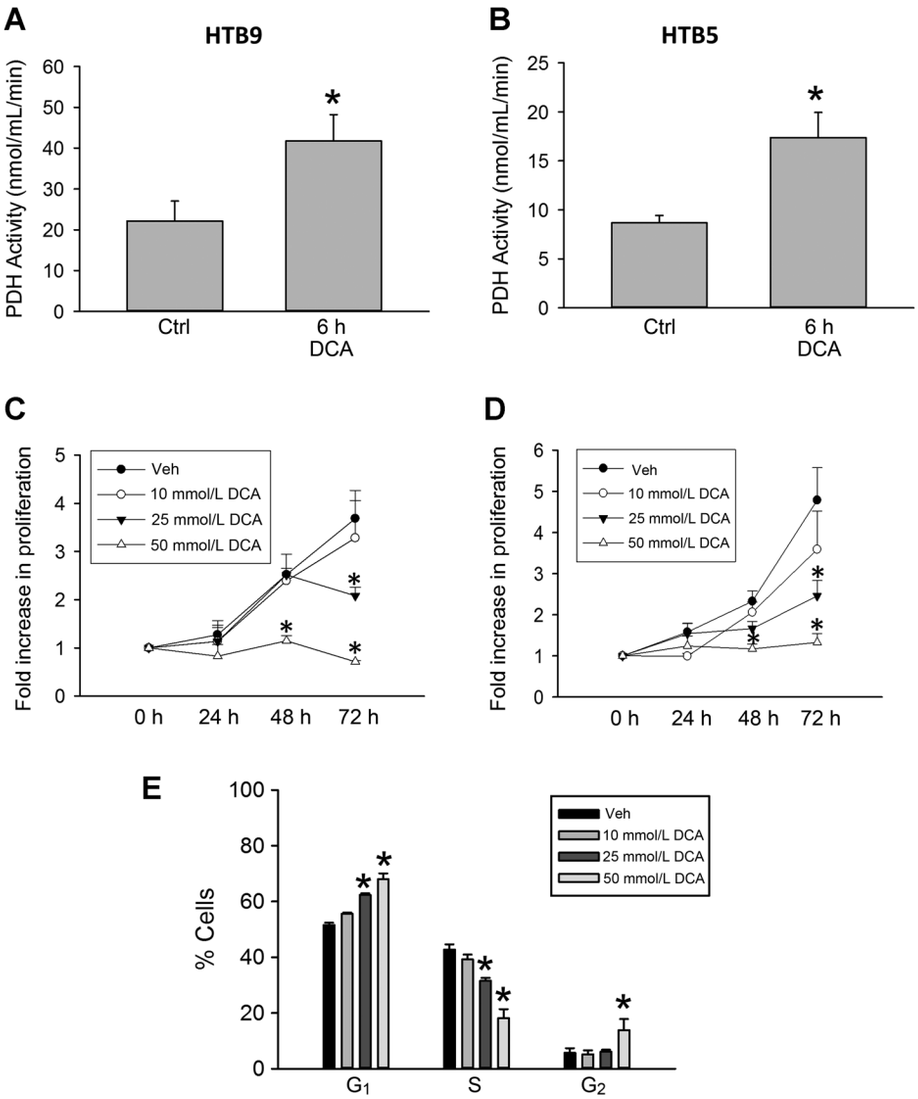

Figure 2.
DCA prevents bladder cancer cell proliferation. HTB-9 (**A, C,** and **E**) and HTB-5 (**B** and **D**) cell lines were treated with DCA and PDH activity was evaluated after 6 hours (**A** and **B**). HTB-9 and HTB-5 cell lines were treated with DCA for up to 72 hours and proliferation was measured by analyzing cell counts (**C, D**). Cell cycle was evaluated by flow cytometry in HTB-9 cells after DCA treatment (**E**). *, $P < 0.05$.

In vitro: effect of DCA on cisplatin-induced cell death

Data from other laboratories have indicated that inhibition of PDKs might sensitize cells to cisplatin (6, 19, 20). Treatment of HTB-9 cells with 50 mmol/L DCA or 5 μmol/L cisplatin resulted in increases in caspase-3 activity, indicating activation of apoptosis by either DCA or cisplatin (Fig. 4). Notably though, caspase activity was not increased in the DCA + cisplatin-treated cells versus either DCA or cisplatin alone (Fig. 4). When total cell death was assessed by LDH activity, cisplatin + DCA treatment led to significant increases above either DCA or cisplatin alone. As there was an increase in LDH release which measures all cell death, but no increase in caspase-3 activity, we hypothesized that combined treatment with DCA and cisplatin led to increases in total cell death (Fig. 4). To confirm this, cell death for both apoptosis and necrosis was assessed simultaneously by flow cytometry for propidium iodide (PI)/Annexin V. DCA + cisplatin-treated cells had significantly higher levels of overall cell death than cisplatin treated alone, although the increase was largely mediated by increases in the Annexin V^+/PI^+ cells, with minimal increases in Annexin V^+/PI^- or Annexin V^-/PI^+. As such, DCA and cisplatin combination treatment increases cell death through multiple mechanisms potentially involving necrosis in addition to apoptosis. We further assessed the capacity of DCA to enhance cisplatin-induced cell death using Chou–Talalay synergy analysis. Cells were treated with a range of doses of cisplatin (0.625–10 μmol/L) or DCA (312.5 μmol/L–50 mmol/L) and the hexosaminidase assay was used to assess synergy via combination index analysis. In HTB-9 cells, concentrations of DCA 10 mmol/L and above synergistically enhanced concentrations of cisplatin 2.5 μmol/L and above after 48 or 72 hours. In HTB-5 cells, similar results were observed at 48 hours, although this effect was lost somewhat after 72 hours (Supplementary Fig. S2; Supplementary Table S1). As such, DCA can synergistically and dramatically enhance cisplatin-induced chemotherapy, making it a potentially useful therapeutic adjuvant.

In vivo: Impact of PDK inhibition on tumor growth

To assess whether DCA could prevent tumor growth *in vivo*, a mouse xenograft model was utilized with HTB-9 cells as they were

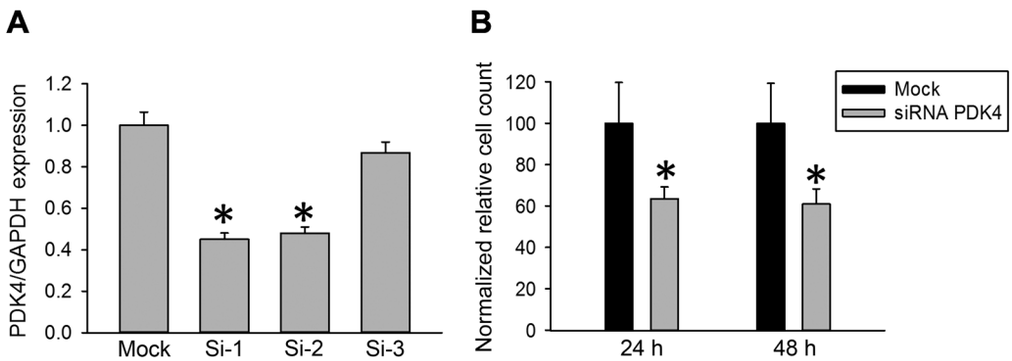

Figure 3.

Knockdown of PDK4 inhibits cellular proliferation. PDK4 was knocked down using different siRNA constructs (Si-1 to Si-3) and knockdown was confirmed by gene expression of PDK4 per manufacturer's instructions (**A**). Cell counts were measured after 24 or 48 hours of exposure to siRNA-1 (**B**). *, $P < 0.05$.

previously established as sensitive to DCA. HTB-5 cells were also assessed but were found to be nontumorigenic. HTB-9 cells were implanted into the flanks of Crl Nu/Nu mice and allowed to grow until tumor volumes reached an average of 150 mm. At this point, DCA was given at 200 mg/kg daily by oral gavage. Tumor volumes were measured weekly. DCA as a nonspecific PDK inhibitor did not alter tumor growth rates in the flank model as assessed by caliper measurement (Supplementary Fig. S3). However, tumor liquefaction due to central tumor necrosis resulted in a ∼60% reduction in viable tumor burden in cisplatin + DCA-treated mice

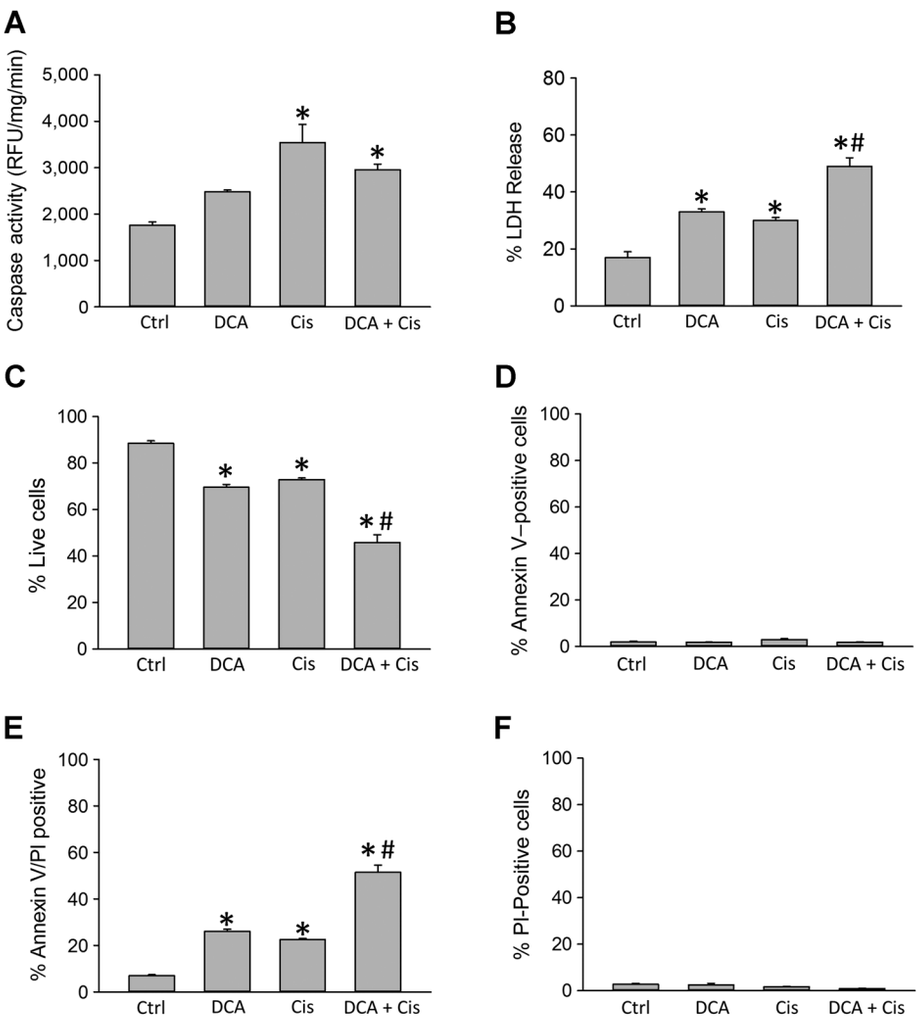

Figure 4.

DCA treatment enhances cisplatin efficacy *in vitro*: HTB-9 cells were treated with DCA, cisplatin, or both. Caspase-3 activity was assessed to measure apoptosis (**A**) and LDH release was measured to assess total cell death (**B**). Flow cytometry for propidium iodide positive and annexin V positive cells was assessed in control cells (**C**) after DCA (**D**), cisplatin (**E**), or DCA and cisplatin treatment (**F**). *, $P < 0.05$; #, $P < 0.05$ vs. DCA or cisplatin groups.

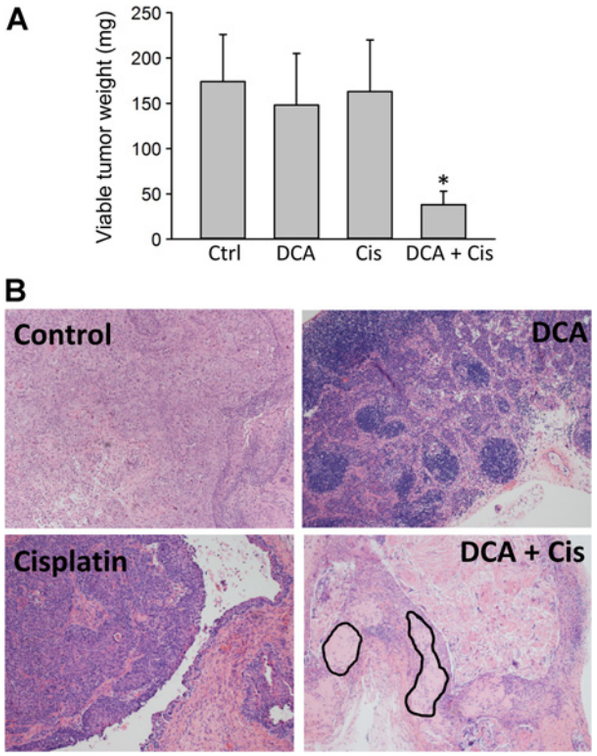

Figure 5.
DCA treatment improves cisplatin therapy. Viable tumor weights post excision (**A**) and histology (**B**). Areas of frank acellularity (necrosis) are indicated with circles. *, $P < 0.05$.

(Fig. 5a). Regions of obvious acellularity were present in DCA + cisplatin-treated tumors, consistent with increased cell killing (see area depicted in blue circles in Fig. 5b). This would be expected given the previous data *in vitro* indicating DCA + cisplatin increased overall cell death without increasing caspase activity and likely indicate increased necrosis in the DCA + *cis* treated animal consistent with the histology. To confirm these data, we used TUNEL staining to evaluate cell death. DCA and cisplatin-treated animals had increased active tumor cell death as assessed by increased TUNEL-positive cells in DCA and cisplatin-treated animals, indicating an increase in cell death (Fig. 6).

In summary, these data indicate PDK4 is substantially upregulated in high-grade bladder cancers, which PDK4 inhibition blocks cellular proliferation, and inhibition of PDKs induces cell death at high concentrations of DCA administration. Furthermore, PDK inhibition may work in concert with cisplatin therapy to further reduce tumor volumes *in vivo*.

Discussion

Advanced bladder cancer remains a highly lethal disease, seeing few impactful therapeutic advances in decades. Five-year survival rates can be as low as 2% to 6%. Radical cystectomy was introduced as the cornerstone of treatment in the 1950s. Cisplatin-based chemotherapeutic regimens were defined by in the 1980s (2). Although this improved disease-specific survival to some degree, the numbers are still underwhelming with half of patients having no response and a significant number of initial responder's ultimately developing resistance during treatment. Chemoresistance remains a critical problem in patients with bladder cancer (21) as there are no accepted or efficacious second-line therapies. Early excitement with immunotherapy and checkpoint blockade has been tempered by the modest response rates of ~20% and the recent report from the only long-term study which revealed no improvement in disease-specific survival (Merck press release, 2017; ref. 22). It is clear based on outcomes of both standard and novel treatments that improvement in current therapies, and development of novel therapeutic strategies are needed.

Cancer cells are known to undergo "hallmark" changes that enhance their ability to survive, invade, and metastasize (23). Included in these alterations is a fundamental shift in energy metabolism first noted by Warburg (3). In the normal cell, energy production is primarily accomplished via oxidative phosphorylation producing 36 ATP. However, cancer cells have been shown to preferentially utilize cytoplasmic aerobic glycolysis, producing two ATP, even in the presence of oxygen. This requires increased glucose metabolism, which is facilitated by upregulation of the transmembrane glucose transporter GLUT1, and other glycolytic metabolizing genes (24). It has been suggested that this alteration proves beneficial for two reasons: (i) continued energy

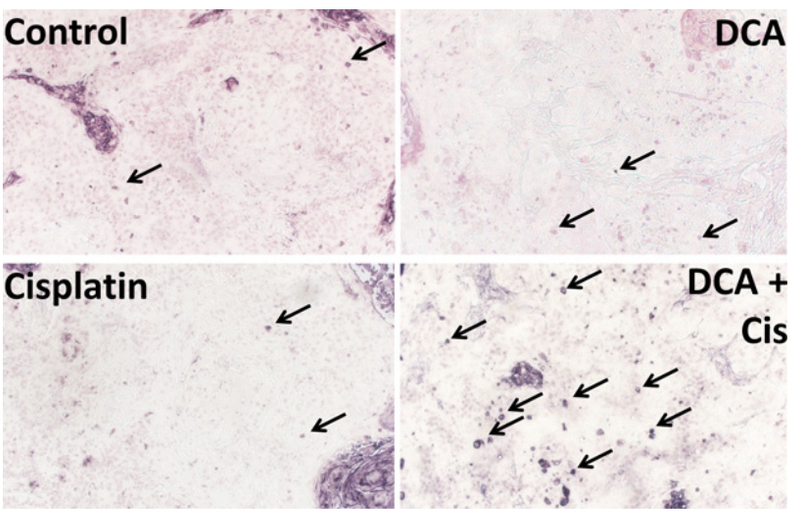

Figure 6.
Increased active TUNEL staining in DCA and cisplatin-treated animals. Increase in active tumor cell death is present after combined treatment with DCA and cisplatin. Arrows represent TUNEL-positive cells.

production in the hypoxic/acidic tumor microenvironment and (ii) sustained production of biosynthetic intermediates for nucleoside and amino acid production which are the building blocks of cellular proliferation (25). In addition, aerobic glycolysis has been shown to enhance cancer chemoresistance through multiple mechanisms, including increased ATP production and resistance to mitochondrial depolarization necessary for cell death (6, 7). Confirming our previous data in human samples, we found that PDK4 is upregulated in bladder cancer cell lines, and that inhibition of PDK4 resulted in reduced proliferation after siRNA knockdown. Inhibition of all PDKs with DCA increased cancer cell death, and enhanced cell killing *in vivo* when combined with cisplatin in a bladder cancer xenograft model, largely consistent with previous reports on the efficacy of DCA as an adjuvant therapy.

Regulation and role of PDK4 in bladder cancer

The physiologic role of the pyruvate dehydrogenase kinase (PDK) family is phosphorylation of the PDH complex (15). Phosphorylation inactivates the PDH complex, reducing metabolism of pyruvate to acetyl-CoA. Increased cellular pyruvate levels is a hallmark of the metabolic switch to aerobic glycolysis (25). DCA administration to cells or mice normalizes cellular levels of both pyruvate and lactate, indicating inhibition of PDKs can restimulate mitochondrial metabolism of pyruvate in cancer cells (17). Bladder cancers have been shown to have elevations of both pyruvate and lactate intracellularly as compared with normal tissue, and as such, likely undergo some degree of aerobic glycolysis to produce energy potentially due to overexpression of PDKs such as PDK4 (26). As such, aberrant upregulation of PDKs likely contributes to the switch to aerobic glycolysis both by reducing oxidative phosphorylation of downstream metabolites of acetyl-CoA and increasing glycolysis (7, 17). Targeting specific PDKs responsible for this switch may be a unique way to limit both cellular proliferation and chemoresistance in tumors.

As some degree of hypoxia is common in most cancers, the primary transcription factor responsible for PDK4 upregulation in cancer is likely HIF1α (24). In addition, numerous studies have reported upregulation of PDK4 by PPARα in response to starvation or diabetes (27, 28). We observed baseline upregulation of HIF1α in our cell lines in the absence of hypoxia, as well as upregulation of PPARα. HIF1α is increasingly recognized as a major mediator of the transcriptional regulation of aerobic glycolysis in cancers, and is associated with an unfavorable outcome in bladder cancer (24, 29). If the switch to aerobic glycolysis is mediated by PDK4 upregulation in bladder tumors, then PDK inhibition may be an effective drug target in hypoxic tumors that overexpress HIF1α, although this needs to be tested further and more directly in the future.

A number of papers have reported selective upregulation of different PDK isozymes in models of disease (6, 30). In our initial screen, we found upregulation of PDK4 in the absence of upregulation of PDKs 1 to 3 (10). It is difficult to directly compare this data to other datasets as our own microarray was performed after laser capture microdissection of tumor cells, thus giving near 100% tumor cells in the samples. Other studies have used 65% to 70% tumor in their samples, yielding considerable contamination from nontumorous cells. Even still, exploration of Oncomine datasets indicates PDK4 was upregulated in other bladder cancer arrays (31, 32). Furthermore, PDK4 can be regulated in immune cells during inflammation, and as such, infiltrating immune cells may substantially alter results (33). Notably, our own findings on PDK4 regulation were largely recapitulated in our cell culture model where PDK4 was substantially overexpressed in all malignant cell lines versus the benign cell line. Although we observed minor upregulation of PDK2 and PDK3 in some cell lines, PDK4 was upregulated 20-fold to more than 100-fold or more in all cell lines tested, indicating the predominant PDK is likely PDK4. One caveat to this study is that we have not yet been able to test effects of direct inhibition of PDK4 in the absence of other PDKs. It remains a possibility that baseline expression of PDK1 to PDK3 could provide sufficient activity such that sole inhibition of PDK4 would not yield a significant effect. Even still, the contribution of PDK4 specifically was partially confirmed using RNA interference as knockdown of PDK4 with siRNA resulted in reduction in cellular proliferation rate on the order of what was observed with DCA. Other reports have indicated DCA can largely act through specific PDK isozymes when they are substantially overexpressed (6, 30, 34). We have hypothesized that targeting PDK4 directly in the future will be paramount to the activity of PDK inhibitors in bladder cancer. As such, we believe therapeutic inhibition of PDK4 should be further explored in bladder cancer. Although we did not confirm an overall increase in PDH activity in siPDK4 expressing cells, future directions include understanding how specific knockdown of PDK4 affects cellular bioenergetics and mitochondrial metabolism and defining mechanisms of how PDK4 knockdown reduces proliferative potential, while also investigating the impact of PDK1 to PDK3.

DCA has been proposed as a potential therapeutic via its ability to inhibit PDKs, reduce cellular proliferation, and induce apoptosis in various cancers (6, 7, 17). DCA is a nonspecific inhibitor of all PDK isozymes but suffers from dose-limiting toxicities (35). Although our data largely confirm DCA as cytotoxic *in vitro*, we did not see direct cell killing or reduction in proliferation *in vivo* with DCA alone in two separate studies. The mechanism as to why PDK inhibition without cisplatin did not result in cell killing or reduced proliferation *in vivo* is unclear but the focus of ongoing work. Even still, novel inhibitors that are specific for PDKs expressed in a tissue-specific pattern may have significant potential benefit relative to DCA in treatment of bladder cancer as a way to minimize toxicity and maximize therapeutic effects of cisplatin. Given that DCA is not specific for any single isozyme, it will be important in the future to determine the relative impact and expression pattern of individual PDKs in normal bladder and in bladder cancer models, and then determine how inhibition of specific PDKs affects cellular proliferation, bioenergetics, and overall outcomes.

PDK4 and cisplatin-based chemotherapy

Chemoresistance remains a major problem in patients with bladder cancer (21). Agents that can enhance cisplatin-based chemotherapy are sorely needed to increase the number of patients who can receive cisplatin, and address resistance to chemotherapy. Canonically, cisplatin induces apoptosis, dependent on depolarization of the mitochondria; however, cisplatin also induces nonapoptotic cell death under multiple varied circumstances (36, 37). DCA also induces apoptosis as part of its therapeutic effect (6, 7, 17). Although we did see increases in apoptosis both with cisplatin and with DCA, we did not see a synergistic increase in caspase activity when we combined these treatments *in vitro*; however, when we observed total cell death (both apoptosis and necrosis) by multiple different assays, it was

clear that DCA and cisplatin enhanced cell death *in vitro*. DCA is known to induce depolarization of the mitochondria, which may benefit cisplatin-induced cell death by enhancing release of endonucleases present in the mitochondria that mediate caspase independent cisplatin-induced cell death (7, 38, 39). *In vivo*, although neither DCA nor cisplatin induced any significant effect alone, the combined DCA + cisplatin treatment resulted in obvious and substantial tumor necrosis in a xenograft mouse model. This was confirmed with TUNEL staining as we observed increased epithelial cell death in mice treated with both DCA and cisplatin. Although the TUNEL assay is commonly used to assess apoptosis, it should be noted that this assay is only specific for DNA fragmentation, and is commonly positive for both apoptotic and necrotic cells (40). As such, this study cannot determine whether cisplatin and DCA-induced necrosis or apoptosis as a primary mechanism of cell death; although, *in vitro* data indicate this is possibly a mix of both cell death types. This study did not directly test the ability of DCA or genetic depletion of PDK4 to address chemoresistance using cisplatin-resistant cell lines; however, the increased cell death when cells are treated with DCA and cisplatin may yield therapeutic advantages, especially if PDK4 inhibition with DCA can overcome hyperpolarization of the mitochondria in bladder cancers. We are currently exploring these mechanisms.

PDK inhibition is a novel strategy in the treatment of bladder cancer with potential therapeutic benefit when combined with cisplatin. Future studies aimed at understanding the mechanisms behind how inhibition of PDKs, particularly PDK4, can enhance cisplatin-based therapies may yield promising results for increasing cisplatin efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B.L. Woolbright, D. Choudhary, A. Mikhalyuk, J.A. Taylor, III

Development of methodology: B.L. Woolbright, D. Choudhary, A. Mikhalyuk, J.A. Taylor, III

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.L. Woolbright, D. Choudhary, A. Mikhalyuk, C. Trammel, S. Shanmugam, E. Abbott, C.C. Pilbeam, J.A. Taylor, III

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.L. Woolbright, D. Choudhary, S. Shanmugam, J.A. Taylor, III

Writing, review, and/or revision of the manuscript: B.L. Woolbright, D. Choudhary, J.A. Taylor, III

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.L. Woolbright

Study supervision: B.L. Woolbright

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