Combination of Taxol® and dichloroacetate results in synergistically inhibitory effects on Taxol-resistant oral cancer cells under hypoxia

QI XIE[1], HAN-FANG ZHANG[1], YING-ZI GUO[1], PENG-YI WANG[1], ZHONG-SHUNG LIU[2], HUA-DONG GAO[1] and WEI-LI XIE[1]

[1]Department of Prosthodontics, School of Stomatology; [2]Institute of Hard Tissue Development and Regeneration, The Second Affiliated Hospital, Harbin Medical University, Harbin, Heilongjiang 150001, P.R. China

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Abstract. Cancer cells preferentially catalyze glucose through the glycolytic pathway in the presence of adequate oxygen. This phenomenon is known as the Warburg effect. As is the case with numerous cancer therapeutic agents, resistance remains a significant problem when using Taxol® to treat malignancies. The present study reported that expression of pyruvate dehydrogenase kinase 1 (PDK1) was induced by Taxol treatment at low toxic concentrations in oral cancer cells. In addition, Taxol-resistant cells exhibited upregulated PDK1 protein and mRNA expression. Elevated PDK1 levels contribute to Taxol resistance under hypoxic conditions. Inhibition of PDK1 expression was observed when oral cancer cells were treated with the PDK1 inhibitor dichloroacetate (DCA). The combination of Taxol with DCA showed synergistic inhibitory effects on Taxol-resistant cells under hypoxic conditions; these effects were not observed in Taxol-sensitive oral cancer cells under normoxic conditions. The present study provides a novel mechanism for overcoming Taxol resistance in oral cancer cells, and will contribute towards the development of clinical therapeutics for cancer patients.

Introduction

Paclitaxel (Taxol®) is an anti-mitotic agent that promotes apoptosis in cancer cells through multiple mechanisms (1); the stabilization of microtubules, mitotic arrest, and eventually cell death (2). The ability of Taxol to inhibit tumor cells has made it an effective chemotherapeutic agent against numerous cancer types, including ovary, breast, oral, colon and lung, as well as malignant melanoma (3-8).

Despite impressive initial clinical responses, the majority of patients eventually develop resistance to Taxol (9). One mechanism known to be involved in cancer cell resistance to Taxol and other microtubule-stabilizing agents, is the high expression of membrane P-glycoprotein which functions as a drug-efflux pump (10). Other cellular mechanisms include alterations of tubulin structure (11-13), changes in the drug-binding affinity of the microtubules (14) and cell cycle deregulation (15,16). The mechanisms of cancer cell intrinsic and acquired paclitaxel resistance are currently not well understood.

Cancer cells, unlike normal cells, use glycolysis with reduced mitochondrial oxidative phosphorylation for energy production (17). Thus, the metabolic switch by cancer cells to undergo anaerobic, rather than aerobic, respiration is preferential for the progression of cancer. In anaerobic respiration the pyruvate generated from glycolysis is converted into lactic acid, whereas in aerobic respiration pyruvate is converted into acetyl-CoA by the action of pyruvate dehydrogenase (PDH). Pyruvate dehydrogenase kinase 1 (PDK1) can inactivate PDH by phosphorylation. Inhibition of PDH prevents the conversion of pyruvate to acetyl-CoA, thus resulting in the switch of metabolism to anaerobic glycolysis, which has proved to be advantageous for tumor growth (18).

Dichloroacetate (DCA), an inhibitor of PDK1, has previously been demonstrated to promote cancer cell apoptosis. DCA is therefore considered to be a promising antineoplastic agent (19). By blocking PDK1, DCA decreases lactate production, and promotes the metabolism of pyruvate, generated from glycolysis, towards oxidation in the mitochondria (20). Clinical trials are underway for the use of DCA in the treatment of disorders of lactic acid accumulation, as well as numerous types of cancer (21). It has been reported that DCA can restore mitochondrial function, thus restoring apoptosis in cancer cells *in vitro*, and shrink tumor size in rats (21). A previous paper demonstrated that PDK1, as well as other enzymes important for glucose metabolism, were upregulated in myeloma, and that a combination of DCA and bortezomib showed cytotoxic effects, indicating that PDK1 inhibition may serve as a novel therapeutic approach (22).

Correspondence to: Dr Wei-Li Xie, Department of Prosthodontics, School of Stomatology, Harbin Medical University, 141 Yiman Street, Harbin, Heilongjiang 150001, P.R. China
E-mail: xiewli1028@sina.com

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In the present study, an essential function of PDK1 in Taxol-induced oral cancer cell apoptosis was reported. Cancer cells displayed resistance to Taxol treatment following a short incubation period under hypoxic conditions. In addition, Taxol treatment upregulated both mRNA and protein expression levels of PDK1. Taxol-resistant oral cancer cells showed higher expression of PDK1 as compared with Taxol-sensitive cancer cells. The combination of the PDK1 inhibitor DCA with Taxol resulted in synergistic inhibitory effects on Taxol-resistant cancer cells under hypoxic conditions. This study proposed novel aspects for overcoming Taxol resistance in oral cancer cells, and will contribute to the development of clinical therapeutics for patients with cancer.

Materials and methods

Cells and cell culture conditions. OECM-1 and SAS human oral squamous cell carcinoma (OSCC) cell lines, were purchased from ATCC (Rockefeller, MD, USA). Briefly, OECM-1 and SAS cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Paisley, UK) containing 10% fetal bovine serum (FBS; HyClone, VT, USA), at 37˚C in a humid atmosphere with 5% CO_2. Hypoxic conditions were induced by incubation of the cells in a sealed Bug-Box anaerobic workstation (Ruskinn Technology, Ltd., Bridgend, UK). For the hypoxia experiments, the oxygen levels were maintained at 2%, with the residual gas mixture being 94.0 to 94.9% N_2 and 5% CO_2, as compared with the normoxia experiments, in which the oxygen levels were maintained at 21%.

Antibodies and reagents. A rabbit monoclonal antibody against PDK1 was used at a 1:1,000 dilution(#3820; Cell Signaling Technology, Danvers, MD, USA); a β-Actin antibody was used as a loading control, at a 1:2,000 dilution (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA). DCA and Taxol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

cDNA synthesis and quantitative polymerase chain reaction (qPCR). RNA was extracted from oral cancer cells using TRIzol™ reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA synthesis was performed using a SuperScript First-Standard Synthesis System for RT-PCR (Invitrogen Life Technologies), according to the manufacturer's instructions. qPCR analyses were performed using Assay-on-Demand primers and the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Foster City, NJ, USA). The samples were analyzed using an ABI Prism® 7700 Sequence Detection System (Applied Biosystems). The primers for the qPCR were: PDK1 forward, 5'-GCTTATCAGAAACTCCAAAGACTGC-3' and reverse, 5'-GCCGGAAAGGTGGGACA-3'. The expression levels of β-actin were used to normalize the relative expression levels. All experiments were performed in triplicate.

Western blotting. Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM phenylmethanesulfonyl fluoride and Protease Inhibitor Cocktail (Sigma-Aldrich) for 20 min on ice. Lysates were centrifuged at 16,000 x 6 at 4˚C for 10 min. The supernatants were collected and the protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). The proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Following blocking of the membranes with phosphate-buffered saline (PBS) with 5% non-fat dry milk for 1 h, the membranes were incubated with the primary antibodies, in PBS with 5% non-fat dry milk, overnight at 4-8˚C. The membranes were extensively washed with PBS and incubated with horseradish peroxidase conjugated secondary anti-mouse antibody or anti-rabbit antibody (1:2,000, Bio-Rad). Following additional washes with PBS, the antigen-antibody complexes were visualized using an Enhanced Chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Cell viability assay. Oral cancer cells were seeded in a 6-well plate, at a density of 10,000 cells/well in 2 ml DMEM containing 10% FBS. Following overnight incubation under the same culturing conditions, each well was refreshed with 2 ml serum-free medium (SFM) for another day. Following this, the cells were then treated with 2 ml SFM containing various concentrations of Taxol or DCA. The drug-containing SFM was refreshed after 2 days, and incubated under the same conditions for a further 2 days. The cell viability was assessed using MTT reagent (Sigma-Aldrich), and by measuring the absorbance at 570 nm using a plate reader. The relative viability was obtained from the absorbance at 570 nm of both the drug-treated and untreated OECM-1 cells. The experiment was repeated three times.

Generation of a Taxol-resistant cell line. OECM-1 Taxol-resistant cells were obtained by gradually increasing the concentration of Taxol in the cell culture medium, followed by selection of Taxol-resistant cells. After successive Taxol treatments for a duration of 3 months, several resistant cell clones were generated.

Statistical analysis. The data was analyzed using the unpaired Student's t-test. All the data are expressed as the means ± standard error. A $P<0.05$ was considered to indicate a statistically significant difference.

Results

Oral cancer cells acquire resistance to Taxol under hypoxia. Since the switch from mitochondrial oxidation to anaerobic glycolysis renders cancer cells resistant to chemotherapy (23), OECM-1 and SAS human oral cancer cell lines were cultured under hypoxic conditions for 24 h followed by measurements of Taxol sensitivity. Under hypoxic conditions, both cell lines acquired resistance to Taxol at multiple concentrations, as compared with cells cultured in normoxic conditions (Fig. 1A and B). It has previously been reported that low oxygen stabilizes hypoxia inducible factor-1 α (HIF-1α) which promotes the transcriptional expression of enzymes that are required for glycolysis (24). The results of the present study indicated that HIF-1α-mediated upregulation of glycolysis may contribute to Taxol resistance in oral cancer cells.

Hypoxia induces PDK1 expression in oral cancer cells. The protein and mRNA expression levels of pyruvate

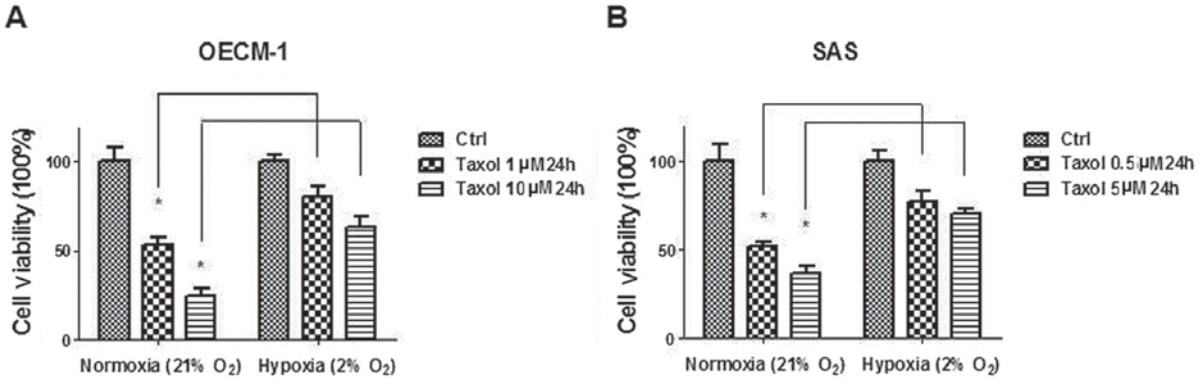

Figure 1. Effects of hypoxia on Taxol resistance in OECM-1 and SAS human oral squamous cell carcinoma cell lines. (A) OECM-1 cells were incubated in a 2% oxygen anaerobic workstation overnight, followed by Taxol treatments of 1 and 10 μM for 24 h. MTT assay was performed to measure cell viability. (B) SAS cells were incubated in a 2% oxygen anaerobic workstation overnight followed by Taxol treatments of 0.5 and 5 μM for 24 h. An MTT assay was performed to measure cell viability. The data represent the means of the three independent experiments ± standard error. *P<0.05. Ctrl, control; OECM-1 and SAS, human oral squamous cell carcinoma cell lines.

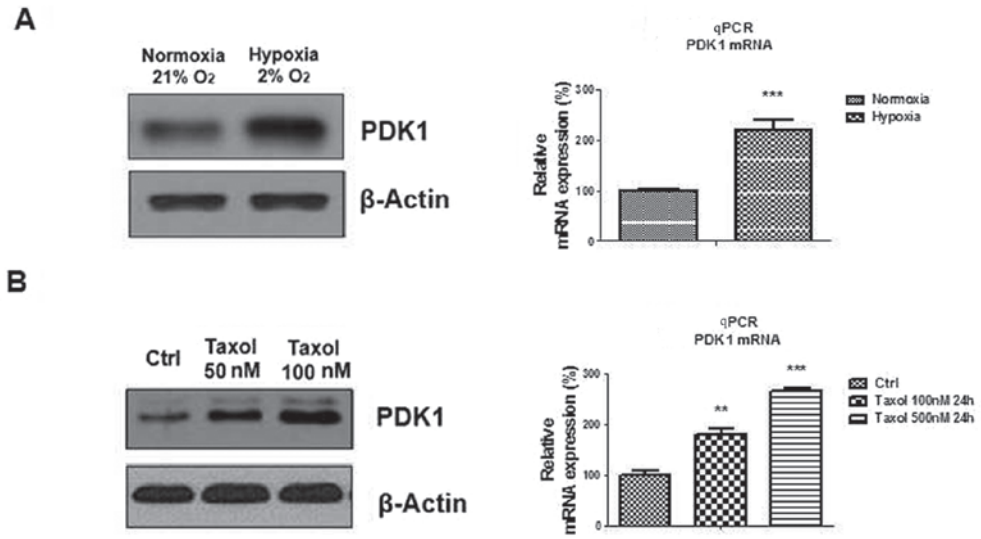

Figure 2. Hypoxia and Taxol treatments induce PDK1 mRNA and protein expression in OECM-1 human oral squamous cell carcinoma cells. (A) OECM-1 cells were incubated in 2% oxygen for 24 h, after which cells were collected and whole cell lysates were subjected to western blot analysis. β-actin was used as a loading control. The total mRNA of the cells, which had been cultured in both normoxic and hypoxic conditions, was isolated and used for cDNA synthesis and quantitative PCR. (B) OECM-1 cells were treated with Taxol at 50 and 100 nM under normoxic conditions for 24 h. The cells were collected and the whole cell lysates were subjected to western blotting. β-actin was used as a loading control . The total mRNA of Taxol-treated cells was isolated followed by cDNA synthesis and quantitative PCR. The data represent the means of the three independent experiments ± standard error. **P<0.01; ***P<0.001. Ctrl, control; PDK1, pyruvate dehydrogenase kinase 1; PCR, polymerase chain reaction; mRNA, messenger RNA.

dehydrogenase kinase 1 were measured. Both PDK1 mRNA and protein expression was upregulated by hypoxia (Fig. 2A), suggesting that PDK1 may be involved in Taxol sensitivity in oral cancer cells.

PDK1 is induced by Taxol in oral cancer cells. It has been reported that treatment with Taxol can upregulate and increase the activity of lactate dehydrogenase A (LDHA) expression, which contributes towards Taxol resistance in breast cancer cells (25). Since both LDHA and PDK1 are key enzymes of glycolysis, it was hypothesized that PDK1 may participate in Taxol resistance. PDK1 protein and mRNA expression was measured in oral cancer cells in response to Taxol treatments. PDK1 was significantly upregulated by Taxol treatment at multiple concentrations (Fig. 2B), indicating that PDK1 may be induced by Taxol treatment and hypoxia. These results may provide a potential mechanism for Taxol resistance.

Taxol resistant cells exhibit upregulated PDK1. OECM-1 Taxol-resistant cells were generated by gradually increasing concentrations of Taxol in the cell culture medium, followed by selection of Taxol-resistant cells. Several resistant cell clones were developed after successive Taxol treatments for 3 months. OECM-1 Taxol-resistant cells had ~70% viability under 5 and 10 μM Taxol treatments, which was much higher as compared with Taxol-sensitive cells (Fig. 3A). The mRNA and protein expression of PDK1 was measured in both Taxol-sensitive and Taxol-resistant cells. Fig. 3B and C showed that both the PDK1 mRNA and protein expression levels were increased in Taxol-resistant cells, as compared with Taxol-sensitive cells.

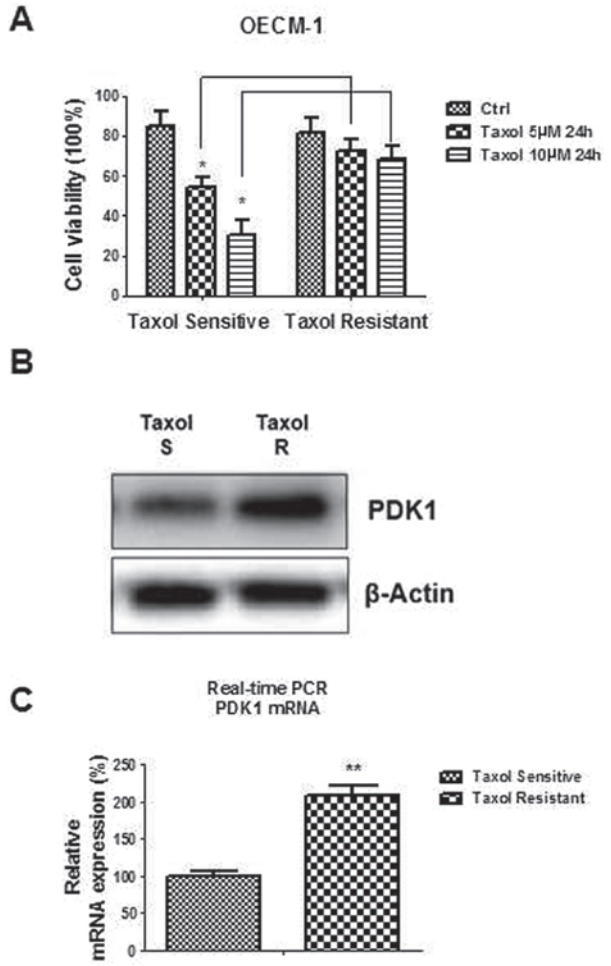

Figure 3. Taxol resistant cells exhibit upregulated PDK1 mRNA and protein expression levels. (A) The Taxol-resistant cells were generated from OECM-1 human oral cancer cells. Taxol-sensitive and resistant cells were treated with Taxol at 5 and 10 μM for 24 h, followed by MTT assay to measure cell viability. (B) The Taxol-sensitive and resistant cells were collected and the whole cell lysates were subjected to western blotting. β-actin was used as a loading control. (C) Total mRNA from Taxol-sensitive and resistant cells was isolated, followed by cDNA synthesis and quantitative PCR. The data represent the means of the three independent experiments ± standard error. *P<0.05; **P<0.01. PDK1, pyruvate dehydrogenase kinase 1; Ctrl, control; mRNA, messenger RNA; OCEM-1, human oral squamous cell carcinoma cell line; PCR, polymerase chain reaction; S, sensitive; R, resistant.

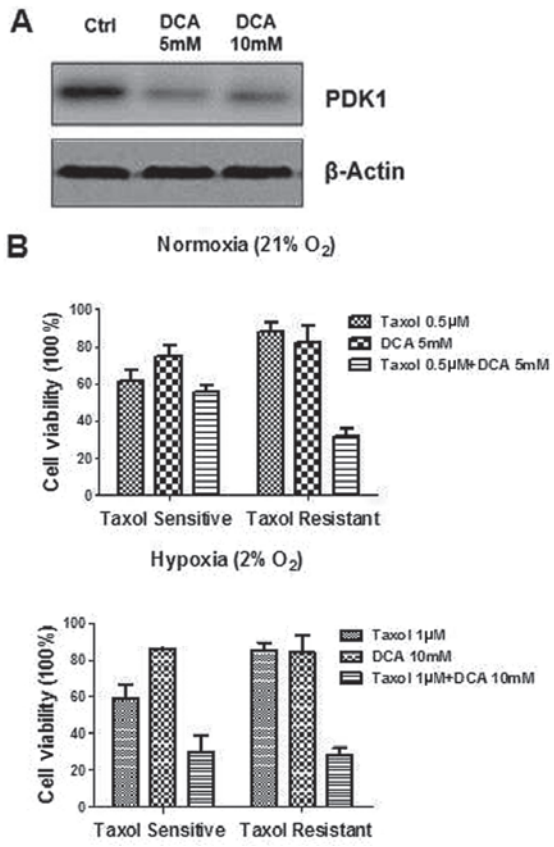

Figure 4. Combination treatment of DCA and Taxol exhibits a synergistic inhibitory effect on Taxol-resistant cells. (A) DCA treatments were shown to inhibit PDK1 protein expression, as compared with the control. OECM-1 human oral cancer cells were treated with DCA at 5 and 10 mM for 24 h followed by western blotting. β-actin was used as a loading control. (B) Taxol-sensitive cells, cultured in normoxic conditions, were not affected by the enhanced inhibition resulting from the combination of DCA and Taxol. Taxol-resistant cells showed synergistic effects from the combination of DCA and Taxol treatments. Under hypoxic conditions, the combination of DCA and Taxol produced synergistic effects on both Taxol-sensitive and resistant cells. The data represent the means of the three independent experiments ± standard error. PDK1, pyruvate dehydrogenase kinase 1; DCA, dichloroacetate; Ctrl, control.

These results suggest that PDK1 is important in cancer cell Taxol resistance.

The combination of DCA and Taxol has synergistic effects on Taxol-resistant oral cancer cells. DCA, an inhibitor of PDK, has been demonstrated to inhibit glycolysis and promote cancer cell apoptosis. OECM-1 cells were treated with 5 and 10 mM concentrations of DCA for 24 h, after which, the PDK1 protein and mRNA expression levels were measured. Figure 4A shows protein expression levels of PDK1 were significantly suppressed by DCA at 5 and 10 mM, as compared with the controls. To further explore the possible biological significance of DCA in Taxol resistance, oral cancer cells were treated with Taxol alone, DCA alone, or a combination of Taxol and DCA under hypoxia. The combination of Taxol and DCA demonstrated a synergistic inhibitory effect on both Taxol-sensitive and resistant cells under hypoxia (Fig. 4B). However, a combination of Taxol and DCA did not exhibit a synergistically inhibitory effect in Taxol-sensitive cells under normoxia. (Fig. 4B). A possible explanation for this may be because the PDK1 expression in Taxol-sensitive cells was not altered when the cells were conditioned under the normal oxygen conditions, resulting in a non-synergistic effect.

Discussion

Taxol is a widely used chemotherapeutic agent for the treatment of several types of cancers, including breast cancer (26). Taxol resistance may result in the recurrence and metastasis of cancer, ultimately increasing the risk of mortality (27). Although extensive research has been carried out in regards to the resistance of cancer cells to Taxol, the specific mechanisms involved are still poorly understood (28). The ability to reduce Taxol resistance would be of benefit to cancer patients, demonstrating the importance of research into the underlying mechanisms of how chemoresistance may arise. Numerous studies support the concept that dysregulated cellular metabolism is linked to

drug resistance in cancer therapy (23,29). It has been previously reported that Taxol-resistant breast cancer cells have an upregulation of LDHA expression and activity, as compared with Taxol-sensitive cells (25), indicating hyperactive glucose metabolism in Taxol-resistant cells.

The Warburg effect refers to the difference in metabolism between normal and cancerous tissues; tumors consume more glucose and produce lactate, even in the presence of adequate oxygen (17). It has been understood that energy metabolism in cancer cells depends on glycolysis, followed by lactate production, rather than oxidative phosphorylation in mitochondria (30). It has been previously reported that several oncogenes, including AKT, MYC and ErbB2, as well as HIF-1 are able to regulate aerobic glycolysis, which contributes to chemoresistance (24,29,31,32). Myc induces the expression of genes regulating glycolysis, such as GLUT1, HK2, Enolase 1 and LDHA (32). Under hypoxic conditions, HIF-1 also induces the expression of glycolytic enzymes, such as GLUT1, Enolase 1, LDHA and PDK1 (24). PDK1 is a serine/threonine kinase which phosphorylates and inhibits mitochondrial PDH, the enzyme responsible for the conversion of pyruvate to acetyl-CoA. PDK1 is a key regulator of the Warburg effect (33). DCA, a PDK inhibitor has been proposed as an anticancer agent which targets glycolysis. As well as the regulation of glycolysis, DCA has been shown to possess other anti-cancer activities, including induction of cell cycle arrest, and depolarization of the hyperpolarized inner mitochondrial membrane (34). Oral administration of DCA was previously reported to show good bioavailability and encouraging phase I/II clinical trials for its use in patients with brain and non-small cell lung cancer (35). A new selective PDK1 inhibitor, AZD7545, is also expected to undergo a clinical trial (36).

In the present study, PDK1 was reported as a novel, putative target for overcoming Taxol resistance in oral cancer cells cultured under hypoxic conditions. Both low oxygen and treatment of cells with Taxol-induced PDK1 expression, rendering cancer cells more vulnerable to the PDK1 inhibitor, DCA. When Taxol-resistant cells were pre-incubated in a low oxygen environment, PDK1 expression was induced, resulting in a synergistic therapeutic effect with Taxol. The present study was focused on PDK1, which is one of the key metabolic enzymes in glycolysis. It remains to be determined whether other enzymes, such as GLUT1, HK2 and LDHA, are involved in Taxol resistance in oral cancer cells. More importantly, the mechanism by which Taxol induces PDK1 expression is still under investigation. Our future studies will focus on the selection of other PDK1 inhibitors, and will explore the mechanisms by which Taxol induces PDK1 expression in human oral cancer cells. In conclusion, the results of the present study highlight the importance of PDK1 in its role in Taxol resistance, and may allow for possible therapeutic interventions in patients that have developed a resistance to Taxol.

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