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The Impact of Hypoxic Treatment on the Expression of Phosphoglycerate Kinase and the Cytotoxicity of Troxacitabine and Gemcitabine[S]

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ABSTRACT

β-L-Dioxolane-cytidine (L-OddC, Troxacitabine, BCH-4556), a novel L-configuration deoxycytidine analog, is under clinical trials for treating cancer. The cytotoxicity of L-OddC is dependent on the amount of the triphosphate form (L-OddCTP) in nuclear DNA. Phosphoglycerate kinase (PGK), a downstream protein of hypoxia-inducible-factor-1α (HIF-1α), is responsible for the phosphorylation of the diphosphate to the triphosphate of L-OddC. In this study, we studied the impact of hypoxia on the metabolism and the cytotoxicity of L-OddC and β-D-2′,2′-difluorodeoxycytidine (dFdC) in several human tumor cell lines including HepG2, Hep3B, A673, Panc-1, and RKO. Hypoxic treatment induced the protein expression of PGK 3-fold but had no effect on the protein expression of APE-1, dCK, CMPK, and nM23 H1. Hypoxic treatment increased L-OddCTP formation and incorporation of L-OddC into DNA, but it decreased the uptake and incorporation of dFdC, which correlated with the reduction of hENT1, hENT2, and hCNT2 expression. Using a clonogenic assay, hypoxic treatment of cells made them 2- to 3-fold more susceptible to L-OddC but not to dFdC after exposure to drugs for one generation. Dimethyloxallyl glycine enhanced the cytotoxicity of L-OddC but not dFdC in Panc-1 cells under normoxic conditions. Overexpression or down-regulation of PGK using transient transfection of pcDNA5-PGK or inducible shRNA in RKO cells affected the cytotoxicity of L-OddC but not that of dFdC. The knockdown of HIF-1α in inducible shRNA in RKO cells reduced the cytotoxicity of L-OddC but not dFdC under hypoxic conditions. In conclusion, hypoxia is an important factor that may potentiate the activity of L-OddC.

β-L-Dioxolane-cytidine (L-OddC, BCH-4556, Troxacitabine) is a novel L-configuration deoxycytidine analog with anticancer and antiviral (hepatitis B virus and human immunodeficiency virus) activity (Kim et al., 1992; Grove et al., 1995; Grove and Cheng, 1996; Gourdeau et al., 2004). It was shown in clinical evaluation to be effective against both leukemias and solid tumors. Phase II clinical studies have shown that L-OddC has significant antileukemic activity in patients with acute myeloid leukemia or patients with chronic myelogenous leukemia in the blastic phase (Giles et al., 2002) and modest activity in advanced pancreatic adenocarcinoma (Lapointe et al., 2005). It is currently undergoing phase I/II clinical trials for the treatment of solid tumors.

In previous studies, we demonstrated that L-OddC can be phosphorylated by deoxycytidine kinase (dCK) to its monophosphate metabolite, which is further phosphorylated to the di- and triphosphate metabolites by cellular kinases (Grove and Cheng, 1996). The triphosphate metabolite of L-OddC can be incorporated into DNA in vitro by DNA polymerases α, β, δ, γ, and ε (Kukhanova et al., 1995). Because L-OddC lacks a hydroxyl group at the 3′-position, once incorporated into DNA, it causes premature termination of DNA replication and eventually leads to cell death. Therefore, the cytotoxicity of L-OddC is dependent on the steady-state level of the incorporated L-OddC in nuclear DNA. This level is affected by the amount of L-OddCTP formed by phosphoglycerate kinase (PGK) (Krishnan et al., 2002a,b, 2003) as well as the removal

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Y.-C.C. is one of the inventors of L-OddC (troxacitabine) which was licensed to SGX Pharm by Yale University for the treatment of cancer. Y.-C.C. could have a financial stake in this compound.

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ABBREVIATIONS: L-OddC, β-L-dioxolane-cytidine (troxacitabine); dFdC, β-D-2′,2′-difluorodeoxycytidine (gemcitabine); PGK, phosphoglycerate kinase; APE-1, apurinic/apyrimidinic endonuclease; dCK, deoxycytidine kinase; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter; HRE, hypoxia response element; DMOG, dimethyloxallyl glycine; shRNA, short hairpin RNA; PBS, phosphate-buffered saline; HIF-1α, hypoxia-inducible factor-1α; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction.

of L-OddCMP from the 3′ termini of DNA by APE-1 (Chou and Cheng, 2000; Lam et al., 2006).

The primary function of PGK, a 46-kDa glycolytic protein, is to use 1,3-biphosphoglycerate as a phosphate donor to generate one molecule of ATP during glycolysis. In hypoxic conditions, ATP synthesis depends on glycolysis in the cytoplasm instead of occurring via oxidative phosphorylation in mitochondria. The up-regulation of glycolytic enzymes such as PGK under hypoxic conditions has been postulated to be one of the compensatory mechanisms for ensuring enough ATP generation. PGK was identified as one of the HIF-1α target genes based on the presence of hypoxia response elements (HRE) (acgtg or gcgtg) in the promoter region (Kress et al., 1998; Okino et al., 1998).

Because hypoxic conditions (oxygen partial pressure 5–10 mm Hg; 0.7%–1.4% oxygen in gas phase) are commonly detected in solid tumors (Brown and Wilson, 2004) and hypoxia has been shown to enhance only PGK expression and not PGK excretion in tumor cells (Daly et al., 2004). We hypothesize that hypoxic conditions in tumors may facilitate the synthesis of the triphosphate metabolite of L-OddC through the action of PGK activated by HIF-1α. L-OddC is therefore a potential drug for preferential inhibition of hypoxic tumor growth.

We report here the effect of hypoxic treatment (1% O_2, 5% CO_2, 94% N_2) on the expression of PGK and APE-1, and the cytotoxicity of L-OddC and dFdC in human tumor cell lines, including HepG2, Hep3B, A673, Panc-1, and RKO, and the metabolism of L-OddC and dFdC in Panc-1 cells.

Materials and Methods

Cell Culture. HepG2 (human hepatocellular carcinoma), Hep3B (human hepatocellular carcinoma), Panc-1 (human pancreatic carcinoma), A673 (human rhabdomyosarcoma), and RKO (human colon carcinoma) cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in a 37°C humidified incubator with a 5% CO_2 atmosphere or in a 37°C hypoxic chamber that was perfused with a gas mixture of 1% O_2, 5% CO_2, and 95% N_2.

Western Blotting. Cells were lysed in 2× SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.05% bromphenol blue) and sonicated for 10 s to shear DNA. The whole-cell extracts were then electrophoresed through 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) with a Mini Trans-Blot cell (Bio-Rad). The membranes were blocked and probed in 1× PBS buffer with 0.2% Tween 20 containing 5% nonfat milk. Monoclonal anti-HIF-1α (1:2000), polyclonal anti-PGK (1:5000), polyclonal anti-APE-1 (1:7500; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-nM23 H1 (1:1000; Santa Cruz Biotechnology), polyclonal anti-CMPK (1:2000; Liou et al., 2002), and polyclonal anti-dCK (1:2000; Hatzis et al., 1998) were used to detect the corresponding proteins. β-Actin was used as an internal control to ensure equal protein loading and detected with a monoclonal actin antibody diluted 1:2500 (Sigma, St. Louis, MO). The membranes were then further incubated with horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (1:5000; Sigma), The immunoreactive bands were visualized by enhanced chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Waltham, MA), and densitometry scanning was performed with the densitometer (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Confocal Microscopy. Cells with or without hypoxia pretreatment were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X-100 in PBS. Bovine serum albumin (1%) in PBS was used to block nonspecific binding. Cells were then exposed to monoclonal anti-HIF-1α, polyclonal anti-PGK, or monoclonal anti-APE-1 (1:3000; Novus, Littleton, CO) at room temperature for 1 h, followed by fluorescein isothiocyanate-conjugated anti-mouse/rabbit IgG at 1:200 dilution. Cytoplasmic actin was counterstained with 0.25 μg/ml rhodamine phalloidin (Invitrogen, Carlsbad, CA). The cells were then sealed in antifade reagent (Invitrogen). Confocal micrographs were scanned by a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, NY).

Clonogenic Assay. Cells (1×10^3) were plated in six-well plates and pretreated with or without hypoxic conditions for 24 h. Cells were treated with L-OddC or dFdC for one generation followed by an exchange with fresh culture medium. The colonies were counted after incubating 7 days and then stained with methylene blue. The results include the means and S.D. obtained from three independent experiments.

Metabolism of Nucleoside Analogs. The cells were treated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 20 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 2 h. For up-regulation of PGK, the open reading frame of PGK was cloned into pcDNA5/TO vector to form pcDNA5-PGK. The pcDNA5-PGK was transfected into RKO cells (5 μg of DNA/10^6 cells) using Lipofectamine 2000 (Invitrogen) for 48 h before hypoxic treatment. For down-regulation of PGK and HIF-1α, inducible shRNA cell lines were established as described previously (Lam et al., 2006). The DNA targeting sequences for PGK was 5′GCTTCTGGGAACAAGGTTAAA3′ and for HIF-1α, 5′TACGTTGTGAGTGGTATTATT3′. The cells were harvested in ice-cold phosphate-buffered saline containing 20 μM dipyridamole (Sigma) and extracted with 15% trichloroacetic acid for 10 min on ice. The supernatant containing the nucleoside and its phosphorylated forms was extracted with a 45:55 ratio of trioctylamine/1,1,2-trichlorotrifluoroethane. The trichloroacetic acid-insoluble pellet representing the nucleotide incorporated into the DNA was washed twice and resolubilized in dimethyl sulfoxide before evaluation using a scintillation counter (LS5000TD; Beckman Coulter, Fullerton, CA). The nucleoside analog metabolites were analyzed by high-pressure liquid chromatography (Shimadzu, Braintree, MA) connected to a detector (Radiomatic 150TR Flow Scintillation Analyzer; PerkinElmer Life and Analytical Sciences) using a Partisil SAX column (Whatman, Clifton, NJ). All results are means and standard deviations obtained from at least three experiments.

Chromatin Immunoprecipitation (ChIP) Assay to Study the Interaction of HIF-1α and PGK Promoter. The cells cultured under either normoxic or hypoxic conditions for 24 h were incubated with 1% formaldehyde at 37°C for 15 min. Cells were washed with ice-cold PBS and lysed in ChIP buffer (1% Triton X-100, 0.1% deoxycholate, 50 mM Tris 8.1, 150 mM NaCl, and 5 mM EDTA) followed by sonication to produce approximately 1 kilobase of DNA fragments. The samples were precleared with salmon sperm DNA-saturated protein A/G-agarose (Calbiochem, San Diego, CA) before a 24-h incubation with anti-HIF-1α antibody (BD Bioscience Pharmingen, Franklin Lakes, NJ) at 4°C. Salmon sperm DNA-saturated protein A/G-agarose was added to precipitate the protein-DNA complexes. The complexes were sequentially washed once with ChIP buffer, ChIP buffer containing 500 mM NaCl, and then LiCl wash buffer. The beads were washed twice with Tris-EDTA buffer before elution at 65°C in 1% SDS and Tris-EDTA buffer. Supernatants were incubated overnight at 65°C to reverse cross-links, and the DNA was purified using a gel extraction kit (QIAGEN, Valencia, CA). The presence of the PGK promoter DNA was determined by PCR. APE-1 promoter was used as the control. The sequences of PGK primer pairs were 5′-gaaggttccttgcggttc-3′ (forward) and 5′-ctagtgagacgtgcggctt-3′ (reverse), and the sequences of APE-1 primer pairs were 5′-gtctccgtcacgtggtgtcag-3′ (forward) and 5′-ggttaggaggagctaggctg-3′(reverse).

Luciferase Reporter Assay. The DNA fragment with PGK-HRE×5 [(gccgg**acgtg**acaaacgg)×5agatctagcggagactatagagggtatataagggcctcggcggcc] or controlx5 [(gccgg**aattg**acaaacgg)×5agatctagcggagactatagagggtatataagggcctcggcggcc] was cloned into pGL4.20 ve-

ctor (Promega, Madison, WI) via the NheI and HindIII restriction sites. The cells were transiently transfected for 24 h using the control-pGL4.20 vector or the PGK-HRE×5-pGL4.20 vector with FuGENE6 transfection reagent (Roche, Indianapolis, IN). The cells were then incubated under normoxic and hypoxic conditions for 24 h. Transcriptional activity was determined by measuring the activity of firefly luciferase in a multiwell plate luminometer (Tecan, Durham, NC) using Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

Quantitative Real-Time PCR. Total RNA was isolated from HepG2 cells, Hep3B cells, Panc-1, and A673 using a high-pure RNA purification kit that includes a step of DNase treatment (Roche). The purified RNA was used as a negative control for indicating the absence of genomic DNA in the PCR reaction. The reverse-transcriptase reaction was performed using Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen), according to the manufacturer's instructions. Assays were performed using the iCycler iQ RealTime thermocycler detection system (Bio-Rad Laboratories). The primers and probes used for the quantitative real-time PCR are shown in Table 1.

Statistical Analysis. Data were analyzed by two-way analysis of variance (Prism 4 software; GraphPad Software, San Diego, CA), Student's *t* test (Microsoft Excel; Microsoft Corp., Redmond, WA), and one-way analysis of variance (GraphPad Prism 4). The difference was considered statistically significant at $P < 0.05$.

Results

The Growth Rate of Tumor Cell Lines in Normoxic Conditions and Hypoxic Conditions. Under hypoxic conditions, some tumor cell growth can be limited by the availability of glucose for ATP production from glycolysis and the accumulation of lactic acid, the end product of anaerobic respiration. We optimized the growth of tumor cells under hypoxic conditions by increasing the glucose concentration to 4.5 g/liter and by adding 25 mM HEPES buffer, pH 7.4, to the culture medium (results not shown). Under these cell culture conditions, the growth rates of all cell lines (HepG2, Hep3B, Panc-1, and A673) under hypoxic conditions were approximately 50% lower than that under normoxic conditions (Supplemental Fig. 1). Culture medium with 4.5 g/liter glucose and 25 mM HEPES buffer was used in the following experiments.

TABLE 1
Primers and probes for quantitative real-time PCR

Gene name	RT-PCR primer	DNA Product
		bp
hENT1		
Forward	tcagcccaccaatgaaa	215
Reverse	ggcccaaccagtcaaagata	
Probe	SYBR Green	
hENT2		
Forward	gctctttgccgtttctaatgg	172
Reverse	tcagagcagcgccttga	
Probe	SYBR Green	
β-actin		
Forward	ttgccgacaggatgcagaagga	129
Reverse	aggtggacagcgaggccaggat	
Probe	SYBR Green	
hCNT1		
Forward	tctgtggatttgccaatttcag	100
Reverse	cggagcactatctgggagaagt	
Probe	SYBR Green	
hCNT2		
Forward	gtccattgctctgtccacagtgg	298
Reverse	ccagtgccctctgggaaattc	
Probe	SYBR Green	
MRP5		
Forward	cccaggcaacagagtctaacc	112
Reverse	cggtaattcaatgcccaagtc	
Probe	SYBR Green	
APE-1		
Forward	agctcaggacagagccagag	144
Reverse	tcttgagtgtggcaggtttg	
Probe	FAM-acagggctgggccctctcct-TAMRA	
PGK		
Forward	gggcaaggatgttctgttct	95
Reverse	tctccagcaggatgacagac	
Probe	FAM-cctgtgccaacccagctgct-TAMRA	

FAM, 5-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

Induction of HIF-1α in Tumor Cell Lines under Hypoxic Conditions (1% O_2, 5% CO_2, 94% N_2). HIF-1α is the key isoform of the hypoxia-inducible factors for activating the transcription of most hypoxia-inducible genes, including PGK. We tested whether hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) could induce HIF-1α expression. As shown in Fig. 1A, HIF-1α was induced in different tumor cell lines after 24 and 48 h of hypoxic treatment. The basal levels of HIF-1α in different cell lines are very low under normoxic conditions. The induced HIF-1α was translocated into nuclei in different cell lines as shown in the immunofluorescence micrographs (Fig. 1B).

The Induction of PGK mRNA and Protein Expression by Hypoxic Treatment. The effect of hypoxia on the mRNA expression levels of PGK responsible for the L-OddCTP formation, and APE-1 exonuclease activity, which can remove the incorporated L-OddCMP from the 3′ DNA terminus and cause L-OddC resistance in cell culture was determined by quantitative real time RT-PCR. After 24- and 48-h hypoxic treatment, PGK mRNA expression levels in different cell lines increased 4- to 5-fold (Fig. 2A). The expression of APE1 mRNA was not affected by the hypoxic treatment (data not shown). There were interactions between HIF-1α protein and the promoter region of PGK gene (−352 to − 264 base pairs, where two HRE sites could be identified), under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) as determined by chromatin immunoprecipitation assay (Fig. 2B). Results from luciferase reporter assays indicated that the HRE of the PGK promoter mediates the transcriptional response to hypoxia (Fig. 2C). The results of the chromatin immunoprecipitation assay and the luciferase reporter assay suggest that the induction of PGK mRNA could be due to increased binding activity between the HIF-1α transcription factors and the HRE of the PGK promoter under hypoxic conditions. The effect of hypoxia on the levels and the subcellular distributions of PGK protein and APE-1 protein in different cell lines were also studied. Results from Western blotting indicated that the protein levels of only PGK and not APE-1 were induced approximately 2- to 3-fold in different cell lines (Fig. 2D). Immunofluorescence micrographs showed that hypoxia had no effect on the subcellular distributions of PGK protein, which was present mostly in cytoplasm, and the APE-1 protein, which was present in nuclei (Fig. 2E).

The Expression of nM23 H1, CMPK, and dCK Protein under Hypoxic Conditions. In addition to PGK protein, we examined the impact of hypoxia on several key enzymes, dCK (L-OddC and dFdC to L-OddCMP and dFdCMP), CMPK (L-OddCMP and dFdCMP to L-OddCDP and dFdCDP), and nM23 H1 (dFdCDP to dFdCTP), responsible for different phosphorylation steps of L-OddC and dFdC. Western blot results indicated that the levels of these proteins in different cell lines were quite different. How-

ever, hypoxia had no effect on the level of these proteins in all the cell lines studied (Fig. 3).

The Effect of Hypoxic Treatment on the Phosphorylation and Incorporation of L-OddC and dFdC in Panc-1 Cells. The accumulation rates of the total metabolites of L-OddC were similar under both normoxic and hypoxic conditions. Hypoxic treatment significantly increased the intracellular amount of L-OddCTP (approximately 40%) ($P < 0.0001$) after 24-h incubation with L-OddC (Fig. 4A); however, hypoxic treatment had no effect on the amount of L-OddC, L-OddCMP, and L-OddCDP ($P > 0.05$) (Supplemental Fig. 3, A–C). This result is consistent with the PGK protein induced in the hypoxic conditions, but not dCK protein and CMPK protein. The hypoxic conditions induced PGK protein 3-fold but only increased the amount of L-OddCTP by 40%. This suggests that PGK protein may be above the rate-limiting state. The hypoxic treatment also increased the L-OddC incorporation into cellular DNA by 25% ($P < 0.05$) in Panc-1 cells after 24 h incubation with L-OddC (Fig. 4B). Again, this result correlates with the increase of L-OddCTP under hypoxic conditions. To show that the increase in L-OddCTP level and the L-OddC incorporation into DNA was not due to a change in the cellular redox status under hypoxic conditions, dimethyloxallyl glycine (DMOG), a prolyl hydroxylase inhibitor, was added to induce HIF-1α under normoxic conditions. The induction of HIF-1α by addition of 500 μM DMOG increased the PGK expression under normoxic conditions (Fig. 4E). DMOG also increased the level of L-OddCTP and the incorporation of L-OddC into DNA significantly ($P < 0.05$) (Fig. 4, F and G). The addition of DMOG under hypoxic conditions did not affect the level of L-OddCTP and the incorporation of L-OddC into DNA.

The accumulation of the dFdC metabolites increased from 2 to 8 h under normoxic conditions (Fig. 4C and supplemental Fig. 3, D–F). The amount of dFdC incorporation into DNA over time correlated with the level of dFdCTP at different time points (Fig. 4, C and D). Hypoxic treatment decreased all of the phosphorylated forms of dFdC by approximately 50% (dFdCTP is shown in Fig. 4C, and the other forms of dFdC are shown in supplemental Fig. 3, D–F) and also decreased the dFdC incorporation into cellular DNA approximately 50% within 8 h of incubation (Fig. 4D). The difference in the levels of dFdC metabolites and DNA incorporation under normoxic and hypoxic conditions was decreased after 24-h exposure to dFdC. The addition of DMOG has no significant effect on the level of dFdCTP or the incorporation of dFdC into DNA in both conditions (Fig. 4, H and I, and supplemental Fig. 4B).

The Effect of Hypoxic Treatment on the mRNA Expression of Nucleoside Transporters hCNT1, hCNT2 hENT1, hENT2, and MRP5. Because hypoxic treatment decreased the uptake of dFdC that is dependent on nucleoside transporters, but not L-OddC, uptake of which is not dependent on nucleoside transporters (Gourdeau et al., 2001), we examined whether hypoxic treatment affected the mRNA expression of nucleoside transporters, which can reflect the protein level to some extent. RT-PCR results indicated that the mRNA expression of hENT1, hENT2, and hCNT2 was repressed to different extents in different cell lines after hypoxic treatment from 24 to 48 h (Fig. 5A). The mRNA expression of hENT1 decreased approximately 50% in different cell lines under hypoxic conditions. The mRNA expression of hENT2 decreased by 50% in Hep3B cells and only 20% in the other three cell lines. The mRNA expression of hCNT2 decreased by 70% in HepG2 and Hep3B and 50 to 60% in Panc-1 and A673 cells under hypoxic conditions (Fig. 5B). The mRNA expression of hCNT1 and MRP5 was not affected by hypoxic treatment. Under hypoxic conditions, the underexpression of hENT1, hENT2, and hCNT2 decreased on thymidine uptake and incorporation in Panc-1 cells (Supplemental Fig. 2).

The Effect of Hypoxia on the Cytotoxicity of L-OddC and dFdC in Different Cell Lines. We studied whether the increase of L-OddCTP after hypoxic treatment would increase the cytotoxicity of the cancer cells. Under normoxic conditions, different cell lines have different susceptibilities

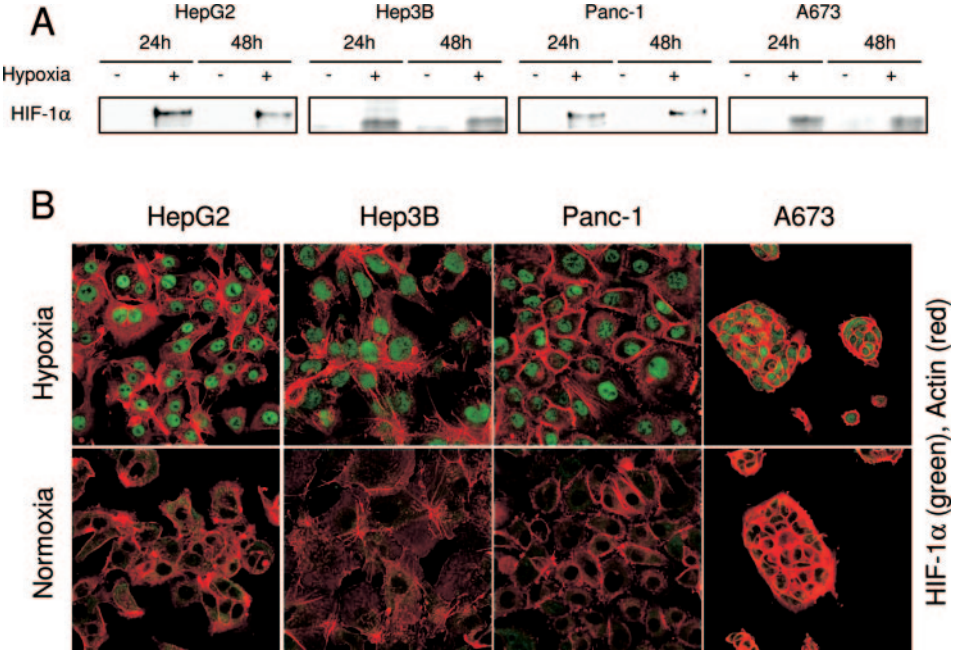

Fig. 1. Induction of HIF-1α in tumor cell lines under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2). A, Western blotting for detecting the induction of HIF-1α of HepG2, Hep3B, Panc-1, and A673 cells after exposure to the hypoxic condition for 24 and 48 h (Ponceau protein staining was used for normalizing total protein loading). B, immunofluorescence micrographs for determination of the subcellular localization of induced HIF-1α of HepG2, Hep3B, Panc-1, and A673 cells after 24-h hypoxic treatment.

to L-OddC and dFdC. Because the DNA synthesis rate and cell growth were reduced by 50% under hypoxic conditions, cells were treated with drugs according to their generation time under different conditions. Clonogenic assays results indicated that hypoxic treatment could sensitize HepG2, A673, and RKO cells by approximately 2-fold and Hep3B and Panc-1 cells by approximately 3-fold (Table 2). For Panc-1 cells, with the normalization of the incorporation rate of L-OddC (25% increase) against the DNA synthesis rate under hypoxic conditions, the incorporation rate of L-OddC per DNA synthesis increased to approximately 2.5-fold, and this was consistent with the increased L-OddC cytotoxicity under hypoxic conditions. Because the incorporation rate of dFdC and DNA synthesis rate dropped to the same extent (50%) under hypoxic conditions, the normalization of the incorporation rate of dFdC and DNA synthe-

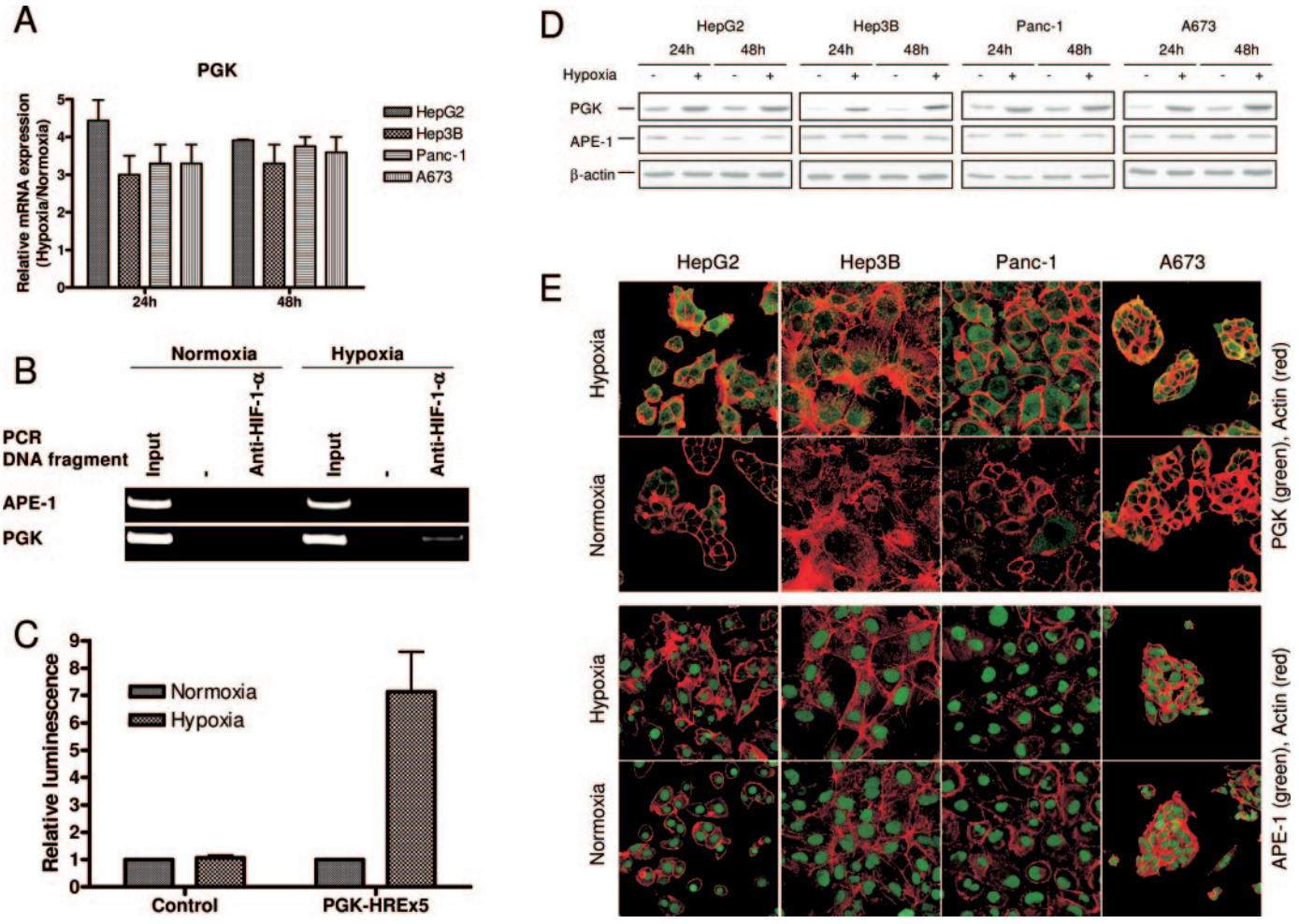

Fig. 2. Characterization of the induction of PGK under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2). A, quantitative real-time RT-PCR for quantifying the expression levels of PGK mRNA under normoxic and hypoxic conditions. B, ChIP for showing the interaction of HIF-1α and PGK promoter of Panc-1 cells after treating the cells in the hypoxic condition for 24 h. C, luciferase reporter assay indicates the hypoxia response element of PGK promoter mediates transcriptional response to hypoxia. D, Western blotting for detecting the level of PGK protein and APE-1 protein of HepG2, Hep3B, Panc-1, and A673 cells after exposure to hypoxic conditions for 24 and 48 h (β-actin staining was used for normalizing total protein loading). E, immunofluorescence micrographs for determination of the subcellular localization of PGK protein and APE-1 protein of HepG2, Hep3B, Panc-1, and A673 cells after 24-h hypoxic treatment.

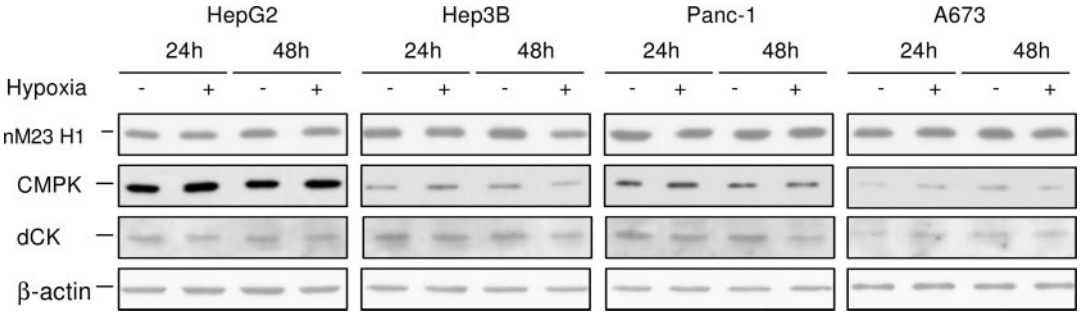

Fig. 3. Western blotting for detecting the levels of H1 protein, CMPK protein and dCK protein of HepG2, Hep3B, Panc-1, and A673 cells after exposure of cells to hypoxic conditions for 24 and 48 h (β-actin staining was used for normalizing total protein loading).

sis showed no difference, and the cytotoxicity of dFdC was not affected by hypoxia (Table 2).

DMOG (500 μM; showing no cytotoxicity up to 2 mM) induced expression of HIF-1α and PGK, and increased the level of L-OddCTP and the incorporation of L-OddC into DNA (Fig. 4, E–G), and also sensitized Panc-1 cells to L-OddC approximately 2.8-fold under normoxic conditions (Table 2). The importance of PGK and HIF-1α in L-OddC cytotoxicity under normoxic and hypoxic conditions was further demonstrated in RKO cells. First, overexpression of PGK using transient transfection increased the level of L-OddCTP and the incorporation of L-OddC into DNA but not dFdC under normoxic conditions (Supplemental Figs. 5–7). The overexpression of PGK also sensitized RKO cells to L-OddC approximately 1.6-fold but not dFdC under normoxic conditions (Table 2). The transient transfection of PGK did not further increase the cytotoxicity of L-OddC under hypoxic conditions; perhaps the level of PGK had reached the maximum level under hypoxic conditions. Second, down-regulation of HIF-1α using shRNA by adding doxycycline decreased the levels of PGK and L-OddCTP and the incorporation of L-OddC into DNA but not that of dFdC under hypoxic conditions (Supplemental Figs. 5–7). The knockdown of HIF-1α reduced the cytotoxicity of L-OddC approximately 1.6-fold but not that of dFdC under hypoxic conditions (Table 2). Third, down-regulation of PGK using shRNA by adding doxycycline decreased the PGK level and L-OddCTP and incorporation of L-OddC into DNA but not that of dFdC under normoxic and hypoxic conditions, respectively (Supplemental Figs. 5–7). The knockdown of PGK reduced the cytotoxicity of L-OddC approximately 1.4- and 1.8-fold under normoxic and hypoxic conditions, respectively (Table 2).

Discussion

Most solid tumors have areas of hypoxia because of the abnormal development of a blood vascular system. Hypoxic conditions induce hypoxia-inducible factors that interact with hypoxia response elements of the target gene promoters, resulting in the expression of many proteins such as glycolytic enzymes and angiogenic factors. Activation of this transcriptional program can result in more aggressive and metastatic cancer phenotypes and is associated with resistance to radiation therapy, chemotherapy, and poor treatment outcomes. To overcome this problem, different approaches have

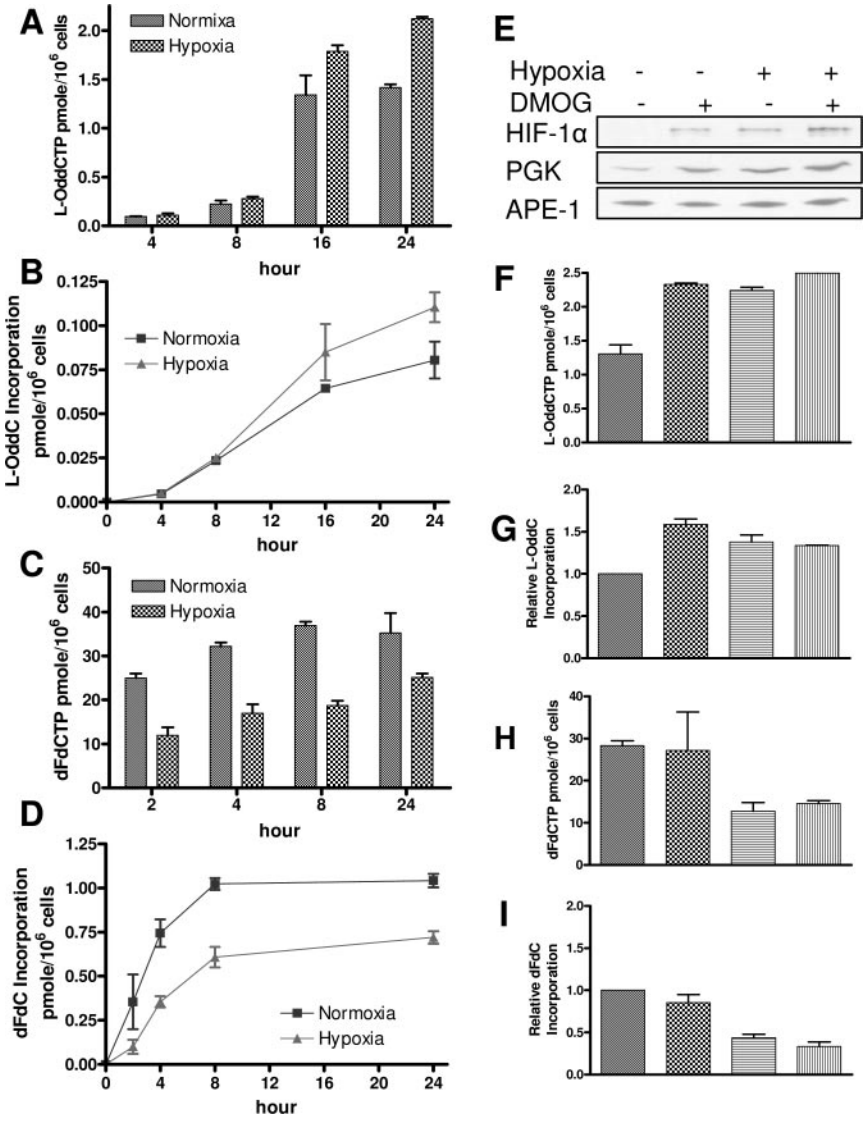

Fig. 4. The metabolism of L-OddC and dFdC of Panc-1 cells under normoxic and hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) with or without addition of DMOG 500 μM. Cells pretreated under hypoxic conditions for 24 h were incubated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 4, 8, 16, and 24 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 2, 4, 8, and 24 h. Trichloroacetic acid (15%) was used to precipitate macromolecules in the cells, the L-OddCTP and dFdCTP in the Trichloroacetic acid-soluble fraction were separated by high-performance liquid chromatography (A, C, F, and H). The amount of L-OddCMP and dFdCMP present in DNA was determined by scintillation counting (B, D, G, I). Western blot for determining the level of HIF-1α, PGK and APE-1 (E). The rest of the procedures were as described under *Materials and Methods*.

been proposed. Here, we report that L-OddC has some advantages in treating solid tumors.

We showed that the induction of PGK in different solid tumor cell lines, including HepG2, Hep3B, A673, Panc-1, RKO, KB, and HeLa (results not shown) under hypoxic conditions are under the control of HIF-1α. Along with other

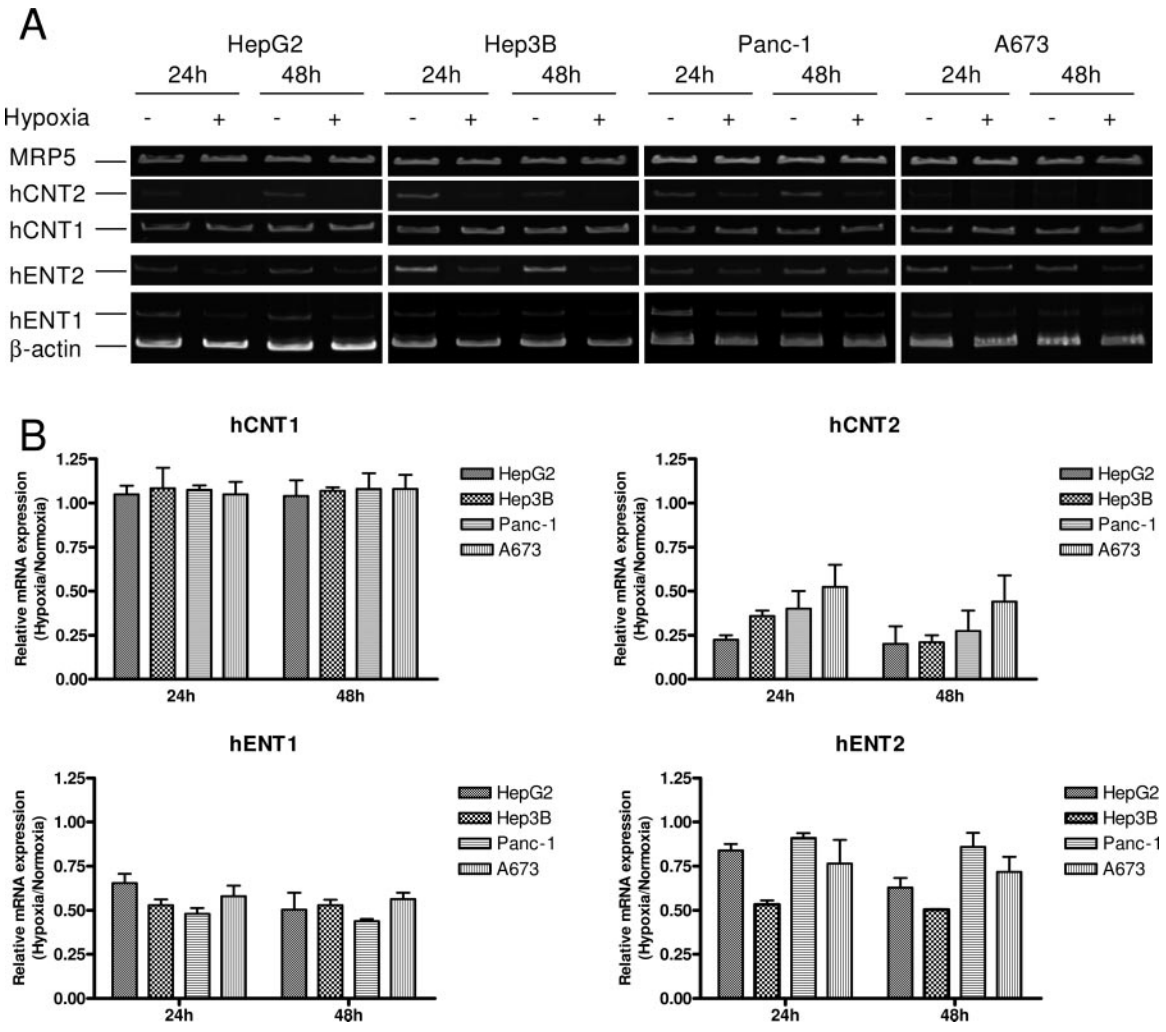

Fig. 5. Effect of hypoxia on the mRNA expression of nucleoside transporters. A, conventional RT-PCR for showing the expression of the hENT1, hENT2, hCNT1, hCNT2, and MRP5. B, quantitative real time RT-PCR for quantifying the expression levels of the nucleoside transporters; hENT1, hENT2, hCNT1, hCNT2 of HepG2, Hep3B, Panc-1, and A673 cells after exposure to hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) for 24 and 48 h.

TABLE 2

Inhibition of clonogenicity (ID_{50}) by L-OddC and dFdC in HepG2, Hep3B, A673, Panc-1, and RKO cells under normoxic and hypoxic conditions

Cells were treated with drug for one generation time under normoxic conditions (N) or cells were treated with drug for one generation time under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) (H). ID_{50} was defined as the concentration of drug required to achieve 50% of surviving fraction and values are means ± S.D. of three experiments, with each data point done in triplicate. N/H indicates -fold change (ID_{50} of normoxia/ID_{50} of hypoxia).

Cell	Additive	L-OddC			dFdC		
		N	H	N/H	N	H	N/H
		nM				*nM*	
HepG2		122 ± 15	60 ± 17	2	2.4 ± 0.2	2.0 ± 0.3	1.2
Hep3B		460 ± 48	150 ± 22	3.1	16 ± 2.5	12 ± 3	1.3
A673		600 ± 50	305 ± 25	2	23 ± 3	19 ± 2	1.2
Panc-1		195 ± 13	57 ± 2.6	3.4	18 ± 1.4	15 ± 1.7	1.2
Panc-1	DMOG	68 ± 7.6	37 ± 8.2		21 ± 0.7	17 ± 1.4	
RKO	pcDNA5	199 ± 8	97 ± 11	2.1	3.5 ± 0.6	2.7 ± 0.4	1.3
RKO	PGK	126 ± 27	81 ± 12		3.6 ± 0.9	2.8 ± 0.2	
RKO-shCon		169 ± 21	84 ± 20	2	3.5 ± 0.8	2.7 ± 0.2	1.3
RKO-shCon	dox	168 ± 3.5	85 ± 24		3.4 ± 0.5	2.5 ± 0.3	
RKO-shHIF-1α		181 ± 23	63 ± 10	2.8	4 ± 0.9	2.9 ± 0.4	1.3
RKO-shHIF-1α	dox	200 ± 31	102 ± 4.7		3.8 ± 0.6	2.6 ± 0.2	
RKO-shPGK		168 ± 2.9	83 ± 17	2	3.2 ± 0.7	2.7 ± 1.4	1.3
RKO-shPGK	dox	228 ± 19	146 ± 25		3.1 ± 0.3	2.4 ± 0.4	

>DMOG, dimethyloxallyl glycine 500 μM; pcDNA5, control vector; PGK, PGK overexpressing vector; dox, doxycycline 5 ng/ml.

reports, the induction of PGK under hypoxic conditions is a common phenomenon. When we compared the cytotoxicity of L-OddC and dFdC in HepG2, Hep3B, A673, Panc-1, and RKO cells under normoxic and hypoxic conditions, we found that the tumor cells were sensitized to L-OddC to different extents depending on the different cell types under hypoxic conditions but not to dFdC. The results from the metabolism of L-OddC demonstrated that the increase in sensitivity to L-OddC was due to the increase of L-OddCTP formation and L-OddC incorporation into DNA under hypoxic conditions. Our results also indicate that hypoxic conditions in culture do not alter the expression of APE-1, which preferentially excises L-OddCMP and misincorporated nucleotides from the 3′-termini of DNA in all of these cell types. Under normoxic conditions, DMOG induced PGK through the stabilization of HIF-1α and sensitized Panc-1 cells to L-OddC. This result suggests that the change in the cellular redox status under hypoxic conditions is not the primary factor in the cytotoxicity of L-OddC. Furthermore, the cytotoxicity of L-OddC in RKO cells correlates with the expression level of PGK in both normoxic and hypoxic conditions and to the level of HIF-1α under hypoxic conditions. Collectively, our results demonstrate that hypoxia can induce PGK expression through the control of HIF-1α and causes sensitization of tumor cells to L-OddC in culture. These findings provide a rationale for clinical development of L-OddC as a treatment for solid tumors, because clinical reports indicate that the development of hypoxia resulting in the expression of HIF-1α in solid tumors is common (Brown and Wilson, 2004). Hypoxia may be the reason for the overexpression of PGK in some solid tumors [e.g., pancreatic ductal adenocarcinoma (Hwang et al., 2006), HER-2/neu-positive breast tumors (Zhang et al., 2005) and lung adenocarcinoma (Chen et al., 2003)]. Because most renal cell carcinomas have defects in the von Hippel-Lindau gene and HIF-1α and its downstream proteins are continuously overexpressing (Patel et al., 2006), testing of the sensitivity of L-OddC in renal cell carcinomas is under investigation.

It is noteworthy that dFdC uptake was severely reduced under hypoxic conditions, but not L-OddC uptake, which is not dependent upon the nucleoside transporters (Gourdeau et al., 2001). We studied the effect of hypoxia on the mRNA expression of the nucleoside transporters. Our results indicate that mRNA expression of hENT1, hENT2, and hCNT2 in different cell lines can be repressed to different extents. The expression of hCNT1 and MRP5, however, were not repressed. These results could partially explain the decrease in dFdC uptake in Panc-1 cells. This is consistent with recent reports stating that hypoxia can repress the expression of hENT1 and/or hENT2 in different cell lines [i.e., human microvascular endothelial cells (Eltzschig et al., 2005) and human umbilical vein endothelial cells (Casanello et al., 2005)]. Clinical reports indicate that decreased expression of hENT1 can be correlated with treatment failure of dFdC in cancers, including mantle cell lymphoma (Marcé et al., 2006), pancreatic cancer (Spratlin et al., 2004; Giovannetti et al., 2006), and non–small-cell lung cancer (Achiwa et al., 2004). Because hENT1, hENT2, and hCNT2 are also involved in the uptake of many other anti-cancer nucleoside analogs [i.e., arabinofuranosyladenine, arabinosylcytosine, 5′-deoxy-5-fluorouridine (Kong et al., 2004 and Podgorska et al., 2005; Hubeek et al., 2005), and clofarabine (King et al., 2006)], hypoxia is likely to be a key factor contributing to the anticancer nucleoside analog resistance via the down-regulation of nucleoside transporters. Currently, the combined use of anticancer nucleosides and antiangiogenic compounds that are likely to increase the hypoxic status in tumors are being tried in the clinic. Based on our findings, we suggest that anticancer nucleosides in which uptake is dependent on the nucleoside transporters should be applied before antiangiogenic compounds. The retention of these nucleosides may be increased because antiangiogenic compounds may induce hypoxia and reduce the expression of nucleoside transporters. However, in the clinical trials of L-OddC, the antiangiogenic compounds should be applied before L-OddC to create more hypoxic conditions and thereby induce the expression of PGK. In conclusion, hypoxia is an important factor that may potentiate the activity of L-OddC.

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