Effect of hypoxia on the expression of phosphoglycerate kinase and antitumor activity of troxacinabine and gemcitabine in non-small cell lung carcinoma

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Abstract

β-L-Dioxolane-cytidine (L-OddC; BCH-4556; troxacinabine), a novel L-configuration deoxycytidine analogue, was under clinical trials for treating cancer. The cytotoxicity of L-OddC is dependent on its phosphorylation to L-OddCTP by phosphoglycerate kinase (PGK) and its subsequent addition into nuclear DNA. Because PGK is induced with hypoxia, the expression of hypoxia-inducible factor-1α and PGK of H460 cells (human non-small cell lung carcinoma) in vitro and in vivo was studied. In culture, hypoxic treatment induced the protein expression of PGK by 3-fold but had no effect on the protein expression of other L-OddC metabolism-associated enzymes such as apurinic/apyrimidinic endonuclease-1, deoxycytidine kinase, CMP kinase, and PGK of A

Introduction

β-L-Dioxolane-cytidine (L-OddC; BCH-4556, troxacinabine) is a novel L-configuration deoxycytidine analogue with anticancer and antiviral (hepatitis B virus and HIV) activity (1–4). It was shown in clinical evaluations 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 and chronic myelogenous leukemia in the blast phase (5) and modest activity in advanced pancreatic adenocarcinoma (6), renal cell carcinoma (7), and non-small cell lung carcinoma (8). It is currently under consideration for beginning phase I/II clinical trials for the treatment of solid tumors.

The metabolism of L-OddC is very different from D-configuration nucleoside analogues. Unlike many D-configuration nucleoside analogues, the cellular uptake of L-OddC is more dependent on passive diffusion than nucleoside transporter uptake (9). Tumor cells that are resistant to D-configuration nucleoside analogues due to underexpression of nucleoside transporters are sensitive to L-OddC (9). L-OddC is not catabolized by cytidine deaminase, which deaminates many D-configuration deoxycytosine nucleoside analogues, such as araC and gemcitabine (dFdC). Intracellularly, L-OddC is phosphorylated by deoxycytidine kinase (dCK) to its monophosphate metabolite that is further phosphorylated to the diphosphate and triphosphate metabolites by cellular kinases (2). The triphosphate metabolite of L-OddC can be incorporated into DNA in vitro by different DNA polymerases (2, 11) and is an inhibitor of L-OddCMP from the 3′-termini of DNA by apurinic/apyrimidinic endonuclease-1 (APE-1; refs. 14, 15).

In culture, the up-regulation of PGK by hypoxia-inducible factor under hypoxic conditions can make cells more susceptible to L-OddC (16). Hypoxic conditions, with oxygen partial pressures of 5 to 10 mm Hg: 0.7% to 1.4% oxygen in gas phase, are also commonly detected in solid tumors including lung cancer (7.5 mm Hg;
ref. 17). We hypothesize the hypoxic conditions in lung tumors could facilitate the synthesis of the triphosphate metabolite (the cytotoxic metabolite) of l-OddC through the action of PGK induced by HIF-1α. l-OddC could be a unique chemotherapeutic agent for the treatment of hypoxic solid tumors such as non-small cell lung carcinoma. The purpose of this investigation is to test this hypothesis.

Materials and Methods

Cell Culture
H460 (non-small cell lung carcinoma) cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 4.5 g/L glucose and 25 mmol/L HEPES (pH 7.4) in a 37°C humidified incubator with a 5% CO2 atmosphere or in a 37°C hypoxic chamber that was perfused with a gas mixture of 1% O2, 5% CO2, and 95% N2.

Western Blotting
Details of the Western blotting procedure are published in our previous report (16). Polyclonal anti-HIF-1α (1:2,000; BD Bioscience), polyclonal anti-PGK (1:5,000), polyclonal anti-APE-1 (1:7,500; Santa Cruz Biotechnology), polyclonal anti-nM23 H1 (1:1,000; Santa Cruz Biotechnology), polyclonal anti-CMP kinase (CMPK; 1:2,000; ref. 18), and polyclonal anti-dCK (1:2,000; ref. 19) were used to detect the corresponding proteins. h-rhodamine phalloidin (Molecular Probes) were made and incubated with Hypoxyprobe 1MAb1 (IgG1), a monoclonal antibody that detects protein adducts of Hypoxyprobe in hypoxic cells and then subjected to streptavidin peroxidase/3,3'-diaminobenzidine tetrahydrochloride development according to the Hypoxyprobe protocol.

Immunohistochemistry
Formalin-fixed, paraffin-embedded H460 xenograft tumor was cut into 4 mm slices. The sections were mounted on Superfrost slides, dewaxed with xylene, and gradually hydrated. Antigen retrieval was achieved by 0.05% citraconic anhydride (pH 7.4) for 1 h. The primary antibodies for against HIF-1α (Cayman Chemical), PGK, and APE-1 were diluted 1:200 using Tris-HCl containing 1% bovine serum albumin and 0.5% Tween 20. The primary antibodies were incubated at room temperature for 1 h. As a negative control, slides were processed without primary antibody. Detection was carried out by biotin-labeled secondary antibody and horseradish peroxidase-conjugated streptavidin with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The slides were counterstained with hematoxylin and mounted. Hypoxyprobe (pimonidazole; Chemicon International) was used for detecting hypoxia in tumors. Briefly, 0.5 h before H460 tumor-bearing mice were killed, Hypoxyprobe was given i.p. at a dose of 60 mg/kg. Tissue was placed in 10% neutral buffered formalin. Tissue was sectioned and slides were made and incubated with Hypoxyprobe 1MAb1 (IgG1), a monoclonal antibody that detects protein adducts of Hypoxyprobe in hypoxic cells and then subjected to streptavidin peroxidase/3,3'-diaminobenzidine tetrahydrochloride development according to the Hypoxyprobe protocol.

Animal Studies
Male NCr-nude mice (average body weight of 20 g), age 4 weeks, were obtained from Taconic Farms and acclimated to laboratory conditions 1 week before tumor implantation. Nude mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Human H460 tumor xenografts were established by s.c. injection. l-OddC (gift of David C.K. Chu, Ph.D., University of Georgia School of Pharmacy) and dFdC were dissolved in PBS to 30 mg/mL. Both l-OddC and dFdC (Eli Lilly) were administered by i.p. injections.
(300 mg/kg), whereas t-OddC was also given by oral gavage (300 or 600 mg/kg). Tumor growth was measured daily using an electronic digital caliper (Fisher Scientific). Tumor volume was estimated by using the formula: length × width² × π / 6. Mice were weighed daily using a standard scale (Ohaus-Scout II) SC2020.

**Statistical Analysis**

Data were analyzed by two-way ANOVA (GraphPad Prism 4), Student’s t test (Microsoft Excel), and correlation analysis (GraphPad Prism 4). The difference was considered to be statistically significant when P < 0.05.

**Results**

**Antitumor Activity of t-OddC and dFdC In vivo**

As illustrated in Fig. 1A, we compared equivalent (300 mg/kg) dosages of t-OddC and dFdC on H460 tumor growth in NCR nude mice. During the first treatment (days 1-8), t-OddC (300 mg/kg i.p.) decreased the size of H460 tumor xenografts by 60%, whereas dFdC (300 mg/kg i.p.) stabilized the H460 tumor xenografts until day 6 and the H460 tumor regrew at day 7 and 8. The second treatment of t-OddC (300 mg/kg i.p.) decreased the size of H460 tumor xenografts to almost immeasurable and the H460 tumor growth was repressed until day 20. The second treatment of dFdC (300 mg/kg i.p.) stopped the H460 tumor growth for 3 days and the H460 tumor rebound with a similar growth rate of control (Fig. 1A). In the two-cycle treatment protocol, t-OddC and dFdC delayed the H460 xenograft reaching 400% initial tumor volume by 15 and 5 days, respectively (Fig. 1B). Our data clearly indicated that the antitumor activity of t-OddC is stronger than that of dFdC in a H460 xenograft tumor model in vivo. No weight loss was observed in either treatment group (data not shown).

Oral activity is one of the unique features of t-OddC, and dFdC is not orally active (3). Here, we examined whether t-OddC was orally active against H460 xenograft tumor

**Figure 1.** Anti-H460 xenograft tumor activity of t-OddC and dFdC in vivo. A, inhibition of H460 tumor growth by t-OddC (300 mg/kg i.p. or p.o. at days 1 and 9; arrow) and dFdC (300 mg/kg was delivered i.p. at days 1 and 9; arrow). B, time required for tumor to reach 400% of initial tumor size after different treatments. C, comparison of the effect of i.p. and p.o. delivery of t-OddC on the inhibition of H460 tumor growth. Three experiments with five animals in each group were done and one of the representative experiments is presented.

**Figure 2.** Pharmacokinetics of t-OddC and dFdC in H460 xenograft tumor-bearing nude mice. A, mean ± SD plasma concentration (n = 5) versus time curves of t-OddC (300 mg/kg i.p. or 600 mg/kg p.o.) and dFdC (300 mg/kg i.p.). B, pharmacokinetic variables [area under the curve (AUC), Cmax, and t1/2] of t-OddC and dFdC using i.p. or p.o. delivery.

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Table 1. Effect of equal dosages (300 mg/kg i.p.) on select hematologic variables 4 d after treatment

<table>
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<th>Lymphocyte (k/μL)</th>
<th>Neutrophils (k/μL)</th>
<th>Monocytes (k/μL)</th>
<th>RBC (M/μL)</th>
<th>Platelet (k/μL)</th>
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<tr>
<td>Control</td>
<td>8.98 ± 2.38</td>
<td>1.38 ± 0.37</td>
<td>0.96 ± 0.39</td>
<td>10.43 ± 0.17</td>
<td>1,346 ± 338</td>
</tr>
<tr>
<td>L-OddC</td>
<td>4.88 ± 1.31</td>
<td>0.65 ± 0.16</td>
<td>0.45 ± 0.09</td>
<td>9.56 ± 1.5</td>
<td>1,340 ± 62</td>
</tr>
<tr>
<td>dFdC</td>
<td>4.3 ± 1.24</td>
<td>0.46 ± 0.11</td>
<td>0.35 ± 0.14</td>
<td>10.77 ± 0.24</td>
<td>1,068 ± 79</td>
</tr>
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Pharmacokinetics of L-OddC and dFdC in H460 Xenograft Tumor-Bearing Nude Mice

Comparison of the pharmacokinetics of L-OddC (300 mg/kg i.p.) and dFdC (300 mg/kg i.p.) was examined (Fig. 2A and B). L-OddC reached a maximum plasma concentration \( C_{\text{max}} \) of 1.2 mmol/L with an elimination half-life \( t_{1/2} \) of 21 min (Fig. 2A and B). The \( C_{\text{max}} \) of dFdC was 0.62 mmol/L and the \( t_{1/2} \) of dFdC was 17.5 min (Fig. 2A and B). Because L-OddC had a higher \( C_{\text{max}} \) and longer \( t_{1/2} \), it was more effective in inhibiting tumor growth compared to dFdC.

Figure 3. Transcriptional response of HRE in H460 cells in vitro and in vivo. A, luciferase reporter assay indicates the HRE of PGK promoter mediates transcriptional response to hypoxia. B, in vivo luciferase imaging shows that HIF-mediated transcriptional response was present in H460 tumor xenografts. C, pimonidazole was used as a hypoxia probe to detect the presence of hypoxic conditions in H460 tumor xenografts. The rest of the procedures was the same as described in Materials and Methods.

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than that of dFdC, the area under the curve of L-OddC is 0.62 mmol h/L, which is 1-fold higher than that of dFdC.

Oral administration of L-OddC had a very different pharmacokinetic profile than i.p. delivery of L-OddC. Oral administration of L-OddC took 30 min to reach its C_{max} of 0.22 mmol/L, and the t_{1/2} after oral administration of L-OddC was 111 min, which is 5-fold longer than the t_{1/2} for i.p. delivery of L-OddC. The relative bioavailability of L-OddC was 50% of the i.p. amount following oral administration (Fig. 2B). The area under the curve for L-OddC (600 mg/kg p.o.) was about the same as that of dFdC (300 mg/kg i.p.).

**Effect on Hematologic Function**

Data from our experiments support that the toxicities of L-OddC and dFdC are very similar in their suppression of select leukocyte populations when given at 300 mg/kg i.p. and measured on day 4 (Table 1). By day 16, drug-treated
groups leukocyte counts were at least equivalent to pretreatment levels. Control mice had a significant increase of their neutrophil counts from pretreatment levels ($P = 0.0064$) on day 16.

Platelet and erythrocyte counts were not significantly different from control on day 4. On day 16, 1-0ddC platelet levels were well within normal limits but statistically less than control ($P = 0.0198$) or dFdC ($P = 0.0013$). These data suggest a similar acute hematologic toxicologic profile of these compounds in nude mice.

### Transcriptional Response of HRE of PGK Promoter in H460 Cells

In normoxic conditions, similar luciferase activity per cell was detected in H460 cells carrying mutated or wild-type HRE reporters (Fig. 3A). In hypoxic conditions, 10-fold higher luciferase activity per cell was found in H460 cells carrying wild-type HRE reporter than that with mutated HRE reporter (Fig. 3A). In similar-sized H460 tumor xenografts, H460 cells with HRE reporter had much higher luciferase activity than H460 cells with mutated HRE reporter (Fig. 3B). This showed that the HIF-mediated transcriptional response was present in H460 tumor xenografts. Furthermore, hypoxia (pimonidazole) staining clearly indicated that some areas of H460 xenograft tumors were lacking oxygen (Fig. 3C).

### Protein Levels of HIF-1α, PGK, and APE-1 in H460 Tumor Sections

Because hypoxic conditions (oxygen partial pressure 5-10 mm Hg; 0.7-1.4% oxygen in gas phase) are commonly detected in solid tumors, and the induction of HIF-1α in hypoxic conditions can induce the expression of PGK, which can facilitate the synthesis of the triphosphate metabolite of l-0ddC, we also studied the immunostaining of HIF-1α and PGK in H460 tumor xenografts from our nude mouse model. As shown in the micrographs, the HIF-1α-stained area increased with tumor progression (Fig. 4A). Most of the HIF-1α-stained cells were found between necrotic and intact cell populations. The intensity of PGK staining, but not APE-1 staining, increased as the tumor size increased. Western blot analysis indicated that the expression of HIF-1α and PGK, but not APE-1, had a positive correlation with the tumor size (Fig. 4B; Supplementary Fig. S1). Furthermore, PGK protein expression had a positive correlation to HIF-1α (Fig. 4B). The size of tumor had no significant effect on the expression of dCK, CMPK, and nM23 H1 in H460 tumor xenografts (Fig. 4B).

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1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Effects of Hypoxia on the Protein Expression on PGK, APE-1, dCK, CMPK, and nM23 H1

The induction of HIF(s) (heterodimer; HIF-1α/β) activates its downstream target genes through its interaction with the HRE (acgtgor gcgtg) of the promoter of the target genes in hypoxic conditions, and this interaction will result in the alteration of gene expression of many proteins, including PGK. As shown in Fig. 5A, the basal level of HIF-1α in H460 cells was very low in normoxic conditions, but HIF-1α was induced after 24 h of hypoxic treatment. The protein level of HIF-1β was not affected by hypoxia treatment. Immunofluorescence micrographs showed that the induced HIF-1α was translocated into the nuclei of H460 cells (Fig. 5B).

The effect of hypoxia on PGK, which is responsible for l-OddC/TP formation, and APE-1, which has exonuclease activity that can remove the incorporated l-OddCMP from the 3'-DNA terminus and cause L-OddC resistance in cell culture, was determined with Western blotting. Results showed that 24 h of hypoxia treatment increased the protein level of PGK in H460 cells by ~3-fold (Fig. 5A). The protein level of APE-1 was not affected by hypoxia treatment (Fig. 5A). Immunofluorescent micrographs showed that hypoxia had no effect on the subcellular distributions of the PGK protein, which was present mostly in cytoplasm, and the APE-1 protein, which was present in the nuclei of H460 cells (Fig. 5B).

Besides the PGK protein, we also examined the effect 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. Hypoxia, however, had no effect on the level of these proteins in H460 cells (Fig. 5A).

Effect of Hypoxia on the Cytotoxicity and the Metabolism of l-OddC and dFdC in H460 Cells

In cell culture, dFdC is more toxic than l-OddC in H460 cells. Taking into consideration the 50% reduced growth rate under hypoxic conditions, clonogenic assay results indicated that hypoxia treatment could sensitize H460 cells by ~4-fold to l-OddC on the same generation time with exposure to the drug (Fig. 6A). The above result could be attributed to the increase of the triphosphate level and the DNA incorporation of l-OddC in hypoxic conditions (Fig. 6C and E; Supplementary Fig. S2). However, hypoxic treatment did not significantly sensitize H460 cells to dFdC on the same generation time exposure of the drug (Fig. 6B). Indeed, the triphosphate level of dFdC was not affected by hypoxic treatment (Fig. 6D). DNA

![Figure 6. Effect of hypoxic conditions on the cytotoxicity and the metabolism of l-OddC and dFdC in H460 cells. Cytotoxicity (A and B) of l-OddC and dFdC of H460 cells under normoxic conditions and hypoxic conditions. ID50 was defined as the concentration of drug required to achieve 50% of surviving fraction. Mean ± SD of three experiments, with each data point done in triplicate. Triphosphate level (C and D) and DNA incorporation (E and F) of l-OddC and dFdC of H460 cells in the normoxic and hypoxic conditions. The other phosphorylated forms of l-OddC and dFdC were shown in Supplementary Fig. S2.](image-url)
incorporation of dFdC was decreased in hypoxic conditions (Fig. 6F), and this could be the result of a reduction of DNA synthesis in H460 cells after hypoxic treatment (Supplementary Fig. S3).^1^

**Discussion**

The efficacy of l-OddC against solid tumors has been shown in many tumor models including prostate, renal, colon, head and neck, non-small cell lung, hepatocellular, and pancreatic cancer in vivo (2–4). In cell culture studies, dFdC often has a stronger cytotoxicity compared with l-OddC. However, in vivo, l-OddC had been reported to have a stronger antitumor activity than dFdC in several tumor models including Panc-1 (confirmed by us and data not shown; ref. 20), MiaPaCa model (dFdC refractory), and AsPC-1 (22). Here, we also showed l-OddC had a stronger inhibitory effect, than dFdC, on the growth of H460 tumor xenografts in a nude mouse model.

Pharmacokinetic analysis indicated that l-OddC had a higher C_{max} and longer t_{1/2} than that of dFdC using i.p. delivery. The shorter t_{1/2} of dFdC could be caused by cytidine deamination, which eliminates many α-configura-

Effect of Hypoxia on the Cytotoxicity of l-OddC

hypoxic treatment induced the protein expression of PGK and its downstream proteins (PGK, etc.) are likely to be overexpressed (25). Because of this, von Hippel-Lindau minus renal cell carcinoma subpopulations receiving l-OddC may have increased drug activity compared with the von Hippel-Lindau-positive population. Third, l-OddC was also administered to 17 patients with non-small cell lung carcinoma in a phase II trial. After receiving l-OddC as an i.v. infusion (10 mg/m^2 over 30 min on day 1 every 21 days), 8 (47%) patients achieved stable disease with a median duration of 3.6 months (range, 2.0-7.1; ref. 8).
In conclusion, the hypoxic condition present in solid tumors, which increase radiation and chemotherapy resistance, seems to be a favorable factor for the activity of L-OddC. The evaluation of L-OddC anti-solid tumor should be further explored.

**Disclosure of Potential Conflicts of Interest**

Y-C. Cheng, co-inventor of L-OddC with potential financial interest in the compound. No other potential conflicts of interest were disclosed.

**References**

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