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KINETIC STUDIES ON 2′,2′-DIFLUORODEOXYCYTIDINE (GEMCITABINE) WITH PURIFIED HUMAN DEOXYCYTIDINE KINASE AND CYTIDINE DEAMINASE

DAVID Y. BOUFFARD, JOSÉE LALIBERTÉ and RICHARD L. MOMPARLER*
Département de Pharmacologie, Université de Montréal and Centre de Recherche Pédiatrique, Hôpital Ste-Justine, Montréal, Québec, Canada H3T 1C5

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Abstract—Phosphorylation of cytosine analogs by deoxycytidine kinase (dCK) and deamination by cytidine deaminase (CDA) are two important processes in the activation and elimination of these drugs. We have investigated the kinetic parameters of 2′,2′-difluorodeoxycytidine (dFdC) using purified enzymes from human cells. Deoxycytidine (CdR) and dFdC had K_m values of 1.5 and 4.6 μM for dCK, respectively. Feedback inhibition of dCK by deoxycytidine 5′-triphosphate (dCTP) was also studied. Our results show that dCTP produced a greater inhibition of the phosphorylation of dFdC than CdR with concentrations of dCTP ranging from 1 to 25 μM. dFdC was a good substrate for CDA. Kinetic studies with this enzyme gave K_m values for CdR and dFdC of 46.3 and 95.7 μM, respectively. The effect of competitive inhibitors of CDA on the deamination of dFdC was also investigated. Diazepinone riboside was a more potent inhibitor than tetrahydrouridine using either CdR or dFdC as the substrate. Inhibitors of CDA could be useful in clinical trials in patients with cancer to increase the chemotherapeutic effectiveness of dFdC.

2′,2′-Difluorodeoxycytidine (dFdC†, Gemcitabine) is a new and very interesting antimetabolite of deoxycytidine (CdR) in which the two hydrogen atoms in the 2′ position of the deoxyribose sugar have been replaced by two fluorine atoms [1]. Several reports have shown that dFdC is active against many murine solid tumors and leukemic cell lines [2–4] and it is presently undergoing phase I–II clinical trials [5, 6]. The exact mechanism of action of dFdC is not completely understood, but it is related to the incorporation of dFdC into DNA and to its inhibition of ribonucleotide reductase and deoxycytidine monophosphate deaminase [7–10].

Like other CdR analogs, dFdC must be phosphorylated in order to become an active antimetabolite. dFdC is first phosphorylated by deoxycytidine kinase (dCK, EC 2.7.1.74), and then converted to its di- and triphosphate form by other kinases [4, 11, 12]. The phosphorylation of dFdC by dCK is the rate-limiting step in the activation of the drug and cells deficient in this enzyme are resistant to the action of this antimetabolite [11]. Analogs of CdR are also subject to deamination by cytidine deaminase (CDA, EC 3.5.4.5), resulting in a loss of pharmacologic activity [13, 14]. dFdC is deaminated to 2′,2′-difluorodeoxyuridine (dFdU) by CDA and this latter metabolite has much less activity compared to dFdC [15]. The high activity of CDA in the liver and the spleen is responsible for the short half-life of these analogs (between 15 and 20 min) in patients [16]. Resistance of leukemic cells to CdR analogs caused by an elevated CDA activity at time of relapse has been observed in some patients [17, 18].

3,4,5,6-Tetrahydrouridine (THU) was the first inhibitor of CDA to be found [19]. The combination of THU with cytosine arabinoside (ara-C) demonstrated the capacity of this inhibitor to increase the plasma level of ara-C in patients with solid tumors [20]. Since inhibitors of CDA show clinical relevance to the pharmacokinetics of the CdR analogs, more potent inhibitors have been synthesized [21, 22]. Diazepinone riboside (DPR) is one of those new inhibitors. Studies using purified CDA have shown that DPR is a better inhibitor than THU for the deamination of ara-C and 5-aza-2′-deoxycytidine [14].

Since dCK and CDA are two important enzymes in the activation and elimination of CdR analogs, we investigated the kinetic parameters of the new antimetabolite dFdC with purified human enzymes. A better understanding of the enzyme kinetics of dFdC may be useful in the design of future clinical trials on this agent.

MATERIALS AND METHODS

Materials. CdR and [^{3}H]CdR were obtained from Boehringer Mannheim (Laval, Québec) and from Dupont Canada (Mississauga, Ontario), respectively. dFdC was provided by Lilly Research Laboratories (Indianapolis, IN). [^{3}H]dFdC was synthesized by Amersham Canada Ltd. (Oakville, Ontario) and

* Corresponding author: Richard L. Momparler, Ph.D., Centre de Recherche Pédiatrique, Hôpital Ste-Justine, 3175 Chemin de la Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5. Tel. (514) 345-4740; FAX (514) 345-4801.

†Abbreviations: dFdC, 2′,2′-difluorodeoxycytidine; CdR, deoxycytidine; dCTP, deoxycytidine 5′-triphosphate; dFdCTP, 2′,2′-difluorodeoxycytidine 5′-triphosphate; dFdU, 2′,2′-difluorodeoxyuridine; ara-C, cytosine arabinoside; ara-CTP, cytosine arabinoside 5′-triphosphate; THU, 3,4,5,6-tetrahydrouridine; DPR, diazepinone riboside; dCK, deoxcycytidine kinase; and CDA, cytidine deaminase.

Table 1. K_m and V_{max} values for CdR and dFdC of dCK

Substrate	K_m (μM)	V_{max} (pmol/min)
CdR	1.5 ± 0.1	5.5 ± 0.8
dFdC	4.6 ± 0.6	14.9 ± 0.9

Values are means ± SD (N = 3).

purified by HPLC using a Spherisorb ODS C18, 5 μm 0.4 × 25 cm (Chromatography Science, Montréal) with a 5 mM ammonium formate (pH 4.3) buffer containing 5% methanol. THU was obtained from Calbiochem (La Jolla, CA), and DPR was provided by Dr. V. E. Marquez (National Cancer Institute, Besthesda, MD).

Deoxycytidine kinase purification. HL-60 leukemic cells in log growth phase were centrifuged and washed with phosphate-buffered saline (PBS). The pellet was suspended in a buffer of 10 mM HEPES (pH 6.5), containing 100 μM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL leupeptin and 5 mM dithiothreitol (DTT). The cells were then placed in a bomb cavitation under a nitrogen pressure of 800 lb/in^2 for 30 min and cell lysis was performed by rapid decompression. After a centrifugation of 9680 *g* for 40 min (Sorvall), the nucleic acids were precipitated with streptomycin sulfate. The dCK was then precipitated with ammonium sulfate to between 20 and 60% saturation. The enzyme was then suspended in a buffer of 50 mM HEPES (pH 6.5), containing 0.2 M KCl, 5 mM DTT, 5% glycerol, 100 μM PMSF, 2 μg/mL leupeptin, 2 mM ATP and 2.4 mM $MgCl_2$. The enzyme was further purified by gel filtration on an FPLC system using a Superdex 75 HR 10/30 column (Pharmacia) with the same buffer (resulting in a 13-fold purification). The specific activity of the purified dCK was 549 U/mg protein; 1 U of enzymatic activity was defined as the amount of enzyme that catalyzes the phosphorylation of 1 pmol CdR/min at 37°. The protein concentration was determined by the dye-binding procedure of Bradford [23] using the microassay method and bovine serum albumin (BSA) as the standard (Bio-Rad Laboratories, Mississauga, Ontario).

Cytidine deaminase purification. CDA was purified from human placenta, and the method of purification has been described previously [14]. The specific activity of the purified CDA was 11,000 U/mg protein, 1 U of enzymatic activity being defined as the amount of enzyme that catalyzes the deamination of 1 nmol cytidine/min at 37°.

Deoxycytidine kinase assay. The incubation mixture contained 50 mM Tris–Cl (pH 7.0), 2 mM ATP, 2.4 mM $MgCl_2$, 0.5 mg/mL BSA, 5% glycerol, 130 μM THU and a known concentration of [^{3}H]-CdR or [^{3}H]dFdC in a total volume of 100 μL. The reaction was started with the addition of 3.5 μg (2.0 U) of the enzyme. After 30 min of incubation at 37° the reaction was stopped by the addition of 3 mL of cold water and spotted on DE-81 filter discs. The discs were then washed with water and ethanol, dried and counted for radioactivity in Omnifluor (Dupont, Canada).

Cytidine deaminase assay. The reaction mixture (0.2 mL) contained 20 mM $KHPO_4$ (pH 7.5), 100 mM KCl, the indicated concentration of substrate, and about 0.15 μg (1.5 U) of the purified CDA. The mixture was placed in a 10-mm light-path cuvette and incubated at 37° in a Gilford 260 spectrophotometer. The rate of deamination was determined by the decrease in absorbance at 286 nm. A ΔE_M value of 3000 at 286 nm was used to calculate the amount of substrate deaminated.

RESULTS

The K_m and V_{max} values for CdR and dFdC of dCK are shown in Table 1. The K_m value for CdR was three times lower than the one for dFdC (1.5 μM compared with 4.6 μM), showing a better affinity of the enzyme for the natural substrate than for the antimetabolite. The maximal velocity (V_{max}) value for dFdC (14.9 pmol/min) was also about three times higher than the one for CdR (5.5 pmol/min), demonstrating different kinetics for the two substrates. The efficiency of phosphorylation expressed as V_{max}/K_m was approximately the same for CdR and dFdC (3.7 vs 3.2, respectively).

We also investigated the feedback inhibition of deoxycytidine 5′-triphosphate (dCTP) on dCK with CdR or dFdC as substrate for the enzyme (Table 2). At an identical substrate concentration (10 μM), dFdC was more sensitive than CdR to the inhibition produced by dCTP. With increasing concentrations

Table 2. Effect of dCTP on the inhibition of phosphorylation of CdR and dFdC by dCK

dCTP (μM)	dCMP formed* (pmol/min)	Inhibition (%)	dFdCMP formed* (pmol/min)	Inhibition (%)
0	3.70 ± 0.12†	0	8.51 ± 0.22†	0
1	3.04 ± 0.11	18	5.45 ± 0.11	36
2	2.63 ± 0.24	29	4.95 ± 0.04	42
5	2.46 ± 0.09	33	3.27 ± 0.30	62
10	1.84 ± 0.06	50	1.77 ± 0.14	79
25	1.17 ± 0.14	68	0.77 ± 0.08	91

* Substrate concentration 10 μM.
† Mean ± SD (N = 3 in duplicate).

Table 3. K_m and V_{max} values for CdR and dFdC of CDA

Substrate	K_m (μM)	V_{max} (nmol/min)
CdR	46.3 ± 2.0	1.1 ± 0.1
dFdC	95.7 ± 8.4	1.2 ± 0.1

Values are means ± SD (N = 3).

of dCTP from 1 to 5 μM the inhibition of phosphorylation of dFdC (36–62%) was two times higher than for CdR (18–33%). At dCTP concentrations of 10 and 25 μM the inhibition of phosphorylation was still markedly higher for dFdC than for CdR.

The K_m and V_{max} values for CdR and dFdC of CDA are shown in Table 3. The K_m value for CdR (46.3 μM) was 2-fold lower than the K_m value for dFdC (95.7 μM), demonstrating a better affinity of the enzyme for the natural substrate than for the antimetabolite. The V_{max} values for the two substrates were similar (1.1 and 1.2 nmol/min for CdR and dFdC, respectively). The efficiency of deamination expressed as V_{max}/K_m was around 2-fold greater for dFdC than for CdR (0.013 vs 0.024, respectively).

We also examined the effects of two CDA inhibitors, THU and DPR, on the deamination of CdR and dFdC. The effects of the inhibitors on the deamination of different concentrations of CdR and dFdC were evaluated according to the method of Lineweaver–Burk. The plots indicated that the inhibition produced by THU and DPR for CdR and dFdC was competitive. To evaluate the K_i values for the two inhibitors we used the Dixon plot of the reciprocal velocity of the enzyme reaction as a function of changing inhibitor concentration at a constant substrate concentration. Table 4 shows the actual K_i values of THU and DPR for CdR and dFdC. The K_i values of THU for CdR and dFdC were not significantly different (108.5 vs 110.2 nM, respectively). The K_i value of DPR for dFdC (23.2 nM) was slightly lower than for CdR (37.8 nM). These results show that DPR is a better inhibitor of CDA than THU as shown by the lower K_i value of DPR for each substrate.

DISCUSSION

Deoxycytidine kinase and cytidine deaminase are two important enzymes in the anabolism and catabolism of cytosine nucleoside analogs. The objective of our investigation was to evaluate the kinetic parameters of dFdC using dCK and CDA purified from human cells to better understand the metabolism of this CdR analog.

Table 4. K_i values of THU and DPR for CDA with either CdR or dFdC as substrate

Substrate	Type of inhibition	K_i (nM) THU	K_i (nM) DPR
CdR	Competitive	108.5 ± 8.6	37.8 ± 4.3
dFdC	Competitive	110.2 ± 2.9	23.2 ± 2.2

Values are means ± SD (N = 3).

Although our purification of the dCK was not extensive, the K_m values obtained with our preparation were similar to the K_m value reported with the pure protein [24] and thus assured us of the reliability of our experimental approach. With our partially purified enzyme from the HL-60 leukemic cell line we obtained a K_m for dFdC of 4.6 μM. The reported K_m values for dFdC of dCK was 3.6 μM in extracts of Chinese hamster ovary (CHO) cells and 3.1 μM in extracts of K562 leukemic cells [11, 25]. Our K_m value is in accord with these published values.

One of the observations made with dFdC *in vitro* was that the accumulation of its phosphorylated forms was higher than that of ara-C [11, 26]. The K_m values reported for ara-C are between 8.8 and 40 μM [11, 24, 25], which are higher than the K_m values for dFdC. The differences in K_m values of dCK may be one factor responsible for the higher intracellular pool of 2′,2′-difluorodeoxycytidine 5′-triphosphate (dFdCTP), but not the only explanation for the different degree of phosphorylation of the two analogs in neoplastic cells.

dCTP is a potent feedback inhibitor of the dCK enzyme. Our results demonstrated that the inhibition of phosphorylation of dFdC was 2-fold higher than that of CdR at low dCTP concentrations (Table 2). We have reported in a previous study that ara-C (10 μM) phosphorylation is inhibited by 94% with 10 μM dCTP [27]. Together with lower K_m value of dCK and the weaker inhibition by dCTP for dFdC as compared with ara-C could result in a higher accumulation of dFdCTP than cytosine arabinoside 5′-triphosphate (ara-CTP). But the fact that dFdCDP can decrease the pool of dCTP in the different cell lines by inhibition of ribonucleotide reductase gives dFdC a major advantage over ara-C because inhibition by dCTP of dCK is reduced, permitting a higher rate of phosphorylation.

We have also investigated the deamination of dFdC with highly purified CDA from human placenta. The K_m for dFdC of CDA was 95.7 μM. A previous publication from our laboratory reported a K_m value of 140 μM value for ara-C [14]. With a partially purified CDA from the liver of DBA/2 mice, Plunkett *et al.* [12] reported K_m values of 2.0 mM for dFdC and 1.0 mM for ara-C. These differences in the K_m values for dFdC and ara-C could be due to the species and degree of purification. Our results show that dFdC was a better substrate of CDA than ara-C and that CdR was a better substrate than both of these analogs (Table 3). Since the affinity of dCK for dFdC and ara-C is higher than the affinity of CDA, this would favor phosphorylation of the nucleosides over deamination by CDA in the cell. This in accord with results from Heinemann *et al.* [9] who have reported that accumulation of dFdU comprises less than 10% of the cellular dFdC metabolites in CEM cells. Leukemic cells expressing a high level of CDA can show resistance to cytosine antimetabolites and thus limit the antineoplastic activity of these drugs

[17, 18]. To avoid this type of resistance to CdR analogs, inhibitors of CDA could be useful adjuvants in combination chemotherapy.

Our results demonstrated that THU is a good inhibitor of the deamination of dFdC; however, DPR was five times more potent than THU (Table 4). DPR was also found to be a better inhibitor of CDA than THU with ara-C as substrate [14]. Kreis *et al.* [20] demonstrated the clinical importance of THU by showing that it significantly increased the plasma level of ara-C in patients. The combination of ara-C with THU reduces the dose of ara-C needed to obtain plasma levels of 10 μM by a factor of 10 [28].

Grunewald *et al.* [29, 30] reported that the plasma half-life of dFdC in patients was between 0.8 and 26 min (median of 6.7 min). They also observed that in these patients dFdU was the only metabolite found in the plasma. These results demonstrate that deamination of dFdC to dFdU is responsible for the short half-life of this antimetabolite. The use of a CDA inhibitor could increase the half-life of dFdC so as to obtain higher concentrations of the analog in the body fluids, especially in anatomical sanctuaries containing tumor cells resulting in greater clinical effectiveness.

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