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Regulation of Phosphorylation of Deoxycytidine and 2′,2′-Difluorodeoxycytidine (Gemcitabine); Effects of Cytidine 5′-Triphosphate and Uridine 5′-Triphosphate in Relation to Chemosensitivity for 2′,2′-Difluorodeoxycytidine

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ABSTRACT. Deoxycytidine kinase (dCK) and deoxycytidine deaminase (dCDA) are two key enzymes in the activation and inactivation, respectively, of deoxycytidine and its antiviral and anticancer analogues. One purpose of this study was to determine whether or not the deoxycytidine-converting activity of both enzymes would correlate with growth inhibition by 2′,2′-difluorodeoxycytidine (dFdC), a deoxycytidine analogue with established antitumour activity in solid tumours. Another aim of this work was to determine the effects of normal nucleotides on dCK. dCK and dCDA activities were measured with both deoxycytidine and dFdC as substrates in 5 solid tumour cell lines, but no correlation with cellular sensitivity to dFdC was found with either substrate. The normal dCK activities with deoxycytidine as substrate varied between 0.8 and 13 nmol/hr/10^6 cells. The activities determined with dFdC as substrate were remarkably similar in all 5 cell lines (1.1–1.6 nmol/hr/10^6 cells). dCDA activities varied considerably with both substrates (20–30-fold). Because dFdC markedly affected intracellular concentrations of cytidine 5′-triphosphate (CTP) and uridine 5′-triphosphate (UTP), we studied their effects on deoxycytidine- and dFdC-phosphorylating activities in 3 cell lines (i.e., A2780, WiDr and C26-10) with a similar dCK activity but major differences in dFdC sensitivity. 1 mM CTP inhibited deoxycytidine phosphorylation (at 230 μM) by 20–30% in A2780 and C26-10 cells, but increased that of WiDr cells by approximately 70%. CTP did not affect dFdC phosphorylation (at 230 μM) in A2780 cells, but did increase it by 40% in WiDr cells. At 1 and 10 μM of deoxycytidine the effects of CTP on dCK activity in A2780, C26-10 and WiDr cells were less pronounced. 1 mM UTP enhanced deoxycytidine phosphorylation at 230 μM in WiDr cells by approximately 40%, whereas dFdC phosphorylation was increased 40% by UTP in C26-10 cells but decreased by 70–80% in WiDr cells. UTP caused a more pronounced increase in dCK activity at 1 and 10 μM deoxycytidine in C26-10 cells, but provoked a higher inhibition in A2780 and WiDr cells at 10 μM. Because of these complex results, dCK kinetics were studied in greater detail. Biphasic kinetics for deoxycytidine were observed in all 3 cell lines, with K_m values of 23.2 and 0.4 μM for A2780 cells, 15.9 and 1.5 μM for C26-10 cells, and 27.2 and 0.9 μM for WiDr cells. In all 3 cell lines, adenosine 5′-triphosphate (ATP) was the optimal phosphate donor, as compared to CTP and UTP. In conclusion, the efficiency of dCK (V_{max}/K_m ratio) seems to correlate with accumulation of dFdCTP, the active metabolite of dFdC, and with cellular sensitivity. UTP and CTP, which are seriously affected in cells exposed to dFdC, display varying effects in these solid tumour cell lines. Both activation and inhibition have been observed; the physiologically low CTP pools and the relatively minor effect on dCK in A2780 cells seem to favour dFdC phosphorylation in these cells, which are the most sensitive. BIOCHEM PHARMACOL 51;7:911–918, 1996.

KEY WORDS. deoxycytidine kinase; deoxycytidine deaminase; gemcitabine; CTP; UTP; regulation; pyrimidine salvage; deoxynucleosides

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‡ *Abbreviations:* ara-C, 1-β-D-arabinofuranosylcytosine; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; dCDA, 2′-deoxycytidine deaminase; dCK, 2′-deoxycytidine kinase; dCMP, 2′-deoxycytidine 5′-monophosphate; dCTP, 2′-deoxycytidine 5′-diphosphate; dFdC, 2′,2′-difluorodeoxycytidine, gemcitabine; dFdCTP, 2′,2′-difluorodeoxycytidine-5′-triphosphate; TLC, thin layer chromatography.

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dCK‡ (E.C.2.7.1.74), a pyrimidine salvage enzyme, catalyses the phosphorylation of deoxycytidine to its monophosphate dCMP, as well as the phosphorylation of several deoxyribonucleoside analogues that play a role in cancer and antiviral treatment [1–3]. One of the physiological roles of this enzyme is deoxycytidine reutilization, although deoxyadenosine and deoxyguanosine are also good substrates [1, 3]. dCDA (EC 3.5.4.5) catalyses the deamination of cytidine, deoxycytidine, and its analogues [4, 5]. Both enzymes have been associated with sensitivity and resistance to 1-β-D-arabinofuranosylcytosine (ara-C) [6–8], the best known deoxycytidine analogue, commonly used in the treatment of leukemia. But, overall, dCK is considered the most important of the two enzymes with respect to the efficacy of deoxycytidine analogues [3, 6–9]. The regulation of this enzyme is quite complex. In leukemic cells especially, UTP has been reported to play an important role in the regulation of dCK activity. UTP was found to be the optimal phosphate donor in MOLT-4 and Ehrlich ascites cells [10, 11], and could also reverse the inhibition of dCK by its most prominent feedback inhibitor dCTP [12]. Sarup *et al.* [13] showed that both UTP and CTP could inhibit deoxycytidine phosphorylation in leukemic blasts by 25% and 15–28%, respectively. No other effects of CTP on dCK activity have been reported until now. Most studies on dCK kinetics have been performed on enzyme extracts obtained from lymphoid cells or tissues, both normal and malignant [1, 3, 10–13]. Only a few studies on dCK activity and regulation have been performed on enzyme from solid tumours and tissues [3, 14, 15].

dFdC (gemcitabine) is a relatively new deoxycytidine analogue with established clinical activity in solid malignancies, such as ovarian and non-small cell lung cancer [16–18]. The major mechanisms of action of this compound are exerted by the phosphorylated metabolites and include incorporation into nucleic acids [19, 20]. To gain more insight into the role of both dCK and dCDA in the antitumour activity of dFdC in solid tumour cell lines, we used dFdC as a substrate in addition to deoxycytidine to evaluate dCK in a panel of five solid tumour cell lines, characterized previously for their sensitivity to dFdC [21]. One effect of exposing these cell lines to dFdC is a major disturbance in concentrations of CTP and UTP [21], two nucleotides with effects on dCK activity [10–13].

Therefore, the effects of these ribonucleotides on dCK enzyme activity and kinetics with deoxycytidine and dFdC as substrates were studied in three solid tumor cell lines: the human ovarian carcinoma cell line A2780, the human colon carcinoma cell line WiDr, and the murine colon carcinoma cell line C26-10. These cell lines were selected because of their different sensitivity to dFdC (despite similar deoxycytidine phosphorylating activity) and the fact that all three were used for an extensive study of dFdCTP accumulation and the accompanying changes in normal ribonucleotide pools [21].

MATERIALS AND METHODS

Materials

dFdC, dFdU, and [5-^{3}H]-dFdC (16.7 Ci/mmol) were kindly provided by Eli Lilly & Co., Indianapolis, IN. Deoxycytidine, CTP, and UTP were purchased from Sigma Chemical Co., St. Louis MO. Deoxy-[5-^{3}H]-cytidine (25 Ci/mmol) was obtained from Amersham International, Buckinghamshire, U.K. All other chemicals were of analytical grade and commercially available.

Cell Culture

The sources of the human ovarian carcinoma cell lines A2780 and OVCAR-3, the human colon carcinoma cell line WiDr, the human head and neck squamous cell carcinoma cell line UM-SSC-14C, and the murine colon carcinoma C26-10 cells have been described previously [21, 22]. Cells were maintained in exponential growth in Dulbecco's Modification of Eagle's Medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (Gibco), 1 mM L-glutamine (Sigma), and 250 ng/mL gentamicin at 37°C and 5% CO_2. Previously we used A2780, C26-10, and WiDr cells for extensive studies of dFdC metabolism, in which considerable differences in chemosensitivity and metabolism were observed. Given these results and the similarity of deoxycytidine and dFdC phosphorylation, these cell lines were selected for more detailed studies. All enzyme assays were performed with enzyme extracted from cells harvested 2 days after seeding, when cells were in the exponential growth phase.

dCK Assay

The assay for dCK was performed as described previously [14]. Briefly, for determination of dCK activities in cell lines, a minimum of 25.10^6 cells were required. The cell pellets were resuspended in cold dCK buffer (0.3 M Tris-HCl, 50 μM β-mercaptoethanol, pH 8.0), sonicated and centrifuged. The 10,000 g supernatant was immediately used in the enzyme assays. One part of the undiluted supernatant was taken for determining its protein content using the Biorad Bradford protein assay [23]. To 25 μL 10,000 g supernatant (2–14 μg protein/μL), 25 μL of substrate mixture was added. The substrate mixture was prepared by mixing 2 volumes of Mg-ATP (50 mM ATP in 25 mM $MgCl_2$, pH 7.4) (final concentration in the reaction mixture: 10 mM ATP), 2 volumes of [5-^{3}H]-deoxycytidine or [5-^{3}H]-dFdC and one volume of dCK buffer. For activity measurements under saturated substrate conditions, the final concentration of deoxycytidine (0.04 Ci/mmol) and dFdC (41.8 Ci/mol) was 230 μM. The enzyme activity was measured with and without 0.1 and 1 mM final concentration CTP or UTP in three of the five solid tumour cell lines: A2780, WiDr, and C26-10 cells. dCK activity was also determined with CTP or UTP as phosphate donors (10 mM final concentration). The reaction mixture was incubated for 15–60 min at 37°C. The reaction was terminated by heating at 95°C for 3 min and the subsequent addition of 10 μL 5 mM unlabeled deoxycytidine or dFdC to visu-

alize the spots. The substrate (deoxycytidine or dFdC) was separated from the product (dCMP or dFdCMP) by thin layer chromatography on PEI (polyethylene imine) cellulose layers, with distilled water as eluent. The spots could be visualised under UV light, marked and cut out. Radioactivity was estimated in a liquid scintillation counter, after addition of 9 mL Optima Gold (Packard Instrument B.V., Chemical Operations, Groningen, The Netherlands). Enzyme activities were expressed as nmol product formed per hour per 10^6 cells (nmol/hr/10^6 cells) and were linear in time and enzyme concentration. Because dialyzing the samples or adding tetrahydrouridine to inhibit dCDA did not change the outcome of the assay, they were, therefore, omitted. Dialysing would remove intracellular nucleotides and nucleosides that theoretically could interfere with the assay. However, the concentration of UTP is less than 800 pmol/10^6 cells (21). Cells are suspended in 1 mL and subsequently diluted, resulting in a maximum of 2 μM in the enzyme suspension; in the assay mixture this would be diluted at least two-fold, resulting in nucleotide concentrations too low to interfere with the assay. Because dCTP concentrations in cells are at least 100–1000-fold lower than CTP, this possible interference would also be negligible. The separation procedure used (TLC) enabled us to determine how much label was consumed; this would not be possible if filter discs similar to those employed by several other investigators were used. Assays were designed in such a way that deoxycytidine consumption was less than 10%. Due to the higher K_m of deoxycytidine for deamination compared to phosphorylation, it would be unlikely that, under these conditions, deoxycytidine concentrations would be reduced by deamination by more than 10%. ATP measurements showed that no significant degradation occurred during the assay. In other kinase assays, addition of inhibitors of nucleotide degradation did not affect the enzyme activities (data not shown).

For the apparent K_m determinations, the deoxycytidine concentration ranged from 0.4 to 230 μM. The measured activities were linear in time and corrected for >10% substrate conversion. K_m and V_{max} were calculated using the statistical application (linear regression analysis) of the Symphony 2.0 computer program (Lotus Development Corporation).

dCDA Assay

The assay was performed as described previously [14]. Briefly, the reaction was performed in crude cell extracts with a final substrate (deoxycytidine or dFdC) concentration of 500 μM. Product and substrate were separated by means of HPLC with isocratic elution. Deoxycytidine and deoxyuridine were separated on a LiChrosorb 5-RP-18 column (Chrompack, Bergen op Zoom, the Netherlands) with 10 mM ammonium dihydrogen phosphate, pH 6.5 as eluent. dFdC and dFdU were separated using a μBondapack C_{18} column (Waters-Millipore, Etten-Leur, the Netherlands), with $PicB_7$ (Waters) in 15% methanol (final concentration heptane sulfonic acid 5 mM), pH 3.1. Peaks were detected and quantitated using their absorption at 254 and 280 nm.

RESULTS

dCK and dCDA Measurements with Deoxycytidine and dFdC as Substrates

In Table 1, sensitivity to dFdC and dCK, as well as dCDA activities, as determined at saturating substrate concentrations (230 μM) in five different solid tumour cell lines, are summarized. The human ovarian carcinoma A2780 cells were most sensitive to dFdC, both at short (4-hr) and long exposure. The two colon carcinoma cell lines, C26-10 (murine) and WiDr (human), were least sensitive to dFdC. The measured enzyme activities were linear within the incubation time and protein concentration range applied. dCK activities with the natural substrate deoxycytidine varied considerably, from 1.0 to 12.8 nmol/hr/10^6 cells. dCK activities as determined with the deoxycytidine analogue dFdC were remarkably similar between the cell lines, varying only from 1.1 to 1.6 nmol/hr/10^6 cells. For dCDA activities, a considerable variation was observed both with deoxycytidine (30-fold, from 0.1 to 3 nmol/hr/10^6 cells) and dFdC (20-fold, from 0.6 to 12 nmol/hr/10^6 cells). However, the pattern of activity was different for each substrate, with UM-SSC-14C having the highest activity with deoxycytidine as substrate and OVCAR-3 with dFdC.

Apparent K_m and V_{max} Values

Although C26-10, WiDr, and A2780 cells showed very different sensitivity patterns (Table 1), these three cell lines

TABLE 1. Sensitivity to dFdC and deoxycytidine kinase (with ATP as phosphate donor) and deoxycytidine deaminase activities with different substrates

		dCK†		dCDA†	
Cell line	**Sensitivity***	**deoxycytidine**	**dFdC**	**deoxycytidine**	**dFdC**
C26-10	238	1.24 ± 0.25	1.11 ± 0.10	2.15 ± 0.57	0.92 ± 0.18
WiDr	205	1.01 ± 0.10	1.18 ± 0.04	0.22 ± 0.03	1.35 ± 0.15
A2780	7	1.01 ± 0.09	1.24 ± 0.05	0.12 ± 0.04	0.63 ± 0.15
OVCAR-3	36	12.8 ± 1.0	1.21 ± 0.13	2.33 ± 0.08	11.9 ± 3.6
UM-SSC-14C	34	5.28 ± 0.53	1.61 ± 0.04	2.95 ± 0.43	4.66 ± 0.65

* Sensitivity to dFdC is expressed as the IC_{50} in nM after 4-hr exposure followed by a 68-hr drug-free period [21].

† Activities (measured at 230 μM substrate concentration for kinase and 500 μM for the deaminase) are expressed as nmol product formed/hr/10^6 cells and are means ± SEM of 3–6 separate experiments. Protein content was 0.1–0.2 mg/10^6 cells.

TABLE 2. Biochemical characteristics of C26-10, WiDr, and A2780 cells and effect of dFdC on CTP and UTP

	C26-10		WiDr		A2780	
Nucleotide	Nucleotide* (pmol/10^6 cells)	Effect of dFdC (%)†	Nucleotide* (pmol/10^6 cells)	Effect of dFdC (%)†	Nucleotide* (pmol/10^6 cells)	Effect of dFdC (%)†
UTP*	789 ± 105	161	560 ± 41	115	630 ± 210	111
CTP*	458 ± 38	153	168 ± 17	86	219 ± 65	58
dFdCTP‡	31 ± 31		33 ± 18		100 ± 64	

Values are means ± SEM of 3–5 separate experiments [21].

* UTP and CTP concentrations are those of untreated cells. Values are means ± SEM of 3–5 separately harvested cell pellets [21].

† % of control UTP or CTP concentration after 4-hr exposure to 1 μM dFdC.

‡ dFdCTP concentration in pmol/10^6 cells after a 4-hr treatment with 1 μM dFdC.

were remarkably similar in dCK activity with either substrate. Therefore, we selected these three lines to study dCK kinetics to see whether or not differences in apparent K_m and/or V_{max} values would explain the differences in dFdC sensitivity and dFdCTP accumulation. In Table 2, some biochemical characteristics of these cell lines (fully described previously [21]) are summarized. Data as observed at a 4-hr exposure to dFdC are presented, because we also used a 4-hr exposure in the growth inhibition experiments. The general pattern was comparable after 24-hr exposure. In short, UTP concentrations in untreated cells were comparable in the three cell lines, but CTP was higher in C26-10 cells. In contrast, the effect of dFdC was different, resulting in an increase for UTP and CTP in C26-10 but a decrease in CTP in WiDr and A2780 cells. dFdCTP accumulation was higher in A2780 cells compared to the other cell lines. A similar pattern in dFdCTP accumulation was observed at lower (0.1 μM) and higher (10 μM) dFdC concentrations. However, at 0.1 μM it was not always possible to measure dFdCTP accumulation (21).

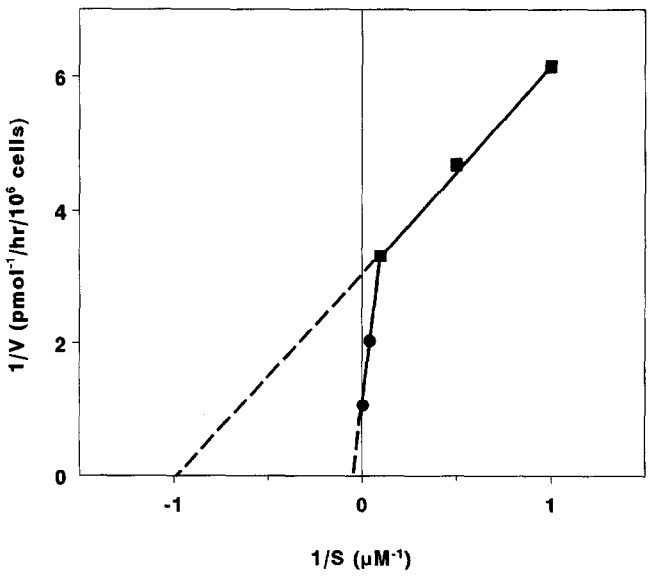

FIG. 1. A typical Lineweaver-Burk plot of dCK activity of an experiment with A2780 cells; with C26-10 and WiDr cells, similar plots were obtained.

Biphasic kinetics for dCK were observed in all three cell lines (with ATP as a phosphate donor) as illustrated in Fig. 1 for A2780 cells. The enzyme kinetic pattern was confirmed using Eadie-Hofstee plots (not shown). In Table 3, the apparent K_m and V_{max} as measured under standard conditions (Pi donor ATP, no addition of CTP or UTP) are summarized. The difference between K_{m_1} and K_{m_2} values of the two human cell lines was more pronounced (30–60-fold) than for the murine C26-10 cells (10-fold). In contrast, in all three cell lines the difference in V_{max_1} and V_{max_2} values was only 3-fold. The V_{max}/K_m ratio, an indication of the efficiency of phosphorylation, was lowest for the murine colon cancer cell line C26-10, both at high and low substrate concentrations. The V_{max}/K_m ratio in A2780 and WiDr cells was comparable at both concentration ranges. Deoxycytidine phosphorylation was most efficient in the low K_m range, because the V_{max}/K_m ratios were 4- to 17-fold higher than at high deoxycytidine concentrations.

Effect of Different Phosphate Donors on Deoxycytidine and dFdC Phosphorylation

Because it has been reported that UTP might be a better phosphate donor than ATP in leukemic cells [10, 11] we measured dCK activity with three different phosphate donors and with deoxycytidine and dFdC as substrates (Table 4). For deoxycytidine as substrate, ATP was the optimal phosphate donor in all three cell lines; the activities of dCK with CTP or UTP as phosphate donor were not more than

TABLE 3. Apparent K_m and V_{max} values as calculated from dCK assays with ATP as phosphate donor

	C26-10	WiDr	A2780
K_{m_1}	1.48 ± 0.28	0.91 ± 0.18	0.38 ± 0.08
K_{m_2}	15.9 ± 2.9	27.2 ± 3.5	23.2 ± 5.7
V_{max_1}	0.18 ± 0.02	0.59 ± 0.06	0.27 ± 0.07
V_{max_2}	0.57 ± 0.15	1.88 ± 0.19	0.90 ± 0.16
V_{max_1}/K_{m_1}	0.13 ± 0.01	0.75 ± 0.14	0.83 ± 0.11
V_{max_2}/K_{m_2}	0.03 ± 0.00	0.07 ± 0.01	0.05 ± 0.01

Apparent K_m is expressed in μM and V_{max} as pmol product formed/hr/10^6 cells. Values are means ± SEM calculated from 4–5 separate experiments.

TABLE 4. dCK activities with deoxycytidine and dFdC as substrates, measured with different phosphate donors

Substrate*	Pi donor†	C26-10	WiDr	A2780
deoxycytidine +	ATP	1.24‡ ± 0.25	1.01 ± 0.10	1.01 ± 0.09
	UTP	0.48 ± 0.03 (39)§	0.44 ± 0.04 (45)	0.54 ± 0.10 (53)
	CTP	0.42 ± 0.03 (34)	0.33 ± 0.03 (33)	0.57 ± 0.13 (56)
dFdC +	ATP	1.11 ± 0.10	1.18 ± 0.04	1.24 ± 0.05
	UTP	1.01 ± 0.03 (94)	0.30 ± 0.03 (25)	0.34 ± 0.04 (30)
	CTP	0.99 ± 0.08 (84)	0.90 ± 0.08 (83)	1.12 ± 0.06 (86)

* Substrate (deoxycytidine or dFdC) concentration was 230 μM.

† Phosphate donor (ATP, UTP, or CTP) concentration was 10 mM.

‡ Activities are expressed as nmol product formed/hr/10^6 cells and are means ± SEM of at least 3 separate experiments.

§ Within parentheses, the relative activity is given as compared with the enzyme activity with ATP as phosphate donor, set at 100%; values are calculated from means of at least 3 separate experiments.

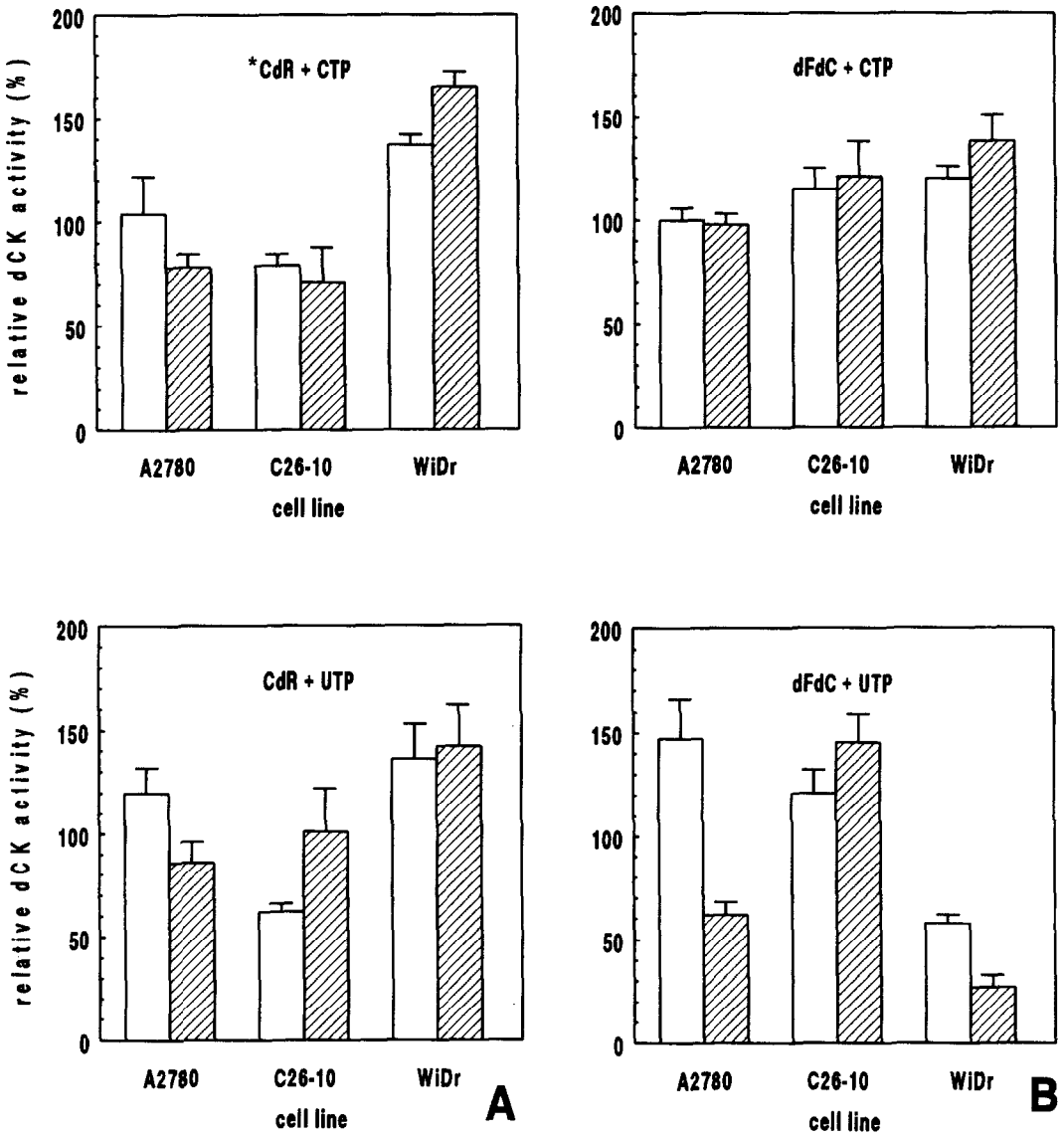

FIG. 2. The effects of CTP and UTP on deoxycytidine phosphorylation (A) and dFdC phosphorylation (B), both at a concentration of 230 μM. The open bars represent the relative phosphorylating activity in the presence of 0.1, the hatched bars at 1 mM CTP or UTP compared to that with only ATP in the reaction mixture, which was set at 100%. Values are means ± SEM of 3–5 separate experiments. *CdR, deoxycytidine.

56% and 62% of the activity with ATP, respectively. ATP was the optimal phosphate donor for dFdC phosphorylation as well, although with CTP enzyme activities almost similar to those with ATP were observed in all three cell lines, and in C26-10 cells UTP was as efficient as ATP as a phosphate donor.

Effect of CTP and UTP on dCK Activity with Deoxycytidine or dFdC as Substrates

Figure 2 summarizes the effects of CTP and UTP on dCK activity using ATP as phosphate donor. 0.1 mM CTP had no effect on A2780 cells, decreased dCK activity slightly in C26-10, and increased it somewhat in WiDr cells. 1 mM CTP decreased dCK activity with deoxycytidine as substrate in A2780 and C26-10 cells by 20–30%, but not with dFdC as substrate. In WiDr cells, however, 1 mM CTP increased deoxycytidine-phosphorylating activity up to 165% and that of dFdC phosphorylation up to 140%. The effects of UTP were more complex; 0.1 mM UTP increased dCK activity in A2780 and WiDr cells and decreased it in C26-10 cells. Although 1 mM UTP increased enzyme activity in WiDr cells, it caused a slight decrease in A2780 cells and did not affect dCK activity in C26-10 cells. UTP, however, had markedly different effects on dFdC phosphorylation. Although 0.1 mM UTP increased enzyme activity in A2780 and C26-10 cells by 50 and 25%, respectively, activity was inhibited by 40% in WiDr cells. At 1 mM UTP only in C26-10 cells, an increase in activity of 50% was observed, and in WiDr as well as A2780 cells enzyme activity was inhibited markedly, by 70 and 40%, respectively.

Based on the biphasic enzyme kinetics we also studied the effects of 1 mM CTP and UTP at 2 other deoxycytidine concentrations: 1 (low) and 10 (intermediate) μM in comparison with 230 μM (saturating) (Table 5). For CTP, the inhibitory effect in A2780 cells was comparable at 1, 10, and 230 μM deoxycytidine and in C26-10 cells at 1 and 230 μM (20–30%). CTP did not affect dCK activity at 10 μM deoxycytidine in C26-10 and WiDr cells. In the latter cell line, CTP increased enzyme activity at 1 and 230 μM more than 40%. For UTP, a similar inhibiting effect on deoxycytidine phosphorylation was observed at all 3 deoxycytidine concentrations in A2780 cells. In contrast, in C26-10 cells, the activity was increased considerably at 1 and 10 μM deoxycytidine, and in WiDr cells a marked stimulation was observed only at 230 μM. At 10 μM deoxycytidine, an inhibition of 35% was found.

TABLE 5. The relative effects of 1 mM CTP and UTP on dCK activity at different deoxycytidine concentrations, with ATP as phosphate donor

Addition	deoxycytidine (μM)	C26-10	WiDr	A2780
CTP +	1	82* ± 8	143 ± 14	88 ± 6
	10	105 ± 3	105 ± 12	75 ± 23
	230	71 ± 17	165 ± 7	78 ± 7
UTP +	1	210 ± 17	91 ± 5	88 ± 14
	10	196 ± 17	65 ± 9	54 ± 13
	230	101 ± 21	142 ± 20	86 ± 10

* dCK activities measured without CTP or UTP at the given deoxycytidine concentrations were set at 100%; values are means ± SEM of 3–4 separate experiments.

DISCUSSION

This study shows that, based on enzyme properties, the metabolism of deoxycytidine and dFdC differs markedly in cell lines of solid tumour origin, even within cell lines derived from a similar organ. Although, in general, dCDA activity was higher with dFdC as substrate than with deoxycytidine, no correlation was evident between sensitivity and dFdC-deaminating activity, as had already been established for deoxycytidine deamination and sensitivity to deoxycytidine analogues [14]. The discrepancies between dCDA activity measured with deoxycytidine compared to activity with dFdC as substrate, are most probably due to differences in K_m and V_{max} for the two substrates. In addition, the substrates may cause a different conformation of the enzyme, resulting in different kinetics and regulation properties.

A remarkable observation was the similarity of dFdC phosphorylation in all cell lines tested, despite considerable variation in deoxycytidine phosphorylation rates. Although it can not be excluded that other deoxynucleoside kinases can use dFdC as a substrate, one can assume that dCK is the major enzyme responsible for dFdC phosphorylation [1, 2, 10, 24]. This would mean that dCK in these cells is sufficiently high to catalyze dFdC phosphorylation. Thus, the observed similarity in the cellular capacity for dFdC phosphorylation cannot account for the differences in sensitivity of the studied cell lines, which varied 1.2 to 34-fold, depending on exposure time (Table 1) [21]. Differences in nucleoside transport do not seem to be limiting because dFdCTP accumulation is rather rapid [21] and chemosensitivity to other nucleoside analogues, dependent on the same carrier, is similar.

This study also shows that CTP as well as UTP can significantly affect dCK-catalysed phosphorylation of both deoxycytidine and dFdC as measured in relatively crude extracts of disrupted cells. Although this does not necessarily reflect the situation in intact cells, it probably gives a better indication of cellular regulation mechanisms than purified enzyme systems. Differences in enzyme regulation may play a role in determining the chemosensitivity of a cell line. We observed biphasic enzyme kinetics for deoxycytidine phosphorylation in all three cell lines studied with apparent K_{m_1} values well below 5 μM. These results, although obtained in cell extracts, are very well in line with those of Bohman and Eriksson [25], who observed similar biphasic kinetics for dCK purified from human leukemic spleen. Most studies on dCK kinetics, however, have been limited to a low deoxycytidine concentration range (<10 μM) [1, 3, 12, 13, 26], precluding measurement of K_m values in a higher range; thus, reported apparent K_m values

vary from 0.6 to 9 μM, depending on the dCK source and experimental conditions. The apparent K_{m_1} values in our cell lines are in this range. For human leukemia HL-60 cells, only Singhal *et al.* [27] reported a very high apparent K_m of 300 μM. Although Stegmann *et al.* [28] measured dCK activity in rat leukemia cells at a broad deoxycytidine concentration range (up to 1.5 mM), these authors reported only one K_m value of 9.4 μM deoxycytidine. Considering the V_{max}/K_m ratio, an indicator for phosphorylation efficiency, dCK from the murine colon cancer cell line C26-10 appears to be the least efficient, both at low and higher deoxycytidine concentrations. The ratio is 6-fold lower than that for A2780 cells. For dFdC and deoxycytidine, an almost similar efficiency was reported for CHO cells [2]. Assuming this also holds for our cell lines, one might extend the differences in V_{max}/K_m, which we found for efficiency of deoxycytidine phosphorylation, to that of dFdC phosphorylation. The pattern in V_{max}/K_m ratio correlates with the accumulation and retention pattern of dFdCTP, as observed in these cell lines [21]. A2780 accumulated the highest dFdCTP levels, closely followed by WiDr (depending on exposure time), both *in vitro* and *in vivo*. C26-10 cells and colon 26 tumours accumulated the lowest dFdCTP levels and retention was less than 24 hr, in contrast to A2780 and WiDr cells and tumours. This pattern follows the chemosensitivity of the cell lines quite nicely [14].

Most studies on dCK enzyme kinetics are based on dCK assays performed with ATP as phosphate donor, because ATP is assumed to be the optimal substrate. For dCK from MOLT-4 cells [10, 29] and Ehrlich ascites tumour cells [11], it was shown that UTP was a better phosphate donor than ATP. In the solid tumour cell lines described in this paper, ATP was the most efficient phosphate donor for phosphorylation of both deoxycytidine and dFdC. Only in C26-10 cells was UTP as efficient as ATP for dFdC phosphorylation, and in the two human tumour cell lines only 30% of the activity with ATP was measured. CTP was a reasonable phosphate donor for dFdC phosphorylation in all three cell lines.

The effects of CTP and UTP on dCK activity were quite pronounced, both with deoxycytidine and dFdC as substrates. The chosen nucleotide concentrations, 0.1 and 1 mM CTP and UTP, are in the range of normal cellular concentrations in the three tested cell lines, including the shifts caused by exposure of the cells to dFdC (Table 2) [21]. Considerable differences were observed for the effects on deoxycytidine and dFdC phosphorylation, possibly related to the mechanism of substrate binding to dCK as proposed by Shewach *et al.* [29]. This sequential Bi-Bi mechanism may involve different conformational changes for deoxycytidine and dFdC as substrates, leading to different affinities for CTP and UTP. At lower substrate concentrations including a more physiological concentration of 1 μM, only for C26-10 cells were considerable differences for the effects of UTP observed, with a 2-fold increase at 1 μM but not at 230 μM. In 2 other studies, an activation (at 2–10 μM deoxycytidine) by UTP was observed [12, 26], and in another, inhibition was found, albeit at a lower deoxycytidine concentration (0.2 μM) [13]. These effects were all found in cells from a myeloid origin (although different species were used as the source), as well as normal and leukemic cells. Habtayesus *et al.* [30] reported a different substrate specificity for murine dCK as compared to human dCK. It seems likely that regulation of dCK is very much species-, tissue-, and proliferation-dependent. Extrapolation of enzymatic characteristics from one source should be done with great caution.

The implications of these results with respect to the shift in CTP and UTP pools as a result of exposure to dFdC are not entirely clear. An increase in CTP seems favourable for dFdC phosphorylation. However, probably as a result of CTP-synthetase inhibition, CTP pools decreased initially in all three cell lines, but were restored to levels higher than control at 24 hr after removal of the drug [21]. The accompanying increase in UTP seems more favourable for deoxycytidine than for dFdC phosphorylation. Because the increase in UTP was lowest in A2780 cells, this may be related to the higher sensitivity of this cell line to dFdC as compared to C26-10 cells. One possibility to clarify the role of CTP and UTP in dFdC phosphorylation would be the combination of dFdC with an antimetabolite capable of affecting CTP and UTP pools. It has been suggested that N-phosphon-acetyl-L-aspartate (PALA), a potent inhibitor of pyrimidine *de novo* synthesis, would increase the effect of ara-C by depletion of CTP and, subsequently, dCTP pools [31, 32]. Indeed, in several cell lines, PALA can potentiate the effect of ara-C [31, 33] but this is not true of all cell lines [33]. Under conditions enabling potentiation of ara-C [33], we could not demonstrate a similar effect for PALA and dFdC (data not shown) in leukemic cell lines. No data are available on solid tumor cell lines.

Our findings on the effects of UTP and CTP are not in line with those of Shewach *et al.* [10]. For dCK purified from MOLT-4 cells, dFdC was phosphorylated most efficiently with UTP as phosphate donor, leading these authors to suggest that high UTP concentrations would be favourable for dFdC phosphorylation. The discrepancy between these and our findings may be explained by the difference in dCK source. Thus, an approach representative of the actual physiological situation in the tumour cells seems to give a better prediction as to the regulation of dCK in that source.

In conclusion, the differential effects of CTP and UTP on deoxycytidine and dFdC phosphorylation seem to potentiate dFdC anabolism more in sensitive A2780 cells compared to the other lines. The efficiency of deoxycytidine phosphorylation may be an indication of dFdCTP accumulation and, consequently, chemosenstivity to dFdC.

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