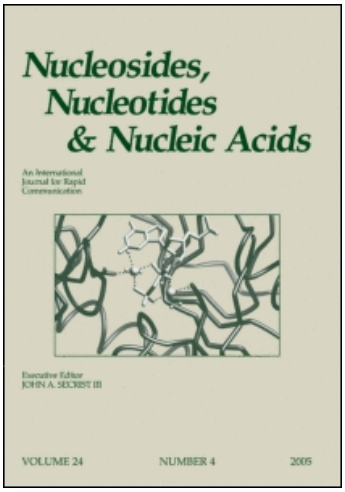

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2',2'-DIFLUORODEOXYCYTIDINE METABOLISM AND MECHANISM OF ACTION IN HUMAN LEUKEMIA CELLS

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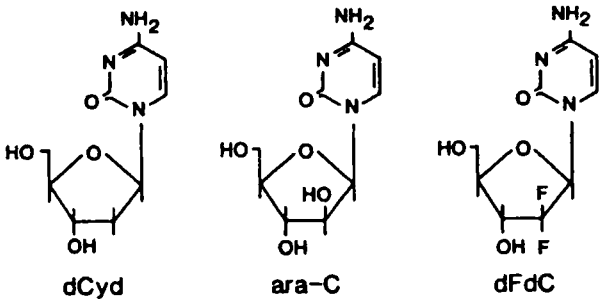
ABSTRACT. The toxicity of 2',2'-difluorodeoxycytidine is due to the inhibition of DNA synthesis by a nucleotide metabolite by either direct inhibition of the process of DNA synthesis and/or to inhibition of ribonucleotide reductase.

The substituents of the 2'-carbon of nucleosides are critical determinants of analogue utilization by enzymes responsible for cellular metabolism of DNA precursors. An hydroxyl group in the ribose configuration, substituted for the natural hydrogen, is not tolerated by either deoxynucleoside kinases[1] or DNA polymerases[2]. The arabinosyl anomers, such as arabinosylcytosine (ara-C), are utilized by deoxynucleoside kinases at somewhat lower efficiency than 2'-deoxynucleosides[1], and can be incorporated into DNA where they function as incomplete chain terminators[3,4].



Substitution of the hydrogen of 2'-deoxycytidine with a fluorine atom in the arabinose configuration produced a 10-fold greater cytotoxicity than when placed in the ribose position[5]. It was therefore of interest to investigate the metabolism and action of a nucleoside in which both hydrogens of the 2'-carbon had been replaced with fluorine atoms. The synthesis of 2',2'-difluorodeoxycytidine (dFdC) provided the opportunity for this investigation[6].

Results and Discussion

The optimal schedule of dFdC in tumor-bearing mice, several day intervals between bolus doses, was unusual for an antimetabolite[7,8]. In contrast, the active antileukemia drug, ara-C, evokes maximum antitumor activity when administered either at close intermittent intervals or by continuous infusion[9]. Thus, the cellular metabolism of dFdC was likely to be different from that of ara-C.

It was possible that a long plasma residence time could facilitate the activity of dFdC by providing an extended exposure of the drug to the tumor. Cytidine/deoxycytidine deaminase is present at high activities in the liver and is known to limit the activity of ara-C[10]. Both cytidine and deoxycytidine were 5-fold better substrates for the enzyme than was dFdC, although dFdC was found to be as efficient as a substrate as was ara-C (TABLE 1). This suggested that dFdC is likely to be deaminated in biological systems, and that it was unlikely that a prolonged residence time of dFdC would explain the unusual dose schedule.

The efficient deamination of dFdC to 2',2'-difluorodeoxyuridine (dFdU) gave rise to the likelihood that dFdU would become a major metabolite in biological fluids. Other uracil nucleoside analogues are known to be substrates for phosphorylase enzymes[11] with the accompanying

TABLE 1. Substrate specificity of cytidine/deoxycytidine deaminase. After the liver of a DBA/2 mouse was homogenized, treated with 1% (w/v) streptomycin sulfate, and the precipitate of the 40-85% ammonium sulfate fraction was dialyzed, the deamination kinetics of radioactive substrates were determined using thin layer chromatography separation.

Substrate	$appK_m$ (mM)	$appV_{max}$ (nmol/mg/h)	Substrate efficiency ($appV_{max}/appK_m$)
cytidine	0.25	16.7	66.8
deoxycytidine	0.27	15.0	55.6
ara-C	1.00	15.4	15.4
dFdC	2.00	29.9	15.0

liberation of the phosphorylated carbohydrates. Because of the uncertainty of the action that 2,2-difluorodeoxyribose might have on intermediary metabolism, it was important to evaluate dFdU as a substrate for pyrimidine phosphorylase. TABLE 2 demonstrates that dFdU is an extremely poor substrate for thymidine phosphorylase. The use of a sensitive HPLC assay failed to reveal detectable activity.

When the human leukemia cell line K562 was incubated continuously over 72 hr with dFdC, the concentration necessary to inhibit growth by 50% (IC_{50}) was 5 nM. This is similar to the IC_{50} value for ara-C, 7 nM. When cells were incubated with dFdC for only 2 hr, however, dFdC was found to be 3 times more potent at growth inhibition than ara-C (90 nM compared to 270 nM, respectively). This finding was consistent with the differences in schedule dependency in the experimental chemotherapy studies. Given the similar susceptibilities to deamination, this result suggested differences in intracellular metabolism of the two drugs.

TABLE 2. Substrate specificity of thymidine phosphorylase. The indicated nucleosides (0.40 mM) were evaluated as substrates for thymidine phosphorylase derived from E. coli (Sigma Chemical Co.) by a spectrophotometric assay described by the manufacturer.

Substrate	Velocity (nmol/min/Unit)	Relative velocity[a]
thymidine	439	100
2'-deoxyuridine	560	128
uridine	41	9
FUdR[b]	389	89
FMAU[c]	<3	<0.6
ara-U[d]	<2	<0.4
dFdU	<0.01[e]	<0.003

a. 0.50 mM nucleoside substrate
b. 5-fluoro-2'-deoxyuridine
c. 2'-fluoro-5-methyl-arabinosyluracil
d. arabinosyluracil
e. Assayed by reversed-phase HPLC.

Cells incubated with various concentrations of dFdC accumulated the proportional levels of the corresponding 5'-triphosphate dFdCTP up to 5 uM dFdC (FIG. 1). Higher concentrations of dFdC did not increase dFdCTP accumulation. In fact dFdCTP accumulation appeared to be inhibited in cells incubated with 50 and 100 uM dFdC. DNA synthesis, measured by [^{3}H]thymidine pulse, was markedly inhibited as the level of dFdCTP rose in cells. Separate experiments that utilized labeled uridine or leucine as indicators of RNA and protein synthesis, respectively, showed no significant inhibition by dFdC (data not shown).

Earlier studies demonstrated that the cellular elimination of dFdCTP was biphasic[12]. Similar kinetic patterns were evident in K562

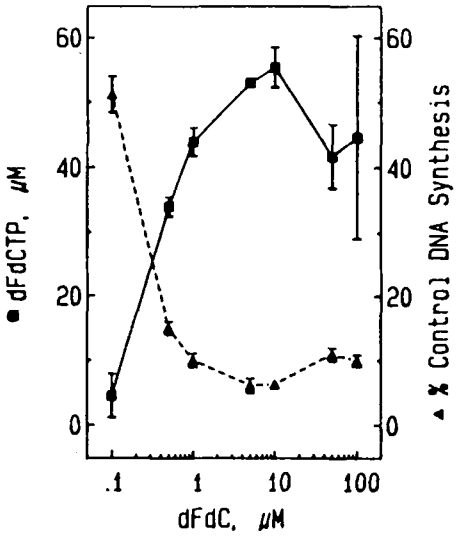


FIG. 1. Effect of a 1-hr incubation with various concentrations of dFdC on dFdCTP accumulation and DNA synthesis in K562 cells.

cells that had accumulated >50 uM dFdCTP (FIG. 2). Cells that accumulated <20 uM dFdCTP, however, eliminated the metabolite with monophasic kinetics. The concentration-dependent cellular kinetics of dFdCTP in K562 cells resemble those reported for human lymphoblasts[12] and differ from the established monophasic elimination of ara-CTP.

To quantitate the possible incorporation of drug into nucleic acids, exponentially growing K562 cells were incubated for 24 h with 0.1 uM [^{14}C]dFdC (spec. act., 1.3 x 10^8 dpm/umol), DNA and RNA were extracted with phenol, and separated by isopycnic centrifugation in a $CsSO_4$ gradient. Radioactivity equivalent to 14.6 fmol dFdC/ug was associated with DNA while none was detected in the RNA fraction. An identical incubation with [^{3}H]ara-C resulted in the incorporation of fmol/ug DNA. Because of the relatively low specific activity of the

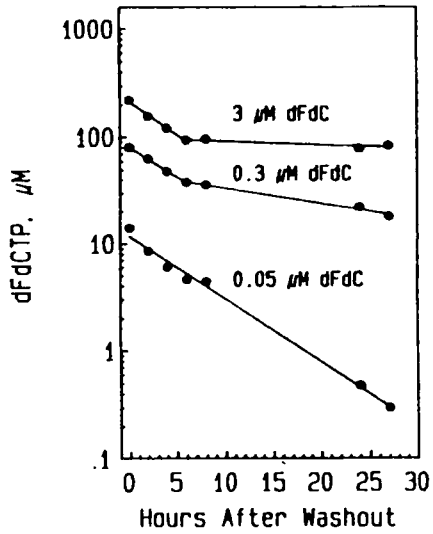


FIG. 2. Elimination of dFdCTP from K562 cells. Cells were incubated with 0.05, 0.3, or 3 uM [^{14}C]dFdC for 1 hr and washed into drug-free medium. Cellular dFdCTP was separated by anion exchange HPLC and eluate fractions were collected to quantitate dFdCTP by scintillation counting.

[^{14}C]dFdC, it was not possible to isolate parent drug from DNA or to determine whether it was located in a terminal position. Experiments utilizing purified mammalian DNA polymerase alpha in a primer extension assay in vitro, however, indicated that dFdCTP is an effective terminator of DNA synthesis[13].

The placement of the two fluorine atoms at the 2'-carbon suggested that a dFdC nucleotide might act as an inhibitor of ribonucleotide reductase. To evaluate this possibility, exponentially growing K562 cells were incubated with 0.1 uM [^{14}C]dFdC and deoxynucleotide pools were quantitated (TABLE 3). Consistent with this hypothesis, this brief incubation caused a 58% decrease in the dATP pool. The levels of dCTP, dTTP, and dGTP were also reduced, by 20-45% of controls.

TABLE 3 Effect of dFdC and ara-C on deoxynucleotide pools. Cells were incubated with 1.0 uM [^{14}C]dFdC or [^{3}H]ara-C for 30 min., and nucleotide pools were extracted. After removal of the ribonucleotides from a portion of the extract by periodate oxidation[14], deoxynucleotides were separated and quantitated by HPLC. Results are the mean ± S.D. of 5 determinations.

	Cellular concentration, uM			
Culture	dCTP	dTTP	dATP	dGTP
control	14 ± 1	22 ± 3	14 ± 4	5 ± 3
1 uM dFdC	8 ± 1	17 ± 2	6 ± 3	4 ± 2
1 uM ara-C	15 ± 1	30 ± 6	19 ± 6	6 ± 1

No effect was seen on the ribonucleoside 5'-triphosphates, which were analyzed separately (data not shown). HPLC quantitation of dFdC nucleotides indicated that the cellular concentrations of dFdCDP and dFdCTP were 3.7 uM and 78 uM, respectively. In contrast, similar incubation of cells with ara-C had no significant effect on deoxynucleotide pools.

Because the model for ribonucleotide reductase regulation by dNTP excludes a role for dCTP[15], it seems unlikely that dFdCTP would be a negative effector. Rather, it is possible that dFdCDP may act as an inhibitory alternative substrate. Investigations with isolated enzyme and pure nucleotide analogs will be necessary to resolve this point.

Deoxycytidine kinase is responsible for the phosphorylation of both ara-C and dFdC to the respective monophosphate[16]. Because the enzyme activity is inhibited by dCTP[17], it is possible that the decrease of dCTP associated with dFdC treatment may affect the metabolism of

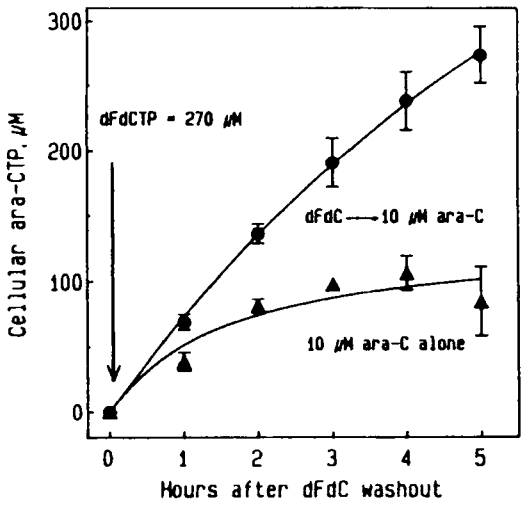
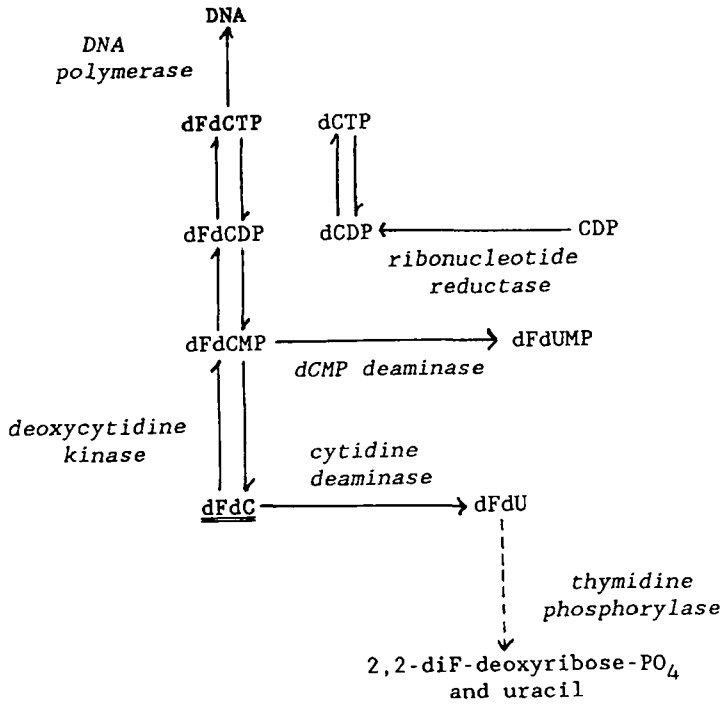


FIG. 3. Modulation of ara-CTP metabolism in K562 cells by pretreatment with dFdC. Details are given in the text.

substrates of deoxycytidine kinase. To evaluate this possibility, K562 cells were incubated with ara-C alone or with ara-C following a 3-hr incubation with 10 uM dFdC. The ability of each culture to accumulate ara-CTP is shown in FIG. 3. Cells that did not receive dFdC pretreatment consistently showed a linear ara-CTP accumulation for 3 hr to about 100 uM before declining during the following 2 hr[14]. The 3-hr incubation with dFdC caused a 80% decrease in the cellular concentration of dCTP. During the first 3 hr of ara-C incubation this was associated with an ara-CTP accumulation rate double that of cells not treated with dFdC. The incubation terminated at 5 hr with 3-fold greater ara-CTP levels in cells that received dFdC pretreatment. Because deoxycytidine kinase also phosphorylates dFdC, the apparent activation of the enzyme in cells after dFdC treatment may serve as a self-potentiating mechanism for enhancing its own anabolism[14].



SCHEME 1.

The pathways of dFdC metabolism are summarized in SCHEME 1. The analogue is a good substrate for cytidine deaminase; it may be expected that like ara-C, this will be the major route of clearance in man. The deamination product dFdU does not appear to be a substrate for thymidine phosphorylase, suggesting the stabilizing influence of the two fluorines on the glycosidic linkage. If future studies of uridine phosphorylase also prove negative, it may be assumed that the difluoro-sugar is not likely to be released to the pathways of intermediary metabolism. Deoxycytidine kinase appears to be required for the initial phosphorylation of dFdC[16]. Since only low levels of the mono- and diphosphate are found in cells, it may be assumed that this step is

rate-limiting to dFdCTP formation. Studies in human lymphoblasts indicated that the changes in the activity of dCMP deaminase are correlated with the biphasic elimination of dFdCTP[12]. Because the human myeloid leukemia cell line, K562, also exhibited concentration-dependent elimination kinetics, it may be expected that the activity of this enzyme will be important to the regulation of cellular dFdCTP metabolism.

It is likely that dFdCTP acts specifically on the process of DNA synthesis; no effects have been observed on either RNA or protein synthesis. This may be due to direct inhibition of the replication process by either inhibition of a DNA polymerase or incorporation and termination of the nascent DNA strand. Alternatively, an indirect action that results from inhibition of ribonucleotide reductase leading to a reduction of cellular deoxynucleotide pools might produce the same effect. The dFdC-induced decrease in cellular dCTP pools, in addition to increasing the activity of deoxycytidine kinase, may also be expected to self-potentiate by enhancing the incorporation of fraudulent nucleotide into DNA. Preliminary evidence indicates a competitive relationship between dFdCTP and dCTP for utilization by DNA polymerase alpha[13]. A better understanding of the specifics of these putative inhibitory actions will require additional experimentation.

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