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Saturation of 2′,2′-difluorodeoxycytidine 5′-triphosphate accumulation by mononuclear cells during a phase I trial of gemcitabine*

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Summary. The plasma and cellular pharmacology of 2′,2′-difluorodeoxycytidine (dFdC, Gemcitabine) was studied during a phase I trial. The steady-state concentration of dFdC in plasma was directly proportional to the dFdC dose, which ranged between 53 and 1,000 mg/m^2 per 30 min. The cellular pharmacokinetics of an active metabolite, dFdC 5′-triphosphate (dFdCTP), were determined in mononuclear cells of 22 patients by anion-exchange high-pressure liquid chromatography. The rate of dFdCTP accumulation and the peak cellular concentration were highest at a dose rate of 350 mg/m^2 per 30 min, during which steady-state dFdC levels of 15–20 μM were achieved in plasma. A comparison of patients infused with 800 mg/m^2 over 60 min with those receiving the same dose over 30 min demonstrated that the dFdC steady-state concentrations were proportional to the dose rate, but that cellular dFdCTP accumulation rates were similar at each dose rate. At the lower dose rate, the AUC for dFdCTP accumulation was 4-fold that observed at the higher dose rate. Consistent with these observations, the accumulation of dFdCTP by mononuclear cells incubated in vitro was maximal at 10–15 μM dFdC. These studies suggest that the ability of mononuclear cells to use dFdC for triphosphate formation is saturable. In the design of future protocols, a dose rate should be considered that produces maximal nucleotide analogue formation, with increased intensity being achieved by prolonging the duration of infusion.

Introduction

The new deoxycytidine analogue 2′,2′-difluorodeoxycytidine (dFdC) is cytotoxic to cells in culture [7, 9] and has therapeutic activity against a variety of murine solid tumors [11]. In each of those models the activity of dFdC was equal to or greater than that of ara-C. Both drugs are structurally related, differing from the natural metabolite deoxycytidine at the 2′-carbon of the carbohydrate moiety by a hydroxyl in the β configuration (ara-C) or geminal fluorines (dFdC) [10]. Phosphorylation by deoxycytidine kinase is required for the activity of each drug [4, 7] and limits the production rate of higher phosphates. Ultimately, cytotoxicity is associated with cellular accumulation of the respective 5′-triphosphates, which, after incorporation into DNA, are associated with the cessation of further synthesis [12]. The diphosphate of dFdC also seems to be an effective inhibitor of ribonucleoside diphosphate reductase [9, 18, 19].

Previous studies have demonstrated that the ability of circulating leukemic lymphocytes and myeloblasts to accumulate ara-CTP is saturated at ara-C dose rates that result in steady-state ara-C concentrations (ara-C_{ss}) of >10 μM [16, 17]. Because the cellular metabolism of dFdC follows the same pathway as that of ara-C [7, 18], we postulated that similar saturation kinetics of dFdCTP accumulation would be observed at a critical plasma $dFdC_{ss}$. Thus, our objective was to determine whether the rate of dFdC phosphorylation was subject to such limitation and if so, to ascertain the $dFdC_{ss}$ at which this stricture takes effect.

A phase I clinical trial of dFdC presented the first opportunity to evaluate this hypothesis. The present report, which relates the plasma pharmacology of dFdC to the cellular metabolism of dFdCTP in circulating mononuclear cells during dFdC infusion, confirms this postulate. This study provides additional evidence that the phosphorylation of nucleoside analogues is a saturable process, the metabolic consequences of which should be considered in protocol design.

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Abbreviations: *ara-C*, 1-β-D-arabinosylcytosine; *ara-C_{ss}*, steady-state concentration of ara-C; *ara-CTP*, 5′-triphosphate of ara-C; *dFdC*, 2′,2′-difluorodeoxycytidine, Gemcitabine; *$dFdC_{ss}$*, steady-state concentration of dFdC; *dFdCTP*, 5′-triphosphate of dFdC

Patients and methods

Patients and therapy. Patients who had histologically proven advanced solid tumors that had been refractory to standard treatment were eligible for these studies. All patients were informed of the study goals and gave their consent. Treatment during the phase I trial consisted of three weekly doses of dFdC, given as an i. v. infusion by pump over 30 min. Pharmacologic data were obtained from 29 patients for dFdC and from 22 subjects for dFdCTP during the first dose of dFdC treatment. The doses studied were 53, 80, 120, 180, 225, 350, 525, 790, and 1,000 mg/m^2. A detailed report of patient characteristics, toxicity, and response has been presented elsewhere [1]. In addition, 5 patients were given infusions of 800 mg/m^2 over 60 min to evaluate the toxicity of prolonged infusions and to compare the dFdC pharmacokinetics in these patients with those in participants who had received 790 mg/m^2 over 30 min.

Plasma pharmacology. Blood samples (10 ml) were drawn from a catheter contralateral to that used for drug infusion and then placed in heparinized tubes containing 5 µmol of the cytidine deaminase inhibitor tetrahydrouridine. Samples were drawn immediately before the start of drug infusion and at 10, 15, and 20 min during the 30-min infusion or at 15, 30, and 45 min during the 60-min infusion. Additional samples were obtained after each infusion had been completed and at 5, 10, 15, 30, and 60 min afterward. Blood samples were placed in an ice bath; plasma was obtained by centrifugation (400 *g* for 5 min at 4° C) and stored at –20° C for subsequent assay.

dFdC analysis. A reverse-phase high-pressure liquid chromatographic method was used to separate and quantitate dFdC. The chromatographic system consisted of two model 6000A pumps, a model 680 gradient controller, a model 730 data module, an automatic sample injector (WISP model 710 B), and a model 481 UV detector from Waters Associates Inc. (Milford, Mass.). The detection wavelength was 275 nm. A reverse-phase analytical µBondapak C_{18} column (Waters Associates) was used. The mobile phase consisted of buffer A, 0.5 M ammonium acetate (pH 6.8), and buffer B, 50% methanol in deionized water. A linear gradient starting at 100% buffer A and going to 60% buffer B over 30 min was run at a flow rate of 1.6 ml/min. For dFdC, the lower limit of detection was determined as 1 nmol (0.265 µg/ml plasma). The standard curve for dose response of detection was linear between 0.05 and 18 nmol. Calculation of the dFdC concentration in plasma was based on the amount of drug detected in a 20 µl sample and was conducted using predetermined absorbance response factors.

An enzyme-linked immunosorbent assay (ELISA) for dFdC, developed by Lilly Research Laboratories and validated in our laboratory, was used to quantitate dFdC in plasma samples from 16 patients who received doses ranging between 53 and 350 mg/m^2. The minimal detectable concentration was 0.02 nmol (0.075 ng/ml plasma). The coefficient of variation ranged between 3% and 14%. The mean of the ratio of peak dFdC values determined in nine patients by ELISA and HPLC was 1.2 ± 0.63.

Cellular pharmacology. After plasma had been removed from blood samples as described, the cell pellet was resuspended in 40 ml phosphate-buffered saline (NaCl, 8.1 g/l; Na_2HPO_4, 1.14 g/l; KCl, 0.22 g/l; KH_2PO_4, 0.27 g/l) and layered over 10 ml Ficoll-Hypaque mixture (sp. gr., 1.077 g/ml). After centrifugation at 400 *g* at 4° C for 20 min, the buoyant mononuclear cells were removed and diluted to 10 ml with phosphate-buffered saline. Duplicate analyses of mean cell number and cell volume were done with a Model ZM Coulter counter equipped with a 100-channel particle-size analyzer (Model C1000). Cellular nucleotides were extracted with $HClO_4$ and stored as described [13, 19].

Analysis of dFdCTP by HPLC. A gradient elution anion-exchange HPLC method similar to that described previously [7] was used to separate and quantitate dFdCTP. The standard curve for dose response of dFdCTP detection was linear between 0.02 and 2 nmol dFdCTP (correlation coefficient, 0.999). dFdCTP was quantitated using a response factor, programmed into the Model 730 data module, for absorbance at 275 nm by authentic dFdCTP, which was synthesized by Dr. A. Sen of this laboratory. The cellular concentration of dFdCTP was calculated by dividing the nanomoles of dFdCTP by the number of cells from which the sample was extracted and the mean cell volume of each sample. This calculation assumes that dFdCTP was uniformly distributed in total cell water.

In vitro incubation of mononuclear cells with dFdC. Mononuclear cells were isolated as described above from blood samples (35 ml) drawn from healthy volunteers. Portions of 5×10^6 cells in duplicate, suspended in 2.5 ml RPMI-1640 and 10% fetal calf serum (Grand Island Biological Co.), were incubated with [5-^{3}H]dFdC (Lilly Research Laboratories) at 2.5, 5, 10, and 15 µM (sp. act., 2×10^7 dpm/µmol) and at 25, 50, and 100 µM (sp. act., 2.4×10^6 dpm/µmol) at 37° C in an atmosphere containing 5% CO_2 for 2 h. Cellular nucleotides were extracted and separated by HPLC as described above, except that the initial conditions included 50% buffer C (0.005 NH_4HPO_4, pH 2.8) and 50% buffer D (0.75 M NH_4HPO_4, pH 3.7) proceeding over 25 min to 100% buffer B on a slightly concave gradient (curve 7) at a flow rate of 1 ml/min. [^{3}H]-dFdCTP, which eluted at 25 min, was quantitated by liquid scintillation counting of 1-ml fractions of the column effluent.

Results

Plasma dFdC

The pharmacokinetics of dFdC in plasma, determined during a phase I study of dFdC in patients with solid tumors, showed that plasma dFdC generally reached a plateau within 15 min after the start of the infusion [1]. Thus, the $dFdC_{ss}$ was taken as the median of plasma dFdC concentrations determined at 15, 20, and 30 min after the beginning of the infusion. Figure 1 shows a linear relationship between the dFdC dose and the $dFdC_{ss}$ over a dFdC dose range of 53–1,000 mg/m^2. The median $dFdC_{ss}$ at the maximum tolerated dose, 790 mg/m^2 per 30 min, was 61 µM [1]. After the infusion ended, plasma dFdC levels decreased rapidly ($t_{1/2}$, 8 min [1]) due to deamination of dFdC to 2′,2′-difluorodeoxyuridine.

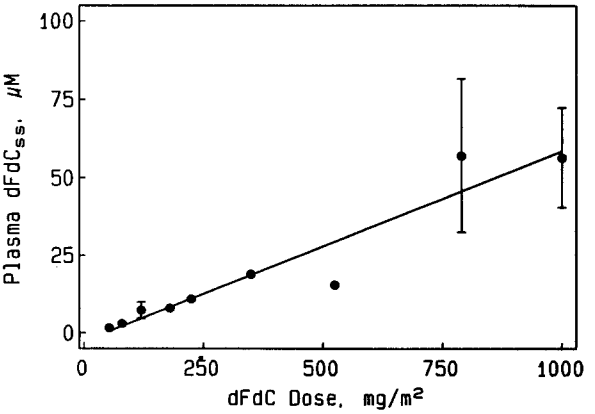

Fig. 1. Steady-state plasma dFdC concentrations in 27 patients during therapy. DFdC was infused i. v. over 30 min. Values represent the average $dFdC_{ss} \pm$ SEM for patients treated at each dose. Dose levels in mg/m^2 and number of patients (in parentheses) were: 53(3), 80(3), 120(3), 180(3), 225(1), 350(3), 525(2), 790(4), and 1,000(5). Correlation coefficient for linearity, $r = 0.954$

dFdCTP in mononuclear cells

The cellular concentration of dFdCTP increased linearly in mononuclear cells during dFdC infusion and generally reached a peak within 30 min after the end of the infusion (data not shown). Both the rate of dFdCTP accumulation and the peak dFdCTP concentration in mononuclear cells increased among patients in response to higher dFdC dose rates until the dose reached 350 mg/m^2 (Fig. 2). The data were plotted as single points for groups of patients who received the same dose. The average $dFdC_{ss}$ was 18 μM for three patients who received dFdC at a dose of 350 mg/m^2 per 30 min. At higher dFdC doses, either no increase in these parameters was observed or the average values decreased. Similarly, no significant increase of the AUC for dFdCTP occurred above a $dFdC_{ss}$ of 20 μM (Fig. 2C).

Effect of the dFdC dose rate on dFdCTP pharmacokinetics

Because a plasma $dFdC_{ss}$ of about 20 μM was sufficient to maximize dFdCTP accumulation in mononuclear cells, we sought to determine the effect that a more prolonged dFdC infusion at this plasma level would have on dFdCTP pharmacokinetics. Extension of the phase I study to 60-min infusions created an opportunity to compare the pharmacokinetic characteristics of similar dFdC doses given over either 30 (790 mg/m^2) or 60 min (800 mg/m^2). Patients who received 790 mg/m^2 over 30 min had a median $dFdC_{ss}$ of 61 μM (Table 1). Although the patients' steady-state levels varied considerably at this dose rate, the median $dFdC_{ss}$ of 20 μM that was observed when 800 mg/m^2 was infused over 60 min may be considered to be in proportion to the higher rates shown in Table 1, as would be expected from data derived from a larger population (Fig. 1).

Despite these differences in dFdC plasma concentrations, the median rates of dFdCTP accumulation were similar at the two dose rates. This is consistent with the observation that dFdCTP accumulation by mononuclear cells in vivo is maximal at a $dFdC_{ss}$ in the range of 20 μM. The duration of infusion had a substantial effect; in patients receiving the longer infusions, peak dFdCTP concentrations were >2-fold and AUC values, 5-fold those determined in patients who received 30-min infusions (Table 1). Thus, a dFdC dose infused at a rate that achieved an optimal $dFdC_{ss}$ maximized the dFdCTP accumulation rate. The accumulation of dFdCTP was therefore higher than when the same dose was given at a rate that greatly exceeded the cells' ability to phosphorylate dFdC. In practical terms, dose rates of >350–400 mg/m^2 per 30 min are unlikely to increase the rate of dFdCTP accumulation in mononuclear cells.

In vitro incubation of donors mononuclear cells from healthy

In vitro incubations of the mononuclear cells from healthy donors with dFdC were conducted to obtain an additional

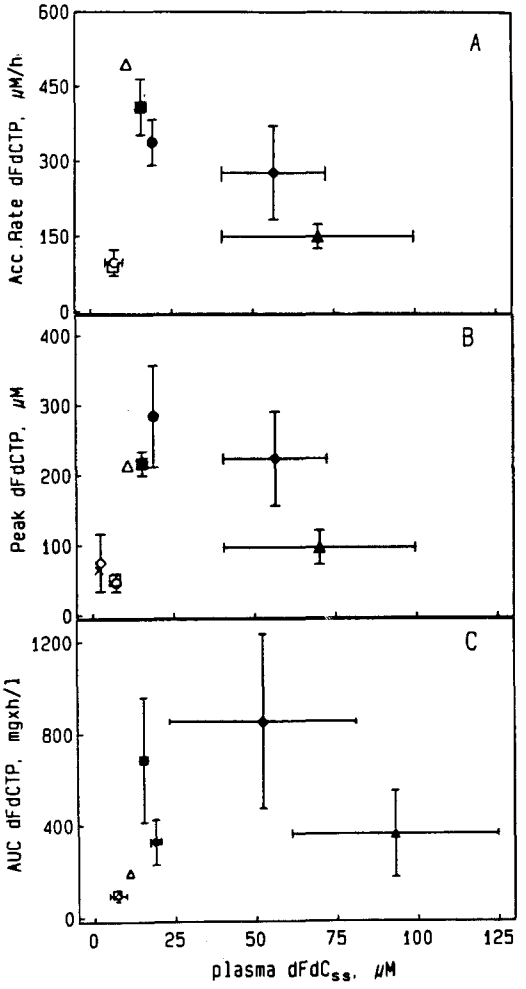

Fig. 2 A–C. Metabolism of dFdCTP by mononuclear cells during dFdC therapy. **A** Accumulation rate. **B** Peak concentration. **C** AUC. Values represent the averages ± SEM for cohorts of patients treated at the same dose rate. *Horizontal bars* indicate the SEM for $dFdC_{ss}$ concentrations, and *vertical bars* indicate the SEM for accumulation rates, peak concentrations, and AUC. The number of patients at each dose were not identical for all three parameters, because inadequate data were obtained for determination of the AUC and/or the accumulation rate. Doses (mg/m^2) are indicated as follows: ×, 53; ◇, 80; ○, 120; □, 180; △, 225; ●, 350; ■, 525; ▲, 790; ◆, 1,000

understanding of the dose response of dFdCTP pharmacokinetics. The objective was to determine the dFdC concentration that would saturate the cells' capacity to accumulate dFdCTP under in vitro conditions and to compare this to results of the phase I study. Mononuclear cells from healthy donors were incubated for 2 h to assure linearity of accumulation and cells from six donors were analyzed (Fig. 3). Although there was interindividual variability in the amount of dFdCTP accumulated, five of the donors had a common feature. Except in the case of donor 1, no increase in cellular dFdCTP levels occurred with >15 μM dFdC in the incubation medium; maximal dFdCTP concentrations were achieved with 15 μM dFdC in donors 5 and 6 but with 10 μM dFdC in donors 3 and 4 and 5 μM in donor 2. Cells from donor 1 achieved a maximal dFdCTP level at 50 μM dFdC. Maximal dFdCTP levels were achieved at a median of 12.5 μM dFdC.

Table 1. Pharmacokinetics of dFdCTP were determined in mononuclear cells during infusion of similar dFdC doses at different rates[a]

Dose rate (mg/m² per 30 min)	$dFdC_{ss}$ (μM)	dFdCTP		
		Accumulation rate (μM/h)	Peak (μM)	AUC (μM/h)
790	125	150	81	18.8
790	61	184	140	23.0
790	24	108	64	13.6
400	11	199	362	99.7
400	17	109	131	54.6
400	20	118	162	58.9
400	23	195	302	97.7
400	83	347	340	173.5

[a] 790 mg/m² dFdC was infused over 30 min; 800 mg/m² was infused over 60 min

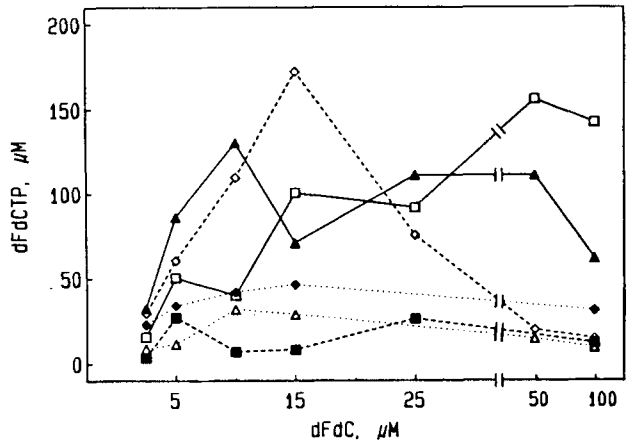

Fig. 3. Accumulation of dFdCTP by human mononuclear cells from healthy donors in vitro. Cells were isolated by Ficoll-Hypaque centrifugation and incubated for 2 h with indicated concentrations of [5-³H]dFdC. Cell extracts were analyzed for dFdCTP as described in Patients and methods. Values represent the average results from duplicate incubations: □, donor 1; ■, donor 2; △, donor 3; ▲, donor 4; ◇, donor 5; ◆, donor 6

Discussion

Strong correlations have been demonstrated between the clinical response and the pharmacokinetics and pharmacodynamics of ara-CTP in leukemia cells during therapy with single-agent, high-dose ara-C given by bolus [13, 15] or continuous infusion [3]. These studies provided the rationale for determining infusion rates that produce a cellular ara-CTP metabolism consistent with efficient cellular phosphorylation of the parent drug [8, 16, 17]. In the present investigation we used a similar approach to determine a dFdC infusion rate that would maximize dFdCTP accumulation in the circulating mononuclear cells of patients with solid tumors. Similarities observed between intracellular exposure of ara-CTP and dFdCTP to biological activity in vitro [7] have suggested the importance of understanding dFdCTP metabolism during therapy. The objective of this study, therefore, was to determine a dFdC dose rate that would achieve $dFdC_{ss}$ levels sufficient to maximize dFdCTP accumulation in circulating mononuclear cells, the only cells currently available for investigation.

The phase I study demonstrated a linear relationship between the $dFdC_{ss}$ levels in plasma and the dFdC dose (Fig. 1). Steady-state levels of dFdC were generally obtained within 15 min of the start of the infusion, a characteristic that has recently been observed in patients with leukemia who received dFdC infusions over 2–4 h [5]. This proportionality provided a basis for comparing dFdCTP pharmacokinetics at different dFdC infusion rates.

Several lines of evidence were obtained to suggest that the accumulation of dFdCTP by mononuclear cells is a saturable process. First, during the phase I study, we determined that a dFdC dose rate of 350 mg/m² per 30 min achieved plasma dFdC levels (about 20 μM) that maximized the accumulation of dFdCTP in mononuclear cells during therapy (Fig. 2). Second, patients infused with 800 mg/m² over 60 min, a rate that achieved a median $dFdC_{ss}$ of 20 μM, exhibited dFdCTP pharmacokinetics similar to those of patients with a $dFdC_{ss}$ of 60 μM (Table 1) or greater (Fig. 2). Finally, incubation of mononuclear cells with various concentrations of dFdC in vitro demonstrated a maximal dFdCTP accumulation rate at 12.5 μM dFdC (Fig. 3). Based on these data, it is reasonable to suggest a $dFdC_{ss}$ of 10–20 μM as being the range of concentrations that is likely to produce maximal rates of dFdCTP accumulation in mononuclear cells. The sample values in these studies showed considerable heterogeneity, demonstrating that the metabolism of dFdCTP varies among the population. However, our data support the conclusion that $dFdC_{ss}$ levels of >20 μM would saturate the rate of dFdCTP accumulation in the cells of a majority of individuals.

The results of in vitro incubation of mononuclear cells from healthy subjects with a spectrum of dFdC concentrations helped to clarify the relationship between dFdC concentration and intracellular nucleotide accumulation. The significance of the in vitro experiment is emphasized by the fact that similar studies [2, 6, 14] have predicted plasma ara-C concentrations that would maximize ara-CTP accumulation during therapy [16, 17].

During the phase I study, dose rates of >350 mg/m² per 30 min (525–1,000 mg/m²) did not increase the rate of dFdCTP accumulation, the dFdCTP peak concentration, or the cellular AUC (Fig. 2). Because the biological activity of dFdC depends on intracellular nucleotide accumulation

[7], dose rates that produce a plasma $dFdC_{ss}$ higher than that which can be used by target cells may contribute to systemic toxicity. This provides a basis for suggesting an upper limit for the dFdC dose rate. As discussed below, however, a key objective of future studies will be to compare the dose-response characteristics of dFdCTP in tumor cells with those in mononuclear cells.

In an extension of the phase I trial, we increased the infusion duration to 60 min in a group of five patients. The dose rate at which these patients were treated, 800 mg/m^2 per 60 min, was roughly twice the dose that gave a $dFdC_{ss}$ of 20 μM (350 mg/m^2 per 30 min) and similar to a dose given over 30 min (790 mg/m^2) that had been studied earlier. As expected from the proportional relationship seen in Fig. 1, 800 mg/m^2 infused over 60 min achieved a median plasma $dFdC_{ss}$ concentration of 20 μM, whereas patients who received 790 mg/m^2 over 30 min had a median $dFdC_{ss}$ of 61 μM. Despite the proportionality of dose rate and $dFdC_{ss}$, the rates of dFdCTP accumulation were comparable. In contrast, the AUC of dFdCTP was increased 4-fold by the longer infusion (Table 1). This observation is consistent with the hypothesis that maximal dFdCTP accumulation would be achieved by more prolonged infusions at a dose rate maintaining a $dFdC_{ss}$ that saturates nucleotide accumulation. However, the length of time for which cells can sustain a linear accumulation of dFdCTP remains to be determined.

The median terminal $t_{1/2}$ of dFdCTP elimination from mononuclear cells in vivo was 5 h ($n = 23$) [1] and was much longer in cell lines [7, 18]. This indicates that the dFdCTP elimination rate is likely to be quite small relative to the rate of dFdCTP accumulation. The rate of dFdCTP accumulation is therefore likely to be a good indicator of the velocity of the rate-limiting step in dFdCTP synthesis. Studies of Chinese hamster ovary (CHO) cells have indicated that, as is true for ara-CTP accumulation, deoxycytidine kinase is rate-limiting for dFdCTP accumulation [4, 7].

To our knowledge, this is the first study to evaluate the dose response of an anticancer drug metabolite in mononuclear cells. We do not know whether dFdCTP metabolism by mononuclear cells is similar to that by either circulating leukemia cells or solid tumors. The bioavailability of dFdC is likely to be similar in mononuclear cells and circulating leukemia cells, but we suppose that penetration of solid tumors may require a greater $dFdC_{ss}$. Although our results provide an indication for more prolonged infusions at a moderate dose rate, the expectation that greater dFdCTP AUC values will be associated with increased therapeutic efficacy depends on a difference in metabolism or inherent sensitivity in tumor relative to host cells. To understand these complexities, it will be important to compare the metabolic characteristics of normal cells with those of different tumor cell types obtained from patients in future investigations. Such studies are required to determine the role that investigations of drug metabolism in mononuclear cells can play with respect to the optimization of drug metabolism by the various target cells in cancer therapy.

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