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The role of deoxycytidine-metabolizing enzymes in the cytotoxicity induced by 3′-amino-2′,3′-dideoxycytidine and cytosine arabinoside*

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Summary. The cellular metabolism of 3′-amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd), a cytotoxic agent previously reported to be a poor substrate for purified Cyd/dCyd deaminase (dCydD), was compared with that of cytosine arabinoside (ara-C) in cells that displayed dCydD activity (HeLa) and in cells that did not (L1210). Growth inhibition induced by 3′-NH_2-dCyd was dependent on the levels of anabolic enzymes, particularly dCyd kinase (dCydK), whereas cytotoxicity induced by ara-C was dependent on the expression of both anabolic and catabolic enzyme activities. Competition kinetics using purified enzyme revealed that the binding affinity of ara-C to dCydK was 5-fold that of the amino analog. However, this binding advantage is apparently offset in cells that contain high levels of dCydD, since the K_i values for this enzyme were 0.2 and 23 mM for ara-C and 3′-NH_2-dCyd, respectively. This was reflected in the decrease in analog sensitivity observed between the two cell lines, whereby the concentrations of ara-C and 3′-NH_2-dCyd required to inhibit growth by 50% were 200 and 7 times higher, respectively, in the dCydD-containing HeLa cells as compared with the dCydD-deficient L1210 cells. The metabolic stability and cytotoxicity of 3′-NH_2-dCyd was independent of cell number. An unexpected finding was the extent to which the effectiveness of ara-C could be mitigated by the number of dCydD-containing cells. A completely cytotoxic concentration of ara-C was rendered nontoxic by a 10-fold increase in cell number. This observation was supported by an increase in I-β-D-arabinofuranosyluracil (ara-U) formation, a decrease in ara-C 5′-triphosphate (ara-CTP) accumulation, and a rise in cell viability with increasing cell number. These findings indicate that unlike ara-C, the effectiveness of 3′-NH_2-dCyd is independent of the level of deaminase, which suggests its possible utility in situations in which high levels of deaminase are manifest.

Introduction

3′-Amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) is a cytotoxic agent that has been demonstrated to be active against murine leukemia in vitro and in vivo [16]. A unique feature of this analog is that it serves as a nominal substrate for human cytidine/deoxycytidine deaminase (dCydD), the enzyme that is responsible for the short biological half-life of cytosine arabinoside ara-C I-β-D-arabinofuranosylcytosine [2, 7, 11] by converting it to noncytotoxic I-β-D-arabinofuranosyluracil (ara-U) [4, 5, 11]. It is known that the plasma half-life of ara-C varies among patients [2, 7, 8, 26] and in some cases of acute myeloblastic leukemia (AML); especially when ara-C is given as a single agent, refractoriness to treatment with this drug has been shown to correlate with a short half-life of ara-C [2] and with elevated levels of deaminase activity in leukemic blasts [25]. Such findings prompted an investigation to explore the possibility of developing a cytotoxic, deaminase-resistant dCyd analog for the treatment of acute leukemia.

Although the antileukemic effect of 3′-NH_2-dCyd is believed to result from an inhibition of DNA replication [16, 19], little is known about the metabolism that occurs in intact cells. Furthermore, in addition to dCydD, cells also contain deoxycytidylate deaminase (dCMPD). If the amino analog is a good substrate for this degradative enzyme, it would be inactivated because the corresponding deoxyuridine (dUrd) derivative is significantly less cytotoxic [16, 19]. The aim of the present investigation was to evaluate the cytotoxic effect of 3′-NH_2-dCyd in cells displaying dCydD activity and in those exhibiting no such activity. Since ara-C is a good substrate for this enzyme, it was studied for comparison.

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Table 1. Cell-line comparison of analog sensitivity and the levels of dCyd-metabolizing enzymes

	IC_{50} (μM)[a]		Enzyme activity (unit/mg)[b]			
Cells	Ara-C	3′-NH_2-dCyd	dCydK	dCMPK	dCydD	dCMPD
L1210	0.02	0.6	0.108±0.004	5.37±0.33	0[c]	2.43±0.17
HeLa	4.4	3.9	0.051±0.005	8.87±0.76	5.90±0.66	5.57±0.37

[a] IC_{50} values were calculated from 72-h dose-response curves [22]. The starting cell density was 1×10^4 and 1×10^5 cells/ml medium for L1210 and HeLa cells, respectively

[b] The conditions for cell growth were the same as those used to determine dose response except that no drug was present. Enzyme activities were determined in duplicate immediately after culture harvest for each experiment. Data represent mean values ± SEM (dCydK and dCydD, n = 3 experiments; dCMPK and dCMPD, n = 2 determinations). One unit is the amount of enzyme which catalyzes the formation of 1 nmol product from substrate per min at 32°C

[c] No enzyme activity detected

Materials and methods

Chemicals. 3′-NH_2-dCyd was kindly provided by Dr. T.-S. Lin of Yale University. Unlabeled nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). [2-^{14}C]-dCyd, [2-^{14}C]-dCMP (56 mCi/mmol) and [5,6-^{3}H]-ara-C (26 Ci/mmol) were obtained from Moravek Biochemicals (Brea, Calif.). [6-^{3}H]-3-NH_2-dCyd (0.75 Ci/mmol) was prepared using the tritium labeling service of New England Nuclear (Boston, Mass.) and subsequently purified as described elsewhere [20]. All other chemicals were reagent grade.

Cells. L1210 cells were obtained from the NCI-Frederick Cancer Research Facility, and HeLa and KB cells were purchased from the American Type Culture Collection. L1210 cells were grown in Fischer's medium (Gibco, Grand Island, N.Y.) supplemented with 10% horse serum, whereas HeLa cells were propagated in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco). For growth-inhibition studies, cells in logarithmic growth were incubated for 72 h with various concentrations of either ara-C or 3′-NH_2-dCyd. Cultures were harvested and the cell number was determined using a model ZM Coulter counter (Hialeah, Fla.).

Enzyme levels. For determination of the dCyd-metabolizing enzyme levels, cultures were propagated under the same conditions used for the growth-inhibition studies, except that no drug was present during the incubation. All harvest procedures were performed at 5°C. After 72 h growth, cultures were collected by low-speed centrifugation and washed twice with phosphate-buffered saline. Extraction buffer [25 mM HEPES (pH 7.5), 3 mM $MgCl_2$ and 4 mM dithiothreitol (DTT)] was added to the pellet prior to three freeze-thaw procedures. Following the addition of KCl (final concentration, 0.15 M), the samples were sonicated with three 5-s blasts. Glycerol was added to 10% of the volume before centrifugation of the samples in an Eppendorf microcentrifuge. The supernatant was immediately used for determination of enzyme levels.

dCMPD was assayed as reported elsewhere [18]. The dCydD assay was carried out essentially as described for dCMPD, except that 1 mM dCyd was used instead of dCMP and the dCTP was omitted from the reaction mixture. The dCyd kinase (dCydK) assay was performed essentially as previously described [17], except that 0.35 mM tetrahydrouridine and 25 mM NaF were included in the reaction mixture for assay of the cell homogenate. The analysis of deoxycytidylate kinase (dCMPK) was carried out as previously described [6]. However, the contents of the assay mixture were modified so as to obtain maximal enzyme activity. The reaction mixture contained 50 mM HEPES (pH 7.5), 5.6 mM phosphocreatine, 0.5 unit (Sigma Chemical Co.) phosphocreatine kinase, 7 unit (Sigma Chemical Co.) nucleoside diphosphate kinase, 75 μg bovine serum albumin, 2 mM DTT, 2 mM adenosine 5′-triphosphate (ATP), 1 mM thymidine 5′-monophosphate (TMP; all obtained from Sigma Chemical Co., St. Louis, Mo.), 2 mM [2-^{14}C]-dCMP (0.37 mCi/mmol), and 2 mM $MgCl_2$.

Enzyme purification. The purification of dCydK from L1210 cells has been reported [17]. Since L1210 cells do not exhibit dCydD activity, this enzyme was isolated from human KB cells. The procedure for the extraction and purification of dCydD up to the thymidine-affinity column step was performed as described elsewhere [19]. For the removal of residual dCydK activity, the preparation was applied to a Blue Sepharose CL-6B (Pharmacia, Piscataway, N.J.) column and eluted with buffer [0.2 M TRIS-HCl (pH 7.5), 2 mM DTT, and 10% glycerol]. Fractions containing dCydD activity were pooled and the sample was applied to a DE-52 (Whatman, Hillsboro, Ore.) anion-exchange column. The enzyme eluted in a gradient [containing 0.05 M TRIS-HCl (pH 7.5), 2 mM DTT, and 10% glycerol] of increasing ionic strength (0 – 0.5 M KCl). The purified enzyme was desalted using a G-25 Sephadex (Sigma Chemical Co., St. Louis, Mo.) column before its use in the kinetics experiments.

Cellular metabolism of analogs. To assess the degree of metabolic degradation by cellular dCydD, HeLa cells were incubated with radiolabeled analogs at the concentrations required to inhibit growth by 50% (IC_{50}). Either 4 μM [5,6-^{3}H]-ara-C or [6-^{3}H]-3′-NH_2-dCyd was incubated with the indicated number of cells for 26 h. Cells were removed by centrifugation and the medium was extracted in 60% methanol. An aliquot of the supernatant was applied to silica thin-layer chromatography (TLC) plates, which were developed in n-butanol, glacial acetic acid, and water (2 : 1 : 1, by vol.). Areas migrating with standard compounds were removed, and radioactivity was quantitated by liquid scintillation spectrometry. Each determination was carried out in triplicate for each experiment.

Determination of ara-CTP levels. For the separation of ribonucleotides from the desired deoxyribonucleotides contained within the methanol extracts, boronate affinity chromatography (Bio-Rad) was used. The eluate containing nucleosides and nucleotides that lacked 2′,3′-hydroxyls in the *cis* configuration was analyzed by HPLC using the anion-exchange matrix [10]. [^{3}H]-Ara-C 5′-triphosphate ([^{3}H]-ara-CTP) which eluted in 0.5 M phosphate (pH 4.6) was quantified using liquid scintillation spectrometry.

Results

Analog-induced cytotoxicity

The ability of 3′-NH_2-dCyd and ara-C to inhibit the replication of L1210 (without dCydD) and HeLa (with dCydD) cells following a 72-h incubation was evaluated. Logit transformation [22] of the dose-response curves established the IC_{50} value, which was found to vary about 7- and 200-fold between the cell lines for 3′-NH_2-dCyd and ara-C, respectively (Table 1). Since both agents are S-phase-specific, it seemed reasonable that differences in drug metabolism may have contributed to this variation.

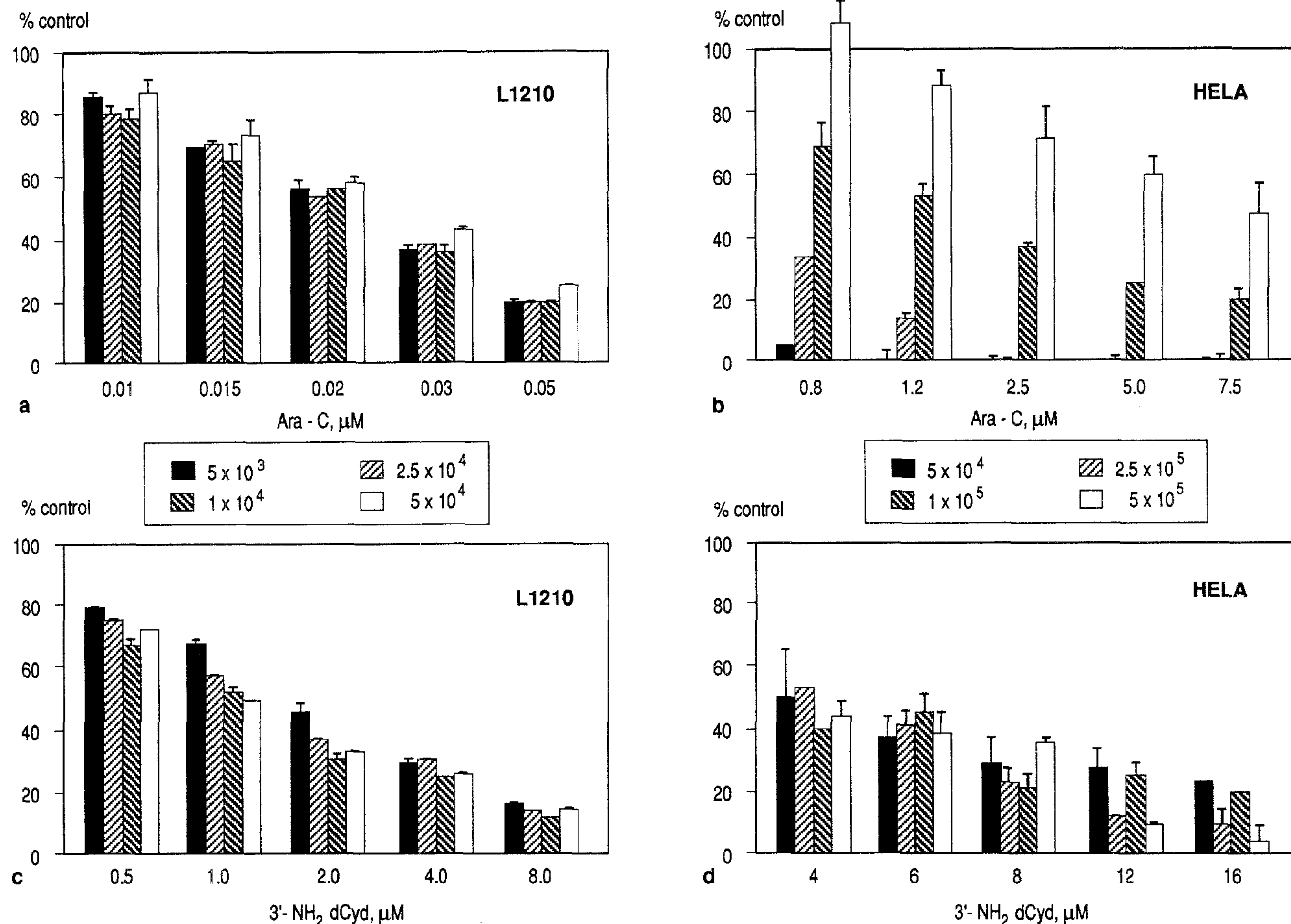

Fig. 1 A–D. Effect of cell number on analog sensitivity. **A, C** L1210 cells that failed to display nucleoside deaminase activity and **B, D** HeLa cells that exhibited such activity were seeded at the indicated cell density in the same volume of medium and were incubated for 48 h in the presence of varying concentrations of **A, B** ara-C or **C, D** 3′-NH_2-dCyd. Each result represents the average value ± SEM for 3 separate cultures

Activities of dCyd-metabolizing enzymes

Table 1 depicts the levels of dCyd-metabolizing enzymes found for each cell line. L1210 cells exhibited about twice the dCydK activity shown for HeLa cells. In addition, the doubling times were 11 h for L1210 vs 25 h for HeLa. These two factors could have accounted for much of the variation in IC_{50} values for 3′-NH_2-dCyd observed between the cells. However, with respect to ara-C, an additional parameter is required to account for the wide variation in drug sensitivity. The presence of high deaminase levels in HeLa cells may have contributed to the difference between these drugs, since ara-C is known to be a good substrate for dCydD.

Table 2. Analog K_i values for dCydK and dCydD

	K_i (mM)[a]	
Enzyme	Ara-C	3′-NH_2-dCyd
dCydK	0.3	1.5
dCydD	0.2	23.4

[a] The dCydK and dCydD reactions were carried out using fixed dCyd concentrations and varying concentrations of inhibitor (ara-C or 3′-NH_2-dCyd). Lineweaver-Burk plots were linear and showed competitive inhibition. Linear regression analysis was used both to calculate the Lineweaver-Burk slopes and to determine the K_i value from the replot (slope vs inhibitor concentration). The correlation coefficients of the replots for ara-C and 3′-NH_2-dCyd, respectively, were 0.9987 and 0.9984 for dCydK ($n = 7$) and 0.9991 and 0.9965 for dCydD ($n = 5$)

K_i determination

The data suggest that the levels of dCyd enzymes may have influenced the observed variation in cytotoxicity. The K_i value for the analogs was determined for the first enzyme involved in both the activation and the degradation pathways. The respective K_i values for ara-C and 3′-NH_2-dCyd were 0.3 and 1.5 mM for dCydK and 0.2 and 23.4 mM for dCydD (Table 2).

Influence of cell number on analog sensitivity

If the presence of deaminase is a major factor in drug-sensitivity variations between cells, then the magnitude of the

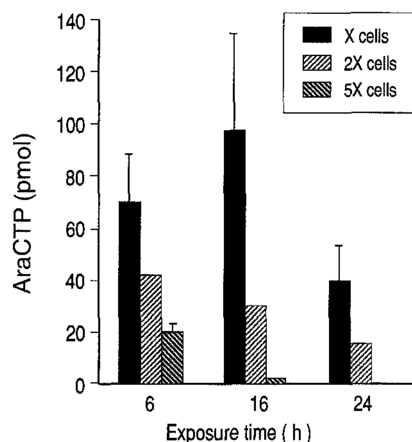

Fig. 2. Effect of cell number on ara-CTP levels. HeLa cells were grown in the presence of 1.2 μM [^{3}H]-ara-C at varying (1-, 2-, and 5-fold) cell densities in the same volume of medium. At the indicated times, cultures were harvested and the amount of ara-CTP (pmol/10^7 cells) was determined by HPLC. The data obtained at 1- and 5-fold cell densities represent average values ± SEM for 2 separate experiments

Table 3. Effect of cell number on the extent of ara-C or 3′-NH_2-dCyd deamination in HeLa cells

Cell number[a]	[^{3}H]-AraU[b] (% total cpm)	[^{3}H]-3′-NH_2-dUrd[b] (% total cpm)
5×10^4	61.2±3.12	
1×10^5	82.2±2.57	0.748±0.61[c]
2×10^5	91.4±0.93	
5×10^5	95.6±0.14	0.540±0.58[c]

[a] Cell number at the time of addition of radiolabeled drug (4 μM) to the medium. Each determination was carried out in triplicate for each experiment. Incubations lasted 26 h

[b] Data represent average values ± SE for 2 separate experiments except for those obtained for ara-C at 1×10^5 and 5×10^5 cells ($n=3$ experiments). Compounds were resolved using TLC [silica supplemented with *n*-butanol, acetic acid, and water (2 : 1 : 1, by vol.)] and the amount of radioactivity was quantitated by scintillation spectrometry. The total counts per minute measured for the drug and its deaminated derivative were approx. 6,000 and 4,000 for ara-C and 3′-NH_2-dCyd, respectively

[c] Level of 3′-NH_2-dUrd formed was not significant according to Student's two-tailed *t*-test ($P<0.001$)

effect may be influenced by the amount of enzyme activity that is expressed at the time at which the drug is added to the medium. One way to test this hypothesis is to expose increasing numbers of cells to a fixed concentration of analog as demonstrated in Fig. 1.

In L1210 cells, which display no detectable dCydD activity, cell number did not influence the cytotoxic effect of either agent (Fig. 1 A, C). Similarly, in the dCydD-containing HeLa cells, the level of cytotoxicity induced by 3′-NH_2-dCyd was not mitigated by a 10-fold increase in cell number (Fig. 1 D). This implies that the amino analog is metabolically stable even in cells that contain high levels of deaminase. In contrast, the influence of the number of HeLa cells on ara-C-induced toxicity was substantial. For example, an ara-C concentration of 0.8 μM completely prevented cell replication at the low cell number, but a 10-fold increase in cell number rendered this concentration completely nontoxic (Fig. 1 B). To rule out the possibility that only growth delay was altered by the change in cell number, cell viability was examined using a clonogenic assay. At 5 and 7.5 μM ara-C, the extent of colony formation as compared with that in untreated cells was 13%±3% and 5.7%±3%, respectively, at the lowest cell number and 76%±7% and 79%±7%, respectively, at a 5-fold higher cell number.

Analog deamination in intact cells

The data suggest that dCydD may play a significant role in reducing the cytotoxic effectiveness of ara-C but not 3′-NH_2-dCyd. Thus far, observations implicating deaminase that have been drawn from experiments using isolated enzymes and intact cells have been indirect. To provide more direct documentation of deaminase involvement in this process, the formation of drug metabolites in cultured cells was examined. HeLa cells were grown in the presence of radiolabeled ara-C or 3′-NH_2-dCyd at their IC_{50} concentrations. After 26 h, the medium was removed and the degree of analog deamination was quantitated (Table 3). No significant amount of 3′-amino-2′,3′-dideoxyuridine (3′-NH_2-dUrd) was detected, even at the highest cell number used (Table 3). This finding is consistent with the previous observation and suggests that 3′-NH_2-dCyd is metabolically more stable than ara-C in intact cells that express dCydD.

In ara-C-treated cells, the amount of radiolabeled ara-U formed correlated with the starting cell number. At the highest cell number, >95% of the total radioactivity was present as the deaminated derivative. This cell-number-dependent degradation of ara-C should be reflected in the level of ara-CTP, since the amount of this metabolite has been shown to be a determinant in predicting the response to therapy [24]. Figure 2 reveals that the formation of ara-CTP decreased with increasing numbers of cells.

Discussion

Ara-C is an important part of our armamentarium in the treatment of acute leukemia in children and adults [3, 9, 12]. A major limitation recognized by many investigators is the short biological half-life of this agent caused primarily by the action of dCydD, which degrades ara-C to noncytotoxic ara-U [4, 5, 11]. Potential ways to circumvent this problem include the use of high-dose ara-C therapy [1, 15, 21, 23] in an attempt to saturate the deaminase and the coadministration of tetrahydrouridine with ara-C to inhibit dCydD [13, 14, 27]. However, in either method, the optimization of clinical therapy may be hindered due to variations in the dCydD levels in the liver, plasma, and tumor. An alternative approach to circumvent the enzymic inactivation of ara-C involves the use of a dCyd derivative that is not a substrate for dCydD but serves as a substrate for the kinases that are essential to the formation of the

active metabolite (triphosphate derivative). Such an agent may be useful because it would enable the clinician to initiate therapy without regard to the interpatient variability of deaminase activity.

3′-NH_2-dCyd has been observed to be cytotoxic to cultured murine L1210 and S-180 cells at an IC_{50} value of 0.7 and 4 μM, respectively [16]. This dCyd derivative has also shown activity in mice bearing L1210 leukemia [16], and four of five mice survived for 60 days after the treatment schedule had been optimized [20]. The compound is converted by cellular enzymes to the triphosphate, which specifically inhibits DNA synthesis [20] but not RNA or protein synthesis [16]. 3′-NH_2-dCyd has been found to be resistant to deamination using an in vitro assay for dCydD [19]. However, the finding that L1210 cells are devoid of dCydD and that the deaminated derivative 3′-NH_2-dUrd is not active in vitro [19] or in vivo [16] prompted an evaluation using deaminase-containing cells. The data obtained in the present investigation using intact cells that contained high levels of both nucleoside and nucleotide deaminase suggest that the biological activity of 3′-NH_2-dCyd is not mitigated by the presence of these enzymes (Table 1).

Although both analogs showed variations in cytotoxicity to the cell types tested, the magnitude of this effect was greater for ara-C (200-fold) than for 3′-NH_2-dCyd (7-fold). The parameters that appear to correlate with this observation are the levels of activity of dCyd-metabolizing enzymes. In the murine cells that contained no dCydD and only low levels of dCMPD, the IC_{50} value for ara-C was 30 times lower than that for the amino analog (Table 1). This may have been due in part to the 5-fold lower binding affinity of ara-C for dCydK. There was little difference in the level of dCMPK between these cell types, but the presence of a 2-fold higher dCydK level coupled with a faster doubling time could have accounted for the greater sensitivity of L1210 cells to both agents.

An unexpected finding in the present study was that the effectiveness of ara-C but not of 3′-NH_2-dCyd was dependent on the number of dCydD-containing cells. In HeLa cells, the presence of high dCydD levels suggests that the 5-fold binding advantage of ara-C for dCydK is apparently offset by the 117-fold lower binding affinity of 3′-NH_2-dCyd for dCydD (Table 2). The result is that the cytotoxic effectiveness of ara-C is greatly reduced, whereas that of 3′-NH_2-dCyd is not affected by these levels of dCydD. This is exemplified in Fig. 1. In cells that did not exhibit dCydD activity (L1210), cell number had no effect on the cytotoxicity of either agent (Fig. 1 A, C). In the deaminase-containing HeLa cells, the IC_{50} value for 3′-NH_2-dCyd was about 4 μM and was independent of cell number (Fig. 1 D); under the same conditions, the IC_{50} value for ara-C varied significantly and cytotoxicity was dependent on cell number (Fig. 1 B). At low concentrations of ara-C, a 10-fold increase in cell number greatly diminished the observed cytotoxicity (Fig. 1 B). Consistent with this finding was the correlation of the extent of ara-C degradation with increasing cell number (Table 3) as well as with a reduction in the level of ara-CTP.

The clinical implications of these findings are that the total number of cells that express dCydD, both normal (i.e., liver) and neoplastic (tumor burden), may play a role in the interpatient variability and the overall response to ara-C therapy. Therefore, under conditions in which high activity of dCydD is manifest, a deaminase-resistant analog such as 3′-NH_2-dCyd may be of potential value, since the effectiveness of this compound would be independent of tissue deaminase levels.

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