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RIBO- AND DEOXYRIBONUCLEOSIDE EFFECT ON 3′-AMINO-2′,3′-DIDEOXYCYTIDINE-INDUCED CYTOTOXICITY IN CULTURED L1210 CELLS

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Abstract—3′Amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) is a potent inhibitor of the replication of cultured L1210 cells, with an IC_{50} of 1 μM. When ribo- and deoxyribonucleosides were examined for their effects on 3′-NH_2-dCyd-induced cytotoxicity, only dCyd could both prevent and reverse these effects. Furthermore, even when the maximum increase in modal cell volume was allowed to develop (24 hr) in the presence of 2.5 μM 3′-NH_2-dCyd, the addition of 25 μM dCyd to the medium containing 3′-NH_2-dCyd reduced the modal cell volume nearly to control levels within 24 hr. Examination of the viability of these cells by colony formation in soft agar, following as much as a 9.5-hr exposure of 1, 2.5 and 10 μM 3′-NH_2-dCyd, revealed that the lethal effects of the 3′-NH_2-dCyd treatment were not observed only when 25 μM dCyd was added to the medium during this time. However, the lethality of a 24-hr exposure of 2.5 and 10 μM 3′-NH_2-dCyd could not be prevented either by removal of the drug from the medium or by a 24-hr exposure of the medium containing 3′-NH_2-dCyd to 25 μM dCyd. When the effect of 3′-NH_2-dCyd on DNA biosynthesis in L1210 cells was examined, it was found that radiolabeled dAdo incorporation decreased by approximately 60, 80 or 90% following a 2.5-hr exposure to 2.5, 10 or 20 μM 3′-NH_2-dCyd respectively. The addition of 25 μM dCyd under the same conditions resulted in a greater amount of dAdo incorporation compared to the unrescued cultures. Deamination of 3′-NH_2-dCyd by partially purified human cytidine-deoxycytidine deaminase was about 2.5% that of either Cyd or dCyd deamination. The deaminated derivative, 3′-amino-2′,3′-dideoxyuridine, was significantly less cytotoxic even at 50 μM.

Ara-C has been used as a clinically effective antileukemic agent for more than a decade. The inhibitory action of ara-C appears to be S-phase specific [1], and the active metabolite, ara-CTP, exerts its action by interfering with the biosynthesis of DNA [2–5]. However, a major problem with effective ara-C therapy is the short half-life of the drug due to metabolic conversion to the inactive ara-U by cytidine-deoxycytidine deaminase [6–8].

Therefore, development of an agent which has cytotoxic properties similar to those of ara-C, but which is resistant to deamination, is being pursued by many investigators. Recently 3′-amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) was synthesized and was found to be effective *in vitro* and *in vivo* against L1210 leukemia [9]. Furthermore, the cytotoxic effects were specific for reducing [2-^{14}C]dThd, but not [2-^{14}C]Urd or [U-^{14}C]amino acid, incorporation into macromolecules. The present study presents a more detailed analysis of the cytotoxic effects of 3′-NH_2-dCyd and preliminary results of its susceptibility to enzymic deamination.

MATERIALS AND METHODS

Materials. [2-^{14}C]Cytidine (43 mCi/mmole) was purchased from the Research Products International Corp., Elk Grove Village, IL, and [8-^{14}C]deoxyadenosine (56 mCi/mmole) was purchased from Moravek Biochemicals, Inc., Brea, CA. The AG 50W-X4 (hydrogen form) cation exchange resin and CH-Sepharose 4B were obtained from Bio-Rad Laboratories, Richmond, CA, and Pharmacia Fine Chemicals, Piscataway, NJ, respectively. Thymidine-3′-(*p*-aminophenyl phosphate) was purchased from Ash Stevens, Inc., Detroit, MI, and 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide HCl, nucleosides and nucleotides were from the Sigma Chemical Co., St. Louis, MO. The details of the synthesis of 3′-NH_2-dCyd and 3′-NH_2-dUrd are described in a separate manuscript [9]. Preliminary results have been presented [10, 11]. All other chemicals were reagent grade.

Cell culture. L1210 cells were seeded at a density of 1×10^4 or 2×10^4 cells/ml in Fischer's medium supplemented with 10% non-dialyzed horse serum or 10% dialyzed horse serum (Grand Island Biological Co., Grand Island, NY) respectively. Growth was linear for 72 hr. All determinations presented are the average of triplicate samples, and all experiments were performed at least twice. Cells were found to be free of mycoplasma contamination using a biological culture method [12].

Cell volume. Modal cell volumes were determined using a Coulter Counter with a Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL) calibrated with polystyrene microspheres, 14.99 and 19.90 μm diameter. Samples were triturated before analysis, and each value is the average of three determinations.

Affinity column. Thymidine-3′-(*p*-aminophenyl phosphate) was coupled to CH-Sepharose 4B essentially as described by Kowal and Markus [13].

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Ethanolamine was used to block unreacted carboxylic acid groups on the Sepharose matrix.

Preparation of cytidine-deoxycytidine deaminase. Human KB cells (mycoplasma free) were grown using Eagle's Minimum Essential medium for suspension culture (GIBCO Cat. No. 410–1400) supplemented with 10% fetal calf serum. Cells were harvested by centrifugation at a density of 5×10^5 cells/ml and were washed twice with phosphate buffered saline. Four milliliters of extraction buffer [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 3 mM dithiothreitol (DTT)] was added to 1 g of packed cells before being frozen and thawed three times. Sonication, 1% streptomycin precipitation, and ammonium sulfate fractionation steps are similar to those described previously [14] except that the cytidine-deoxycytidine deaminase was present in the 20–50% ammonium sulfate pellet which was resuspended in 10 ml buffer [10 mM Tris–HCl (pH 7.5), 10% glycerol, 2 mM DTT] before loading onto the thymidine affinity column. Thymidylate kinase was removed according to Chen and Prusoff [15]. Cytidine-deoxycytidine deaminase was eluted from the column by increasing the ionic strength to 0.2 M Tris–HCl, pH 7.5 (containing 10% glycerol and 2 mM DTT), and the thymidine kinase isozymes were separated according to the procedure of Lee and Cheng [16]. This partially purified preparation of cytidine-deoxycytidine deaminase was used in this investigation.

Enzyme assays. Two assays were used. The first assay using [2-^{14}C]Cyd was utilized to monitor the cytidine-deoxycytidine deaminase activity during the enzyme preparation and is a modification of a previously described method [17] where Cyd was used in place of dCMP. The assay mixture in 85 μl contained: 4.25 μmoles 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.5, at 37°, 85 nmoles [2-^{14}C]Cyd (0.375 μCi/μmole), 270 nmoles $MgCl_2$, 170 nmoles DTT, 85 μg bovine serum albumin and the enzyme preparation. The reaction was terminated by the addition of 50 μl of 1.3 N perchloric acid followed by centrifugation. Supernatant fraction (100 μl) was loaded onto the AG 50W column (0.5 × 2.5 cm) equilibrated with distilled water. [2-^{14}C]Urd was eluted with 1.5 ml water followed by the addition of scintillant and counting.

Since radiolabeled 3′-NH_2-dCyd was not available, a high performance liquid chromatography (HPLC) procedure was developed to separate 3′-NH_2-dCyd from 3′-NH_2-dUrd using a Dupont 830 L.C. and BP AN 6 cation exchange resin (Benson Co. Reno, NV). The enzymic reaction conditions were the same as above except that non-radioactive compounds were used. The substrate, 3′-NH_2-dCyd, which contains two amino groups was easily separated from the one amino-containing product of the reaction (3′-NH_2-dUrd) at a flow of 1.5 ml/min at 60° using an isocratic buffer (0.3 M NH_4ClO_4, 10 mM HEPES: pH 7.7). The retention times of 3′-NH_2-dCyd and 3′-NH_2-dUrd were 12 and 7.7 min respectively. For comparison, the deamination of both Cyd and dCyd (retention times 2.9 and 3.3 min) was monitored (280 nm) by loss of the substrate peak. Deamination of 3′-NH_2-dCyd was determined by directly measuring the amount of 3′-NH_2-dUrd present and comparing that to a standard curve of known concentrations of 3′-NH_2-dUrd (see Fig. 2). To increase the sensitivity of the procedure, detection of the 3′-NH_2-dUrd peak was performed using a variable u.v. detector (Schoeffel SF 770) set at the λ_{max} for 3′-NH_2-dUrd (262 nm).

Isolation of DNA. Radiolabeled dAdo with or without 25 μM dCyd was added 2.5 hr after the addition of 3′-NH_2-dCyd, and the cultures were re-incubated at 37°. At the indicated time, cells were harvested by centrifugation, washed twice with phosphate buffered saline containing 5 mM EDTA and resuspended in a solution containing 5 mM EDTA, 50 mM NaCl and 1% sarkosyl. RNA was hydrolyzed by treating the samples with 0.3 M KOH and incubating at 37° for 18 hr. The samples were then spotted on Whatman No. 1 paper discs, washed twice with 5% trichloroacetic acid and twice with 95% ethanol. After drying, the amount of radioactivity was determined by liquid scintillation spectrometry.

RESULTS

Effect on cell growth. The effect of 3′-amino-2′,3′-dideoxycytidine (3′-NH_2-dCyd) on the replication of L1210 cells *in vitro* was investigated. As shown in Table 1, the concentration of 3′-NH_2-dCyd that inhibited the growth of L1210 cells in culture by 50% was approximately 1 μM while the deaminated derivative, 3′-amino-2′,3′-dideoxyuridine (3′-NH_2-dUrd), was significantly less inhibitory even at a concentration of 50 μM.

Since the inhibitory actions of some nucleoside analogs can be either potentiated [18–20] or mitigated [21–23] in the presence of specific naturally occurring nucleosides, we investigated the effects of various nucleosides on the inhibitory action of 3′-NH_2-dCyd. Significant levels of nucleosides are known to be present in serum which may vary with different batches [24]; therefore, dialyzed horse serum was used. Under these conditions, the IC_{50} for 3′-NH_2-dCyd using L1210 cells was approximately

Table 1. Cytotoxicity of 3′-NH_2-dCyd and 3′-NH_2-dUrd toward L1210 cells grown with or without dialyzed horse serum*

Additive (μM)	Percent of control: Non-dialyzed serum	Percent of control: Dialyzed serum
None	100	100
3′-NH_2-dCyd (0.5)	80†	90
3′-NH_2-dCyd (1)	41†	68†
3′-NH_2-dCyd (2.5)	14†	44†
3′-NH_2-dUrd (25)	79†	95
3′-NH_2-dUrd (50)	67†	92

* The amount of cell growth in the absence of additive (equated to 100) was 7.2×10^5 cells/ml and 4.0×10^5 cells/ml for non-dialyzed and dialyzed serum cultures respectively. Cells were harvested 72 hr after drug treatment. Each determination is the average of triplicate samples.

† Values were significantly different from control ($P < 0.05$).

2.5 μM, while 3'-NH_2-dUrd was not significantly inhibitory even up to a concentration of 50 μM (Table 1). The lower 3'-NH_2-dCyd-induced inhibition observed with dialyzed serum is believed to have been due to the slightly slower doubling time of these cultures (15 hr) compared to cultures grown with non-dialyzed serum (13 hr).

The effects of various ribo- and deoxyribonucleosides alone or in combination with 2.5 μM 3'-NH_2-dCyd are depicted in Table 2. To ensure that the analogue was exerting its action on cells in the exponential phase of growth, cells were allowed to recover from sub-culture for 24 hr before the nucleosides were added to the cell suspension at the same time as the drug. dThd and dGuo were the most effective nucleosides in potentiating the inhibitory action of 3'-NH_2-dCyd. This is not surprising since dThd and dGuo are known to inhibit the replication of L1210 cells in culture [25]. However, only dCyd, which by itself had no observed effect on growth, prevented completely the growth inhibition induced by 3'-NH_2-dCyd. Cyd, dAdo and Ado also showed partial protection.

Table 3 illustrates the ability of these nucleosides to protect the cells from the 3'-NH_2-dCyd-induced inhibition of cell growth when added at various times after the drug. Only dCyd prevented completely the 3'-NH_2-dCyd-induced inhibition when given 6 hr after the drug. This mitigation of 3'-NH_2-dCyd inhibition was substantial even if dCyd was added 24 hr later. That these cells recovered from the 3'-NH_2-dCyd-induced inhibition of cell replication implies that the cells, at least during the early stages of drug treatment, may have been in a static position of growth rather than having been killed by the drug. If the growth rate of these cells was slowed in the presence of the drug, then the generation time should reflect this. In the presence of 2.5 μM 3'-NH_2-dCyd, the generation time determined after 72 hr of growth was approximately 2.6-fold longer than that of untreated cells [11]. This drug-induced increased time needed for the cell population to double was reduced significantly even in the presence of dCyd that had been added 24 hr following drug treatment. Furthermore, when the generation time was determined within the first 24 hr period after dCyd rescue, a value of 20.9 hr was obtained, indicating that the drug-induced growth inhibition had been reversed significantly [11].

Effect on cell volume. The effect of 3'-NH_2-dCyd on the cell volume of L1210 cells was determined. In the presence of 2.5 μM 3'-NH_2-dCyd, the modal cell volume was increased by approximately 100% within the first 24 hr of drug treatment (Fig. 1). This

Table 2. Effects of various nucleosides on 3'-NH_2-dCyd cytotoxicity to L1210 cells *in vitro**

Nucleoside (25 μM)	Percent of control: Without 3'-NH_2-dCyd	With 3'-NH_2-dCyd
None	100†	62‡
dThd	88†	43†‡
dUrd	88†	54†‡
dCyd	98†	101†
dAdo	104†	86†
dGuo	60‡	39†‡
Urd	101†	58‡
Cyd	89†	75
Ado	97†	84†
Guo	92†	61‡

* The amount of cell growth (3.2×10^5/ml) in the absence of both 3'-NH_2-dCyd and nucleoside is equated to 100. Cultures were allowed to grow for 24 hr before nucleoside and 2.5 μM 3'-NH_2-dCyd were added at the same time. Cells were harvested 48 hr after drug treatment. Each determination is the average of triplicate samples.

† Significantly different from 3'-NH_2-dCyd addition without nucleoside ($P < 0.05$).

‡ Significantly different from untreated control ($P < 0.05$).

Table 3. Temporal effect of selected nucleosides on 3'-NH_2-dCyd-induced cytotoxicity to L1210 cells *in vitro**

Nucleoside (25 μM)	Percent of control: Hour of nucleoside addition after 3'-NH_2-dCyd† 0	3	6	24
None	29	31	28	30
Cyd	45‡	40	53‡	47‡
dCyd	97‡§	86‡§	98‡§	71‡
Ado	27	31	44‡	44‡
dAdo	50‡	48‡	56‡	45‡
dThd	20‡	20‡	19	23

* The amount of cell growth in the absence of both 3'-NH_2-dCyd and nucleosides (untreated control) was 2.8×10^5 cells/ml and is equated to 100.

† 3'-NH_2-dCyd (2.5 μM) was added at time zero and was present in all samples except the control. Cells were harvested 72 hr after drug treatment. Each determination is the average of triplicate samples.

‡ Significantly different from 3'-NH_2-dCyd addition without nucleoside ($P < 0.02$).

§ Not significantly different from untreated control ($P < 0.01$).

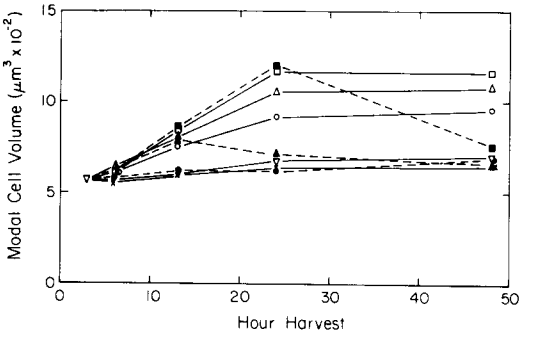

Fig. 1. Modal cell volume changes induced by 3'-NH_2-dCyd. L1210 cells (3.1×10^4 cells/ml) were grown in the absence (▽——▽) or presence of 0.5 μM (○——○), 1 μM (△——△) or 2.5 μM (□——□) 3'-NH_2-dCyd. Cultures treated with 2.5 μM 3'-NH_2-dCyd were rescued (broken lines) with 25 μM dCyd at 0 hr (●- - - -●), 8 hr (▲- - - -▲) and 24 hr (■- - -■) following the addition of 3'-NH_2-dCyd to the medium. The effect of 25 μM dCyd alone (×——×) is shown. At the indicated time, the modal cell volume was measured using a Coulter Channelyzer. Each determination represents the average of triplicate cultures.

increased cell volume was dose-dependent, and at a particular concentration of the drug no further increase was observed after 24 hr of drug exposure. dCyd, which by itself had no significant effect on cell volume, prevented completely the drug-induced cytotoxic effect when both compounds were added to the medium at the same time. Furthermore, dCyd reduced effectively this increased cell volume even when given up to 24 hr following treatment with 3′-NH_2-dCyd. Neither 25 μM Cyd, dThd, dUrd, dAdo nor dGuo prevented this drug-induced increase in cell volume even when added at the same time as the analog (not shown).

Significance was determined using Student's *t*-test ($P < 0.01$). At 0.5 μM 3′-NH_2-dCyd, no significant difference from control was observed in modal cell volume at either the 3- or 6-hr time point. At higher concentrations (1 and 2.5 μM), a significant difference was observed as early as 6 hr post-drug. In the dCyd rescue experiments using 2.5 μM 3′-NH_2-dCyd, significant rescue was observed at 24 and 48 hr when dCyd was added at 8 and 24 hr respectively. When dCyd was added at the same time as 3′-NH_2-dCyd, no increased modal cell volume was observed.

Cell viability. To determine the effect that 3′-NH_2-dCyd had on cell viability, the soft agar cloning technique was employed [26]. L1210 cells at a density of either 1.2×10^5 cells/ml or 3.1×10^4 cells/ml were treated as indicated in Table 4, and the cells were subjected to soft agar cloning in 24 or 48 hr for conditions 1 and 2 (Table 4) respectively. The prevention or reversal of 3′-NH_2-dCyd-induced cytotoxicity was initiated by the addition of dCyd to the growth medium, but dCyd was not present in the cloning medium. As can be seen, 3′-NH_2-dCyd greatly reduced the number of colonies in the absence of dCyd intervention. The addition of dCyd at the same time or 9.5 hr following 3′-NH_2-dCyd yielded a mean number of colonies that was similar to the untreated control. It should be emphasized that this effect was observed without the removal of 3′-NH_2-dCyd. When 3′-NH_2-dCyd was allowed to exert its effect for 24 hr, neither removal of the analogue from the medium nor the addition of dCyd apparently rescued these cells since the number of colonies that survived was not significantly different from those of the unrescued samples.

Effect on DNA biosynthesis. In a separate report [9], we have shown that, in the presence of 3′-NH_2-dCyd, a marked decline in incorporation of radiolabeled dThd (but not of Urd or amino acids) into acid precipitable material results. Since it is possible that the effect of 3′-NH_2-dCyd may have altered the thymidine nucleotide pools, we wanted to examine this in more detail. L1210 cells (3.5×10^5 cells/ml) were treated with various amounts of 3′-NH_2-dCyd as described (Table 5). The presence of a 2.5-hr exposure of the cells to 2.5 μM 3′-NH_2-dCyd reduced dAdo incorporation into DNA by approx-

Table 4. Effect of 3′-NH_2-dCyd on L1210 viability with and without dCyd rescue

3′-NH_2-dCyd (μM)	25 μM dCyd (hr)	No. of colonies* (± S.D.)	% Control†
Condition 1‡			
—	—	56.8 (2.5)	100
—	+ (0)	56.0 (5.2)	99
+ (2.5)	—	29.2‖ (3.9)	51
+ (10)	—	11.0‖ (2.1)	19
+ (1)	+ (0)	55.3 (6.8)	97
+ (2.5)	+ (0)	56.2 (9.6)	99
+ (10)	+ (0)	60.2 (4.4)	106
+ (1)	+ (9.5)	61.1 (6.3)	108
+ (2.5)	+ (9.5)	57.7 (4.1)	102
+ (10)	+ (9.5)	51.2 (7.0)	90
Condition 2§			
—	—	65.5 (9.5)	100
+ (2.5)	—	26.3‖ (4.6)	40
+ (10)	—	10.8‖ (3.8)	17
—	+ (24)	61.8 (7.5)	100
+ (2.5)	+ (24)	31.3‖ (6.5)	51
+ (10)	+ (24)	13.5‖ (2.4)	22

* Each determination represents the average of four soft agar cloning samples.

† The control was the non-drug-treated sample subjected to the same specific experimental conditions as that of the 3′-NH_2-dCyd-treated samples.

‡ 3′-NH_2-dCyd was added where indicated (+) without and with the addition of dCyd to the medium either at the same time or 9.5 hr following drug treatment.

§ 3′-NH_2-dCyd was added where indicated (+). In 24 hr either the 3′-NH_2-dCyd was removed by washing the cells and resuspending them in fresh drug-free medium or dCyd was added directly to the medium containing 3′-NH_2-dCyd.

‖ Significantly different from untreated control ($P < 0.005$).

Table 5. Effect of 3'-NH_2-dCyd on DNA biosynthesis in L1210 cells*

3'-NH_2-dCyd (μM)	25 μM dCyd	Deoxyadenosine incorporation† (net cpm ± S.D.) Hour of harvest after [^{14}C]dAdo 1.5	3.0	4.5
—	—	12,550 ± 70.4	23,762 ± 4,056	39,800 ± 3,555
—	+	13,273 ± 615	29,106 ± 4,204	42,703 ± 1,529
+ (2.5)	−	5,045 ± 1,518	9,620 ± 1,077	16,544 ± 931
+ (2.5)	+	7,568 ± 824	19,579 ± 1,223‡	31,929 ± 1,627‡
+ (10)	−	2,422 ± 62.2	4,344 ± 1,063	6,868 ± 720
+ (10)	+	4,689 ± 169‡	8,754 ± 768‡	23,032 ± 367‡
+ (20)	−	1,468 ± 178	2,810 ± 140	3,138 ± 361
+ (20)	+	3,448 ± 162‡	7,112 ± 518‡	15,489 ± 1,834‡

* L1210 cells (3.5×10^5/ml) were treated as described in Materials and Methods without and with various concentrations of 3'-NH_2-dCyd. [8-^{14}C]dAdo with or without 25 μM dCyd was added to the cultures 2.5 hr after the addition of 3'-NH_2-dCyd.

† Cells were harvested at the indicated times as described (see text for details). Each determination is the average of triplicate samples. Significance using Student's *t*-test was evaluated by directly comparing the effect of the presence of 25 μM dCyd vs its absence under identical conditions.

‡ Significantly different from the same condition without 25 μM dCyd ($P < 0.005$).

imately 60% (Table 5) which is the same as the percentage reported for dThd incorporation into macromolecules [9].

Deamination of 3'-NH_2-dCyd. As demonstrated in Table 1, the deaminated derivative of 3'-NH_2-dCyd, 3'-NH_2-dUrd, was not significantly cytotoxic. Furthermore, we found that L1210 cells had undetectable levels of cytidine-deoxycytidine deaminase (not shown). Therefore, it was important to investigate the potential of 3'-NH_2-dCyd to be deaminated by cytidine-deoxycytidine deaminase. The enzyme was partially purified from human KB cells as described in Materials and Methods. Since radiolabeled 3'-NH_2-dCyd was not available, an HPLC procedure for separation of the substrate and product was developed, as described in Materials and Methods. The standard curve of 3'-NH_2-dUrd (Fig. 2) was used to determine the amount of 3'-NH_2-dCyd that was deaminated by the KB enzyme. The results shown in Table 6 indicate that 3'-NH_2-dCyd was deaminated to about 2.5% that of either Cyd or dCyd under the same assay conditions.

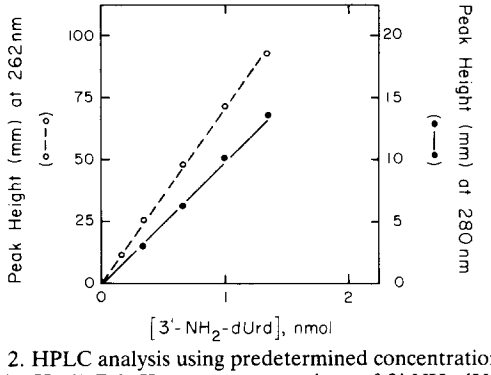

Fig. 2. HPLC analysis using predetermined concentrations of 3'-NH_2-dURd. Known concentrations of 3'-NH_2-dUrd were added in place of 3'-NH_2-dCyd to the cytidine-deoxycytidine assay mixture and the mixtures were treated under conditions identical to those with the natural substrates. The 3'-NH_2-dUrd standards were analyzed by HPLC at two different wavelengths just prior to sample (3'-NH_2-dCyd) resolution.

DISCUSSION

The cytotoxic effect of 3'-NH_2-dCyd was measured in several different ways. Inhibition of cell growth following a 48- or 72-hr drug exposure was used as a direct measure of cytotoxicity (Tables 1–3). However, when the cells were rescued with dCyd, it could not be accurately determined whether the dCyd reversed the drug-induced cytotoxicity or prevented further cytotoxicity. The cell volume analysis (Fig. 1), the cloning in soft agar (Table 4), and the effect on DNA biosynthesis (Table 5) illustrate that the cytotoxic effect observed was probably a reversible phenomenon. The generation time was decreased dramatically when dCyd was added to the 3'-NH_2-dCyd-treated cells, even when given up to 24 hr following the drug [11]. Furthermore, when the generation time was determined during the 48-hr period following dCyd rescue, a value of 15.7 hr was observed for the dCyd rescued cells, compared with 51 hr for the drug-treated cells, illustrating the dramatic reversing effect of dCyd (not shown).

A similar rescuing effect by dCyd was observed in the augmentation by 3'-NH_2-dCyd of the modal cell volume (Fig. 1). When dCyd was given 24 hr following drug exposure, the modal cell volume in

Table 6. Rate of deamination of various cytosine nucleosides by cytidine-deoxycytidine deaminase derived from human KB cells

Substrate (1 mM)	Product formed (nmoles/60 min)
Cyd	37 ± 0.14*
dCyd	36 ± 1.7*
3'-NH_2-dCyd	0.86 ± 0.018†

* The amount of substrate deaminated was estimated, using HPLC, by measuring the decrease in the height of the substrate peak and comparing it to that of known concentrations. The values determined were in good agreement with the assay procedure employing [2-^{14}C]Cyd.

† The amount of product formed was determined, using HPLC, by comparing the height of the 3'-NH_2-dUrd peak to the standard curve (Fig. 2).

the first 24-hr period was decreased from 1196 to 765 μ^3, a value very near to that of the control cells.

The survival of these cells following treatment with 3′-NH_2-dCyd reveals that dCyd both prevented and reversed the lethal effects of 3′-NH_2-dCyd when given up to 9.5 hr after the analogue. Even at this stage of treatment, cytotoxic effects were observed, as evidenced by (1) the slight but significant increase in cell size (Fig. 1), (2) the apparent perturbation of deoxynucleoside metabolism and (3) possible DNA biosynthesis as suggested by a marked decline in radiolabeled dThd incorporation into acid precipitable material following a 2.5-hr exposure of 2.5 μM 3′-NH_2-dCyd to cultured L1210 cells [9] (under the same conditions, no effect was observed on radiolabeled Urd or amino acid incorporation). Further support for an effect on DNA biosynthesis was demonstrated by the marked decrease in radiolabeled dAdo incorporation into DNA (Table 5).

In the present investigation, more incorporation occurred after the addition of dCyd to the 3′-NH_2-dCyd-treated cells (without removal of 3′-NH_2-dCyd), suggesting that the initial perturbation of decreased dAdo incorporation may have been either partially reversed or that further damage was prevented. Since the effects of 3′-NH_2-dCyd were observed even after a few hours of exposure (Fig. 1 and Table 5) and since no loss of cell viability was observed during this time (Table 4), any damage that occurred was either repaired or not lethal. Thus, dCyd not only prevented but also reversed these 3′-NH_2-dCyd-induced cytotoxic effects.

That this rescuing process is time-dependent is suggested from the L1210 cloning results (Table 4). Attempts to rescue cells after a 24-hr exposure to 2.5 or 10 μM 3′-NH_2-dCyd were unsuccessful. Higher concentrations of dCyd, different exposure and rescue schedules, or the presence of dCyd in the cloning medium may create a more favorable environment for an increase in the number of surviving colonies. A more detailed analysis of the precise rescuing conditions is under investigation.

The action of the anti-leukemic agent ara-C is also mitigated by dCyd [1, 23]. Using intact myeloblasts from untreated AML patients, Harris and Grahame-Smith [23] found that 1 μM dCyd decreased the DNA-inhibiting effects of ara-C by inhibition of ara-CTP production. The short plasma half-life (approximately 5 min) of ara-C in AML patients is believed to be due to metabolic inactivation by deamination [27]. These authors also point out that direct inhibition of the deaminase may have adverse effects on ara-C therapy since the plasma level of the competing dCyd could then increase [27]. Therefore, an effective agent which is resistant to deamination may be of clinical value since therapeutic plasma levels may be maintained more easily.

The preliminary results of this investigation show that 3′-NH_2-dCyd is not a good substrate for the KB cytidine-deoxycytidine deaminase (Table 6) under conditions where both Cyd and dCyd are readily deaminated. Since our HPLC procedure was at the limits of sensitivity, kinetic values could not be determined accurately. To overcome this problem, work is in progress, to prepare radiolabeled 3′-NH_2-dCyd.

Although mouse tissues have appreciable levels of the nucleoside deaminase [28], metabolic inactivation by deamination was not a significant problem since 3′-NH_2-dCyd produced a favorable T/C in mice bearing L1210 leukemia while 3′-NH_2-dUrd at the same dosage was not effective [9].

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