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The effect of ara-C-induced inhibition of DNA synthesis on its cellular pharmacology*

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Summary. The cytotoxicity of ara-C is believed to result from incorporation of ara-CTP into DNA and inhibition of DNA synthesis. Since complete inhibition of DNA synthesis would prevent further incorporation of ara-CTP, ara-C may have a self-limiting effect on its own cytotoxicity, particularly at the high concentrations typical of high-dose ara-C clinical protocols. In this study, the incorporation of [^{3}H]-dThd and [^{3}H]-ara-C into DNA were compared. Within 1 h of exposure of L5178Y cells to ara-C, the rate of [^{3}H]-dThd incorporation into the acid-insoluble fraction was reduced by 98%. Despite this nearly complete block in [^{3}H]-dThd incorporation, DNA synthesis was not completely inhibited since [^{3}H]-ara-C continued to be incorporated for up to 6 h, although a plateau in ara-CDNA synthesis was observed between 2 and 3 h exposure when ara-CTP levels were maximal. The effect of ara-C on [^{3}H]-dThd incorporation into DNA was due in part to an indirect effect of ara-C on the metabolism of intracellular [^{3}H]-dThd to [^{3}H]-dTTP. Within 30 min exposure to 10 μM ara-C, the rate of cellular [^{3}H]-dTTP synthesis was slowed to only 15% of the control rate. This was not due to inhibition of [^{3}H]-dThd transport, since the intracellular and extracellular concentrations of the nucleoside were equal. The effect of ara-C on [^{3}H]-dTTP synthesis resulted from significant changes in deoxynucleoside 5'-triphosphate (dNTP) pools. dTTP, dATP, and dGTP levels were increased, whereas the dCTP concentration was decreased. When dThd kinase from L5178Y cells was assayed with increased dTTP levels induced by ara-C vs the dTTP level in control cells, its activity was reduced by 72%. Thus, the [^{3}H]-dThd incorporation experiment overestimated the extent of inhibition of DNA synthesis by ara-C due to increased feedback inhibition of dThd kinase and increased competition for DNA polymerase between the elevated unlabeled dTTP pool and the decreased levels of [^{3}H]-dTTP. In vitro assay of DNA polymerase in the presence of the ara-CTP concentration achieved after 0.5 or 3 h exposure to 10 μM ara-C (60 μM and 200 μM, respectively), plus the mixture of dNTPs found intracellularly at these times, resulted in 57% and 80% inhibition of the polymerase, respectively. This inhibition may account for the plateau in the accumulation of ara-CDNA that was observed at 3 h and suggests that ara-C incorporation may be self-limiting at high cellular concentrations of ara-CTP. The ara-C-induced decline in dCTP noted above was apparently a secondary effect resulting from the inhibition of ribonucleotide reductase by the elevated dTTP and dATP. CDP reductase activity in the presence of dATP and dTTP at the concentrations found in ara-C-treated cells was 58% of the activity observed in the presence of nucleotide levels found in control cells. The decrease in dCTP levels was associated with a reciprocal increase in the rate of [^{3}H]-ara-C phosphorylation following subsequent exposure to unlabeled ara-C. Thus, ara-C self-potentiated its own uptake in these cells. These observations of the self-limiting and self-potentiating effects of high concentrations of ara-C may be relevant to the selection of the optimal dose and the duration of exposure in the clinical use of high-dose ara-C infusions.

Introduction

Cytosine arabinoside (ara-C) is currently the most effective drug for the treatment of acute myeloid leukemia [6]. The predominant metabolite of ara-C, ara-CTP, is a substrate for DNA polymerase and is incorporated into DNA (ara-CDNA). This incorporation, which correlates strongly with cytotoxicity [13, 16, 17], results in inhibition of DNA synthesis due to the chain-terminating effect of ara-CDNA [14, 15].

Inhibition of [^{3}H]-dThd incorporation into DNA has been a standard method for assessing the action of ara-C on leukemia cells [10, 21], and the pattern of inhibition and recovery of [^{3}H]-dThd incorporation in tumor cells after treatment with ara-C may be a useful guide for drug scheduling [10]. Protracted exposure beyond the point of maximal inhibition of DNA synthesis by ara-C could impose a self-limitation, since complete inhibition of DNA synthesis would prevent the incorporation of additional ara-C into DNA.

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Abbreviations: ara-C, 1-β-D-arabinofuranosyl cytosine (cytosine arabinoside); ara-CTP, ara-C triphosphate; NTP, unspecified nucleoside 5'-triphosphate; dNTP, deoxynucleoside 5'-triphosphate; PBS, phosphate-buffered saline

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The intracellular regulation of ara-C metabolism to ara-CTP is complex. In murine leukemia cells, which have very high transport rates [26], the rate-determining step for net accumulation of ara-CTP is the initial phosphorylation to ara-CMP catalyzed by deoxycytidine kinase. The activity of this enzyme is regulated in part by the level of dCTP [8], which acts as a feedback inhibitor; however, other nucleotides are also known to modulate enzyme activity [8, 25]. Inhibition of DNA synthesis by ara-CTP could potentially result in the alteration of dNTP pools, and this could affect the cellular regulation of ara-C anabolism. The present study explores alterations in cellular biochemistry as influenced by the duration of exposure to optimal concentrations of ara-C. Preliminary results have been presented [24].

Materials and methods

Chemicals. Ara-C was provided by the Upjohn Co. (Kalamazoo, Mich). [^{3}H]-Ara-C was purchased from Amersam International Ltd, and [^{3}H]-dThd, [^{3}H]-dTTP, and [^{14}C]-CDP were obtained from New England Nuclear. Dow corning silicone oils 550 and 200 (William F. Nye, Inc., New Bedford, Mass) were blended 84 : 16 to give a density of 1.03. Activated calf thymus DNA, Sephadex A25, and other chemicals were purchased from Sigma Chemical (St. Louis, Mo).

Cell culture and clonogenic assay. The methods for propagation of murine leukemia L5178Y in cell culture and measurement of cell viability by clonogenic assay have previously been described [2]. All studies were carried out with cells in exponential growth.

Rate of [^{3}H]-dThd metabolism to [^{3}H]-dTTP. L5178Y cells were resuspended in Fischer's medium plus 10% horse serum at a density of 3×10^6 cells/ml and incubated at 37° C. At intervals after the addition of 10 μ*M* ara-C, [^{3}H]-dThd was added to a final concentration of 0.14 μ*M* (1 μCi/ml). After incubation with radiolabel for 6 min, 150 μl cell suspension was removed and transferred to a 400-μl microcentrifuge tube containing 50 μl 10% trichloroacetic acid (TCA) overlaid with 100 μl silicone oil. Uptake was terminated by centrifugation at 12,000 *g* for 30 s. The media and oil layers were aspirated, and a 25-μl sample of the TCA layer was applied to a small column (0.5-ml bed volume) of Sephadex A25 in a transfer pipet. The column was washed with 2 ml water to elute unchanged intracellular [^{3}H]-dThd into a scintillation vial, and then with 3 ml 1 *M* ammonium acetate to elute phosphorylated metabolites. Other cell samples were incubated with either $[^3H]_2O$ or [^{14}C]-sucrose and processed similarly, except that a 25-μl sample of the TCA layer was counted directly to determine the total water and the extracellular volume in the sample, respectively.

Rate of net cellular uptake of [^{3}H]-ara-C. The procedure was similar to that described above for [^{3}H]-dThd uptake. At intervals after the addition of unlabeled ara-C, [^{3}H]-ara-C, [^{3}H]-ara-C was added. Samples (150 μl) were removed at 2-s intervals for 12 s and centrifuged through 100 μl silicone oil to separate the cells from the media. After aspiration of the medium and oil layers, the tubes were cut and the tips containing the cell pellets were transferred to 7-ml scintillation vials, where they were digested with 0.5 ml 0.9 *M* NaOH at 60° C until completely dissolved. The digest was neutralized with 0.25 ml 2 *M* HCl and counted in 4 ml scintillation fluid.

Accumulation of ara-CTP and incorporation of [^{3}H]-ara-C or [^{3}H]-dThd into the cold acid-insoluble fraction. Cells were incubated with 10 μ*M* [^{3}H]-ara-C (10 μCi/ml) or 0.14 μ*M* [^{3}H]-dThd (1 μCi/ml) for the period indicated. Samples of the cell suspension (100 μl) were centrifuged at 12,000 *g* through 100 μl oil into 100 μl 10% TCA. After aspiration of the medium and oil layers, the acid layer was transferred to a clean 400-μl tube and mixed with 150 μl 0.5 *M* trioctylamine in freon to remove the TCA and to neutralize the extract [12]. The neutralized acid-soluble extract was analyzed for ara-CTP as previously described [27]. The acid-insoluble pellet was resuspended three times in 1 ml 10% TCA and finally dissolved in 100 μl dimethylsulfoxide and counted. Prior studies have shown that ara-C is incorporated exclusively into DNA [13, 16, 17, 27].

Effect of ara-C on cellular NTP and dNTP pools. A 200-ml suspension of L5178Y cells at 4×10^5/ml was treated with 10 μ*M* ara-C. The cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline [0.14 *M* NaCl, 4 m*M* KCl, 0.2 *M* potassium phosphate (pH 7.2)], and extracted with 100 μl 10% TCA. The TCA was removed by extraction with 150 μl 0.5 *M* trioctylamine in freon as above. The procedure from initial centrifugation to neutralization was completed within 6 min to minimize breakdown of nucleotides in the acid solution. [^{14}C]-Sucrose was added to the TCA to enable a correction to be made for dilution of the TCA by the water associated with the cell pellet. Radiolabel in aliquots of the neutralized extracts was compared with that in the acid solution. NTPs were analyzed directly by HPLC [19]. dNTPs were analyzed by HPLC after periodate oxidation of ribonucleotides [19].

DNA polymerase. A crude enzyme preparation was prepared by a modification of the method of Scolnick et al. [22]. L5178Y cells (175×10^6) were harvested and washed in saline, then resuspended in 1.8 ml 50 m*M* TRIS-HCl (pH 7.8), 1 m*M* $MgCl_2$, 1 m*M* dithiothreitol. Cells were lysed by three cycles of freezing and thawing, after which 2 ml saturated $(NH_4)_2SO_4$ containing 50 m*M* TRIS-HCl (pH 7.8) and 0.1 m*M* EDTA was added and the mixture was homogenized. After centrifugation at 15,000 *g* for 1 h at 2° C, the pellet was suspended in 2 ml 50 m*M* TRIS-HCl (pH 7.8), 50 m*M* KCl, 1 m*M* dithiothreitol. Triton X-100 was added to 0.3% and, after incubation for 15 min at 37° C, the suspension was centrifuged at 15,000 *g* for 1 h. The supernatant was dialyzed against the same buffer, then mixed with an equal volume of glycerol before storage at −20° C. The DNA polymerase kinetic assay was carried out as previously described by Hubscher et al. [7], except that the concentrations of dATP and dGTP were 100 μ*M* and that of [^{3}H]-dTTP was 5 μ*M*. Activated calf thymus DNA from Sigma was used as the template. The apparent K_m for dCTP and the K_i for ara-CTP were determined from a Lineweaver-Burk plot. These determinations were repeated three times; the results are expressed as meanst ± SD.

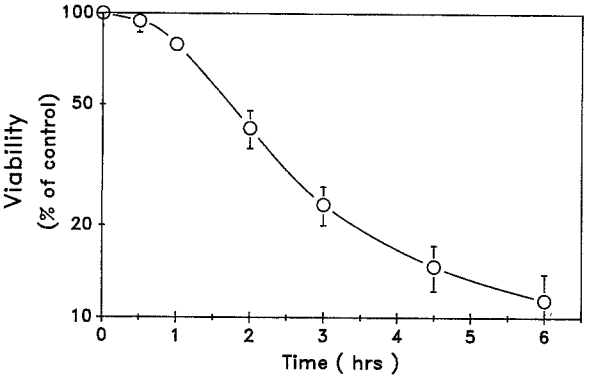

Fig. 1. Effect of exposure time to 10 μ*M* ara-C on the viability of L5178Y cells. Control cloning efficiency = 60%±5%

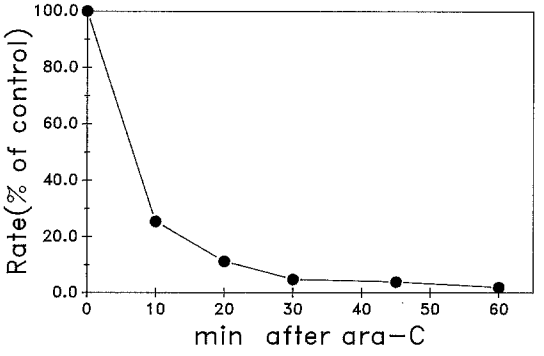

Fig. 2. Effect of ara-C on the rate of incorporation of [^{3}H]-dThd into DNA. L5178Y cells were incubated in growth medium. At the intervals after the addition of 10 μ*M* ara-C indicated on the abscissa, 0.14 μ*M* [^{3}H]-dThd was added to the cell suspension and samples were removed at 5-min intervals for 30 min and processed to determine radiolabel in the acid-insoluble fraction. The rate of incorporation was calculated from the slope. The control rate of [^{3}H]-dThd incorporation was 0.37 pmol/min per 10^6 cells

dThd Kinase. A crude extract was prepared from L5178Y cells and assayed for dThd kinase activity as previously described be Lee and Cheng [11].

CDP reductase. L5178Y cells (700×10^6) were harvested by centrifugation, washed with cold medium without serum, and suspended in 4 ml 50 m*M* TRIS-HCl (pH 8.0), 1 m*M* mercaptoethanol, 2 m*M* $MgCl_2$. The suspension was homogenized by ten strokes of a Teflon pestle in a Potter-Elvehjem homogenizer and centrifuged at 27,000 *g* for 20 min. The supernatant was treated with 4 mg streptomycin sulfate/ml, and the precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to 40% saturation (0.226 g/ml), and the precipitate was redissolved in 1 ml 50 m*M* TRIS-HCl (pH 7.4), 1 m*M* dithiothreitol. This enzyme solution was dialyzed overnight against the same buffer, then stored at −20° C. CDP reductase was assayed by the method of Steeper and Stuart [23].

Results

The curve depicting the relationship between the duration of exposure of L5178Y cells to 10 μ*M* ara-C and loss of viability was sigmoidal (Fig. 1). Although there was minimal loss of viability after only 0.5–1 h exposure, viability

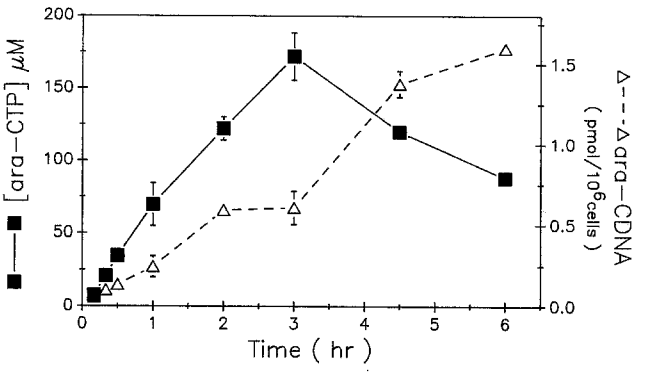

Fig. 3. Net accumulation of ara-CTP and ara-CDNA. At intervals after exposure to 10 μ*M* [^{3}H]-ara-C, [^{3}H]-ara-CTP levels were determined in the acid-soluble fraction. [^{3}H]-Ara-CDNA was taken as being equal to the amount of [^{3}H] in the acid-insoluble fraction. (Bars represent the standard deviation for 3 separate experiments. Where bars are absent, the deviation was smaller than the symbol size.)

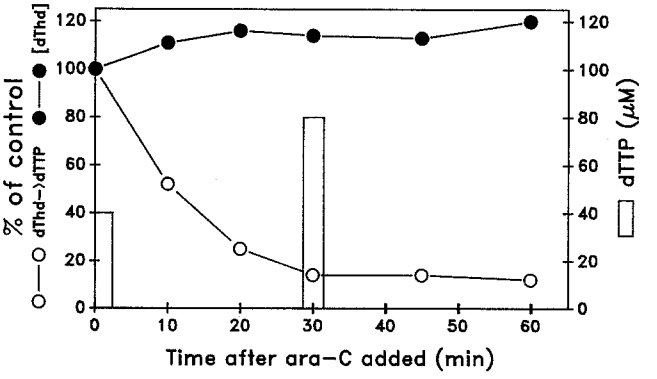

Fig. 4. Effect of ara-C on the rat of cellular metabolism of [3H]-dThd. L5178Y cells were incubated with 10 μ*M* ara-C for the intervals indicated on the abscissa, then [^{3}H]-dThd was added at 0.14 μM. After 6 min, the cells were centrifuged through oil into trichloroacetic acid. The extract was separated into the uncharged fraction (i. e., intracellular [^{3}H]-dThd) and the negatively charged fraction (i. e., dTMP + dTDP + dTTP), the majority of which was dTTP. The control rate of metabolism was 0.56 pmol/min per 10^6 cells; the control intracellular [^{3}H]-dThd concentration was 0.15 μM. The bars at 0 and 30 min indicate the levels of dTTP determined in a separate but similar experiment (from Table 1)

declined sharply between 1 and 3 h, with a tapering off after 4 h exposure to ara-C.

The initial delay in achieving cytotoxicity upon the addition of ara-C could be due to either the time required to accumulate cytotoxic levels of ara-CDNA or the time required to achieve sufficient intracellular ara-CTP to inhibit DNA synthesis. To test the ara-C effect on DNA synthesis, cells were pulsed with [^{3}H]-dThd at intervals after the addition of ara-C (Fig. 2). The rate of [^{3}H]-dThd incorporation into DNA was inhibited by 75% within 10 min after exposure to 10 μ*M* ara-C and by >98% within 1 h. This nearly complete cessation of [^{3}H]-dThd incorporation into DNA would suggest that ara-CTP incorporation into DNA should also be blocked; however, as shown in Fig. 3, [^{3}H]-ara-C continued to be incorporated into DNA for at least 6 h. Very high ara-CTP concentrations did appear to

Table 1. Effect of ara-C on deoxynucleoside triphosphate

	dCTP	dTTP	dATP	dGTP	ara-CTP
Control	57.9 ±9.6	42.2 ± 4.9	20.0 ±2.4	7.5 ±1.1	–
0.5 h	29.8 ±9.1 (51%)	78.5 ±16.3 (191%)	38.1 ±8.1 (190.5)	13.3 ±2.6 (176%)	56.9 ±10.4
3.0 h	23.2 ±3.2 (40%)	68.8 ±10.6 (163%)	49.3 ±3.3 (246%)	10.3 ±1.1 (137%)	196.2 ±20

L5178Y cells were treated with 10 μ*M* ara-C for the indicated times and samples were analyzed for deoxyribonucleotides as described in Materials and methods. The data is expressed as μmol/l cell H_2O (±SD) for 3 separate experiments; each sample was analyzed in duplicate (percentage of control)

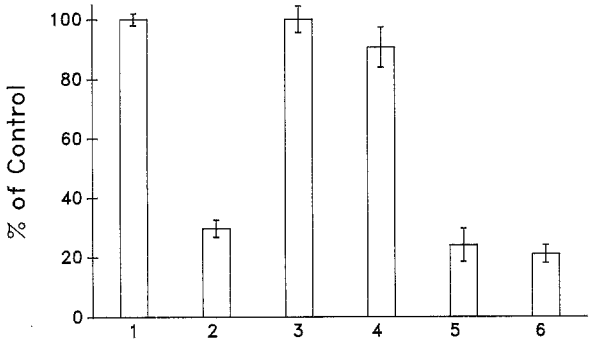

Fig. 5. Effect of altered dTTP levels on dThd kinase activity. L5178Y dThd kinase activity was assayed with dTTP and/or ara-CTP as follows: 1, 40 μ*M* dTTP; 2, 80 μ*M* dTTP; 3, 60 μ*M* ara-CTP; 4, 200 μ*M* ara-CTP; 5, 80 μ*M* dTTP + 60 μ*M* ara-CTP; 6, 80 μ*M* dTTP + 200 μ*M* ara-CTP. The dTTP and ara-CTP levels correspond to those observed in control cells *(bar 1)* or in cells treated with ara-C for 30 min *(bar 5)* or 3 h *(bar 6)* (see Table 1)

block further ara-C incorporation into DNA, since a reproducible plateau was observed in each of three experiments at between 2 and 3 h, when ara-CTP levels were maximal.

The continued incorporation of [^{3}H]-ara-C into DNA despite nearly complete inhibition of the incorporation of [^{3}H]-dThd suggests that the latter assay overestimated the degree of inhibition of DNA synthesis. This reaised the possibility that the apparent ara-C-induced inhibition of [^{3}H]-dThd into DNA occurred by other mechanisms. Figure 4 illustrates the effect of pretreatment with ara-C for 10–60 min on the rate of [^{3}H]-dThd metabolism to phosphorylated metabolites. After only 30 min exposure to 10 μ*M* ara-C, this rate was only 15% of the control value. Thus, a significant portion of the potent effect of ara-C on [^{3}H]-dThd incorporation into DNA appears to be due to inhibition of [^{3}H]-dThd anabolism. This slowing of [^{3}H]-dThd metabolism by ara-C was not due to inhibition of transport by their common membrane carrier, since the concentration of unchanged intracellular [^{3}H]-dThd was the same as the extracellular concentration. L5178Y cells have relatively high nucleoside transport rates [26], and these data indicate that transport of [^{3}H]-dThd must be rapid in comparison with its metabolism to phosphorylated derivatives.

Table 1 depicts the effect of exposure of L5178Y cells to 10 μ*M* ara-C on intracellular dNTP pools. At 30 min after ara-C exposure, dTTP, dATP, and dGTP were substantially increased, presumably due to their accumulation behind the partial block in DNA synthesis by ara-C. In contrast, the dCTP concentration was reduced to about

Table 2. Effect of ara-CTP on DNA polymerase activity

	Rate22 (pmol/min per mg)	% Control	% Expected[a]
Control	52.4 ±4.6	100	
60 μ*M* ara-CTP	22.2 ±6.8	42.7	43.1
200 μ*M* ara-CTP	8.7 ±4.3	16.6	19.8

The incorporation of [^{3}H]-dTTP into DNA by DNA polymerase partially purified from L5178Y cells was measured with 30 μ*M* dCTP, the level observed in cells exposed to ara-C for 30 min. The assay was carried out in the absence of ara-CTP (control) or with either 60 μ*M* ara-CTP, the approximate cellular level after 30 min exposure to 10 μ*M* ara-C, or 200 μ*M* ara-CTP, the approximate level after 3 h (see Table 1)

[a] The "expected" inhibition was calculated from the experimentally determined K_m of 2.1 for dCTP and K_i of 3.6 for ara-CTP;

$$\% \text{ expected} = 100 \times \frac{\dfrac{V_{max} \times [dCTP]}{[dCTP] + K_m (1 + [\text{ara-CTP}]/K_i)}}{\dfrac{V_{max} \times [dCTP]}{[dCTP] + K_m}}$$

50% of the control value. NTPs remained within 20% of control levels. The data in Fig. 5 suggest that this increase in cellular dTTP levels would result in increased feedback inhibition of dThd kinase as reported by Ives et al. [9]. dThd kinase activity was determined in the presence of 40 or 80 μ*M* dTTP, the respective dTTP concentrations found in either control cells or cells exposed to ara-C for 30 min. Kinase activity in the presence of 80 μ*M* dTTP was only 28% of that found in the presence of 40 μ*M* dTTP. The cellular level of ara-CTP achieved after a 30 minute incubation with 10 μ*M* ara-C (60 μ*M* ara-CTP) had no effect on the dThd kinase assay. The effect of the increased dTTP on dThd kinase activity agrees with the data in Fig. 4, which showed a reduction in the cellular metabolism of [^{3}H]-dThd to [^{3}H]dTTP to 15% of control values after 30 min exposure to ara-C. It should be noted that the increase in the unlabeled pool of dTTP combined with the decreased synthesis of [^{3}H]-dTTP would result in a lower specific activity for [^{3}H]-dTTP. The combined effects would result in greatly reduced incorporation of radiolabel into DNA; thus, the [^{3}H]-dThd incorporation assay overestimates the degree of inhibition of DNA synthesis by ara-C.

It should be possible to predict more accurately the true degree of inhibition of DNA synthesis by ara-CTP from a knowledge of intracellular concentrations of ara-CTP and dCTP and the kinetic properties of DNA polymerase. An apparent K_m of 2.1 ±0.1 μ*M* for dCTP and a K_i of 3.6 ±0.8 μ*M* for ara-CTP were determined using

Table 3. Effect of ara-C on CDP reductase

	Rate (nmol/30 min per mg)	% Control mix
Control mix[a]	2.73 ±0.14	100
"ara-C" mix[b]	1.59 ±0.06	58
No dCTP	1.65	60
No dTTP	2.16	79
No dATP	1.96	72
No dGTP	1.52	56
No ara-CTP	1.67	61

CDP reductase from L5178Y cells was assayed in the presence of a mixture of deoxyribonucleotides at levels close to their intracellular concentrations in control cells (Control mix) or in cells exposed to 10 μ*M* ara-C for 3 h ("ara-C" mix) (Table 1). Individual nucleotides were deleted on at a time from the "ara-C" mix to determine which nucleotides contributed to the observed effect

[a] Control mix = 50 μ*M* dCTP, 45 μ*M* dTTP, 20 μ*M* dATP, and 27 μ*M* dGTP

[b] "Ara-C" mix = 27 μ*M* dCTP, 80 μ*M* dTTP, 50 μ*M* dATP, 12 μ*M* dGTP, and 200 μ*M* ara-CTP

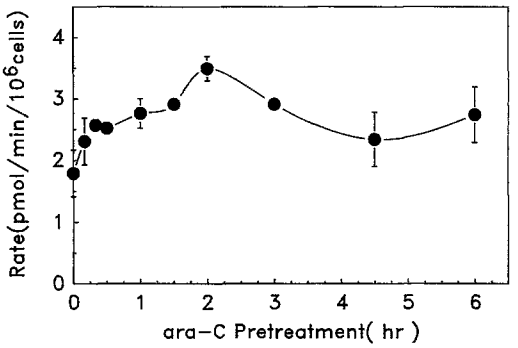

Fig. 6. Effect of preincubation with unlabeled ara-C on the rate of cellular uptake of [^{3}H]-ara-C. Cells were incubated with 10 μM ara-C for the intervals shown on the abscissa, then pulsed for 10 min with [^{3}H]-ara-C. Samples were taken at 2-min intervals and the rate was determined from the slope

DNA polymerase from L5178Y cells and [^{3}H]-dTTP as the substrate as described in Materials and methods. Table 2 illustrates the effect of ara-CTP on DNA polymerase activity when measured at the concentrations of dCTP and ara-CTP found in L5178Y cells after 30 min or 3 h. In this cell-free assay, [^{3}H]-dTTP incorporation was inhibited by 57% by 60 μ*M* ara-CTP, the level obtained after 30 min. This observation agrees closely with the predicted inhibition of 54% that may be calculated from the above kinetic constants, but it is in contrast to the observed 95% inhibition of [^{3}H]-dThd incorporation by intact cells that had been exposed to 10 μ*M* ara-C for 30 min (Fig. 2). It may be calculated from the apparent dCTP K_m and the ara-CTP K_i that 200 μ*M* ara-CTP, the level achieved in L5178Y cells after 3 h exposure to 10 μ*M* ara-C (Table 1), should result in 80% inhibition of dTTP incorporation into DNA in the cell-free assay. This nearly complete inhibition of DNA synthesis by very high levels of ara-CTP is consistent with the observed plateau in [^{3}H]-ara-CDNA that coincided with maximal ara-CTP levels (Fig. 3).

The increase in dTTP, dATP and dGTP following exposure to ara-C is consistent with the partial inhibition of DNA polymerase. The decrease in dCTP may be a more indirect effect that is mediated by the action of the increased dTTP and dATP on CDP reduction [5]. To examine this hypothesis, ribonucleotide reductase from L5178Y cells was assayed in the presence of dCTP, dTTP, dATP, and dGTP at their approximate cellular concentrations in control cells or in the presence of these dNTPs plus ara-CTP at the levels found in cells after treatment with ara-C (Table 3). The rate of CDP reduction in the presence of a nucleotide mixture characteristic of ara-C-treated cells was only 58% of the rate using the dNTP concentrations found in untreated cells. The most significant nucleotides in the mix were dTTP and dATP, since deleting either of these individually resulted in an increase in CDP reductase activity. Deleting ara-CTP, dCTP, or dGTP from the dNTP mix had no effect. Thus, the probable basis for the decline in dCTP pools following the addition of ara-C is inhibition of ribonucleotide reductase by dATP and dTTP that accumulate behind the partial block of DNA polymerase activity.

Since dCTP is a potent inhibitor of the phosphorylation of ara-C to ara-CMP by dCyd kinase, any reduction of cellular dCTP pools should result in an increased rate of cellular uptake of ara-C. Figure 6 shows the effect of preincubation of L5178Y cells with 10 μ*M* unlabeled ara-C on the initial rate of uptake of [^{3}H]-ara-C added at later time points. An enhanced rate of uptake of [^{3}H]-ara-C was seen within 20 min of pretreatment with unlabeled ara-C and was increased to >2-fold over that in control cells after 2 h pretreatment. Thus, ara-C self-potentiated its own uptake. In brief, rapid partial inhibition of DNA synthesis resulted in the cellular accumulation of dTTP and dATP. These nucleotides caused feedback inhibition of ribonucleotide reductase, thus slowing the synthesis of dCTP. The lower dCTP pools enabled the increased ara-C anabolism to ara-CTP.

Discussion

Despite its critical importance in the treatment of acute myeloid leukemia (AML) and extensive laboratory and clinical investigation, the optimal dose and duration of exposure to ara-C in clinical practice remain to be defined. This goal can only be achieved by concurrent correlations of systemic and cellular pharmacokinetic parameters with pharmacodynamic effects such as inhibition of DNA synthesis and loss of cell viability. It is well established that cytotoxicity correlates closely with the extent of ara-C incorporation into DNA [13, 16, 17]. This incorporation has been associated with inhibition of DNA synthesis resulting from the inhibition of DNA chain elongation [14, 15]. However, once DNA synthesis is maximally inhibited, further incorporation of ara-CTP into DNA and, hence, further cytotoxicity may be compromised. Thus, the action of ara-C may be self-limiting.

These competing biochemical effects are relevant to the clinical problem of selecting the optimal dose and duration of drug exposure. Continuous infusion of standard doses of ara-C (100–200 mg/m^2) over 5–7 days, which generates steady-state plasma levels of ara-C in the range of 0.2–0.4 μ*M* [4], produces complete remissions in

approximately 25% of patients [6]. We have shown that for most leukemia cells taken from patients with AML, the rate-determining step for the cellular accumulation of ara-CTP at these ara-C concentrations is transport across the cell membrane [26]. At extracellular concentrations above 1 μM, transport capacity is in excess [26]. This may be part of the basis for the observation that apparent clinical resistance in patients with AML may frequently be overcome by a 15- to 30-fold dose escalation (high-dose ara-C), which generates plasma levels of 50–100 μM [1, 3]. Since systemic catabolism of ara-C is rapid [1], the duration of infusion (i.e., the period for which these higher extracellular levels are sustained) will have a critical bearing on outcome. To date, high-dose infusions have been given empirically for 1–4 h [1]. In contrast to infusions of standard-dose ara-C that are commonly given over a 5- to 7-day period [6], high-dose infusions must be limited to relatively shorter intervals, because prolonged exposure to high systemic ara-C concentrations results in severe multiorgan toxicity [1, 18]. The question remains as to what the optimal duration of high-dose infusion should be and how it should be determined.

The rate of [^{3}H]-dThd incorporation into DNA has been a convenient assay for monitoring the action of ara-C on intact cells [10, 21], but the present data indicate that this assay greatly overestimates the true inhibition of DNA synthesis. Within 30 min after exposure to 10 μM ara-C, the intracellular dTTP and dATP levels were almost doubled. This increase in dTTP presumably was sufficient to inhibit dThd kinase and to slow the anabolism of [^{3}H]-dThd to [^{3}H]-dTTP. Inhibition of [^{3}H]-dThd transport by ara-C was not a factor, since transport capacity was sufficient to maintain intracellular unchanged [^{3}H]-dThd at a level close to the extracellular concentration.

The decreased synthesis of [^{3}H]-dTTP plus the dilution of radiolabel by the increased intracellular pool of unlabeled dTTP resulted in nearly complete inhibition of [^{3}H]-dThd incorporation into DNA. However, [^{3}H]-dTTP incorporation in a cell-free DNA synthesis assay in the presence of 60 μM ara-CTP, the level achieved in cells within 30 min after exposure to ara-C, was inhibited by only 57%. Thus, although 10 μM ara-C caused partial inhibition of DNA synthesis almost immediately, [^{3}H]-ara-C continued to be incorporated into DNA, even when [^{3}H]-dThd incorporation was completely inhibited. The peak ara-CTP level of 200 μM would be sufficient to inhibit DNA synthesis by 80%, as calculated from the apparent K_m and K_i for DNA polymerase. This is presumably the reason why there was no further increase in [^{3}H]-ara-CDNA between 2 and 3 h, when the cellular concentration of ara-CTP was maximal. Later, when ara-CTP levels declined, [^{3}H]-ara-CDNA synthesis increased once again. The basis for the observed decline in ara-CTP levels after 3 h is unexplained and is under investigation. These data, which suggest a self-limiting effect of high concentrations of ara-CTP, are consistent with laboratory investigations with human leukemia cells, which show that a 6-h exposure to 50 μM ara-C is equivalent to the effect of a 3-h exposure [20].

The above observations have bearing on the design of clinical trials using ara-C. The common use of [^{3}H]-dThd incorporation into DNA is not an accurate indicator of drug effect. More precise estimates can probably be obtained by computer simulation using kinetic constants and measured extracellular and intracellular concentrations of the drug and its metabolites. Such efforts are currently under way in our laboratories.

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