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Cytosine Arabinoside Triphosphate Production in Human Leukaemic Myeloblasts: Interactions with Deoxycytidine

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Summary. *The effect of 1 μM deoxycytidine (dC) on Ara-C conversion to Ara-CTP and on inhibition of DNA synthesis by Ara-C was measured in intact leukaemic myeloblasts. dC decreased Ara-CTP production in blasts with high Ara-C phosphorylation, but not those with low activity. The Ki for dC was similar to values found with partially purified deoxycytidine kinase. The change in Ara-CTP concentration was associated with a proportional reduction in inhibition of DNA synthesis. dC decreased the effects of Ara-C by inhibition of Ara-CTP production, rather than by production of dCTP and competition with Ara-CTP. Since low Ara-CTP production in patients' blasts is a predictor of poor therapeutic response to Ara-C, the use of dC with Ara-C may improve the therapeutic index in this group of patients.*

Introduction

Cytosine arabinoside is an effective drug in the treatment of acute myeloid leukaemia (AML) [2]. It is deaminated to an inactive metabolite, uracil arabinoside (Ara-U) by cytidine deaminase [4]. Cytidine deaminase can be competitively inhibited by tetrahydrouridine (THU), an analogue of uridine [28]. Ara-C is converted to its active metabolite Ara-CTP, which competes with dCTP to inhibit DNA polymerase [11]. The rate-limiting enzyme is deoxycitidine kinase, which is most complex in its kinetic behaviour [9, 10]. In human AML, the enzyme has been studied in a partially purified form from the blasts of only two patients [5, 6]. However, the enzyme may have different properties in intact cells, since lysing cells increased deoxycytidine kinase activity 30-fold [15]. In mice with L1210 leukaemia, deoxycytidine (dC), which competes with Ara-C for deoxycytidine kinase, has a protective effect against the toxicity of Ara-C to normal cells, but not the leukaemic cells [3]. The effects in human AML are unknown.

The kinetics of Ara-CTP production in intact myeloblasts, the effect on DNA synthesis, and interactions with deoxycytidine have therefore been studied in AML.

Methods

Preparation of Marrow Myeloblasts. Marrow was obtained from 11 adults with newly diagnosed untreated AML. Aspirates from the posterior iliac crest were collected into 3 ml Eagle's minimal essential medium with Hank's salts (MEM, Flow, Irving, Scotland) plus 2 ml 10% Dextran 110/saline 0.9% (Fison's Ltd., Loughborough, Great Britain) with 200 units preservative-free heparin. The red cells were allowed to sediment, after which the buffy coat was removed and centrifuged at 200 *g* for 5 min. The cell pellet was resuspended in heparin-free MEM and eluted to $0.5-10 \times 10^6$ cells/ml. Marrow infiltration was greater than 85% blasts.

Ara-C Phosphorylation. Cell suspension (100 μl) was incubated with 3–100 μ*M* [5-^{3}H]Ara-C (14–24 Ci/mmol: Radiochemical Centre, Amersham, Great Britain) in MEM in a final volume of 125 μl for 45 min at 37° C. At the end of incubation cells were centrifuged at 2,000 *g* at 0° C for 5 min and the supernatant removed to assay Ara-U. Cell pellets were sonicated with 25 μl 5% ice-cold TCA and 5 μl unlabelled Ara-CTP (1 μg/ml) by means of a Soniprobe (tip 7530A). The suspension was neutralized with 8 μl Tris-acetate buffer pH 8.5, 1.5 *M*, and cleared by centrifugation at 2,000 *g*. The supernatant was chromatographed on PEI cellulose plates 20 × 10 0.1 mm MN 300/UV 254 (Camlab, Cambridge, Great Britain) in 0.5 *M* $(NH_4)_2SO_4$ by ascending chromatography [26]. Recovery of radioacticity was 99%. Ara-CTP was separated from all other labelled Ara-C metabolites (Rf 0.245 ± 0.013 SD, $n = 10$).

The identity of labelled Ara-CTP was confirmed by acid hydrolysis with 10% perchloric acid at 100 °C. All the triphosphate was hydrolysed to a monophosphate that chromatographed with Ara-CMP on DEAE cellulose MN 300 20 × 20 0.1 mm plates in

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0.02 N HCl (Rf Ara-CMP 0.80, Ara-UMP 0.25). The monophosphate was eluted with 0.01 N HCl, which was evaporated to dryness. The residue was redissolved in 0.05 *M* Tris-HCl buffer, pH 8.2, and incubated with 5 units *E. coli* alkaline phosphatase (Sigma, St. Louis, USA) in a final volume of 500 µl for 2 h at 37° C. Protein was precipitated by acetone and Ara-C was shown to be the only labelled nucleoside in the supernatant by ascending chromatography (silica gel plates, butanol : acetone : water : concentrated ammonia : glacial acetic acid; 35 : 25 : 10 : 0.7 : 5 v/v) and descending chromatography (Whatman GFI paper, 94.6 ml 86% N Butanol, 5.4 ml concentrated ammonia). These systems separated Ara-C from Ara-U and from cytosine, uracil, and their deoxy- and ribonucleotides.

After chromatography, the Ara-CTP bands were visualized by UV light, then scraped into scintillation vials and Ara-CTP eluted with 1 ml 0.7 *M* $MgCl_2$–2.0 *M* Tris-HCl pH 7.4 (100 : 1 v/v) [26]. PCS (10 ml) was added and samples counted in a refrigerated counter. Ara-CTP production was calculated after correction for background and quenching. The coefficient of variation was 3%–6.5% for triplicates.

Inhibition of DNA Synthesis by Ara-C. Cell suspension (25 µl) was incubated in a final volume of 125 µl with 100 n*M* [methyl-^{3}H]TdR (41–50 Ci/mmol, Radiochemical Centre, Amersham, Great Britain) and with 1 n*M*–100 µ*M* unlabelled Ara-C for 45 min at 37° C. At the end of incubation 25 µl/100 µ*M* unlabelled TdR was added and the cells washed onto glass fibre filter papers (Whatman Grade GF/C) with water, then with methanol [1]. The papers were dried and counted in PCS. All in incubations were in triplicate. The concentration of [methyl-^{3}H]TdR used has been shown not to affect the rate of DNA synthesis in human leucocytes [7]. Counting [^{3}H]DNA trapped on the filters gave results which did not differ significantly from [^{3}H]TdR incorporation measured by the method of Munro and Fleck [24].

Inhibition of DNA synthesis was maximal at 30–45 min. Preincubation of cells with Ara-C for 45 min before the addition of [^{3}H]TdR did not produce significantly different results. The coefficient of variation of the triplicates was 3%–11%. Inhibition of DNA synthesis was calculated by dividing the counts per minute in the presence of Ara-C by the counts per minute of [^{3}H]TdR incorporated in controls without Ara-C.

Deoxycytidine. A final concentration of 1 µ*M* deoxycytidine (Sigma) in MEM was used in incubations of cells with either labelled Ara-C or labelled TdR plus unlabelled Ara-C. dC was added before the cells, at the start of incubations.

Tetrahydrouridine. Tetrahydrouridine (THU), an inhibitor of cytidine deaminase, was used at a final concentration of 1 m*M*, to prevent Ara-C deamination in some experiments. Ara-U was measured by a method previously described [14] and under the experimental conditions reported here completely inhibited deamination [14].

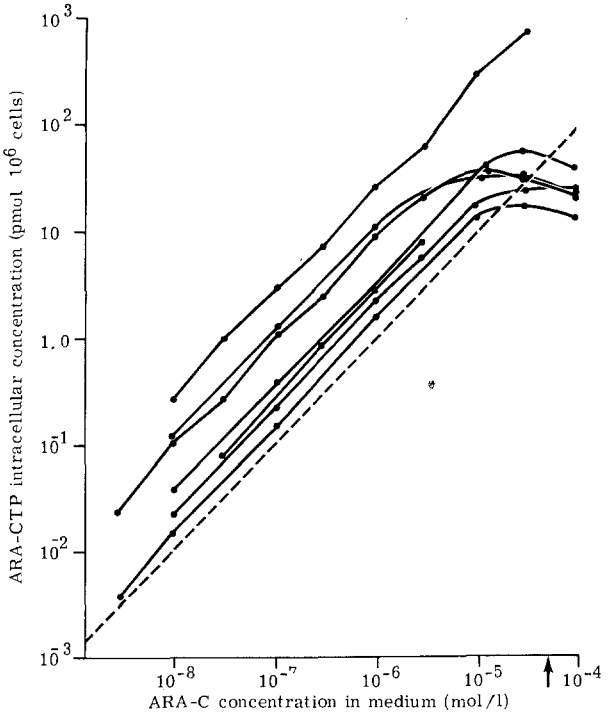

Fig. 1. Ara-C production by bone marrow myeloblasts in AML was measured after 45 min incubation with [^{3}H]Ara-C in concentrations ranging from 3×10^{-8} to 10^{-4} mol/l. Each *point* is the mean of duplicates. The *dashed line* represents an intracellular Ara-CTP concentration equimolar to extracellular Ara-C concentration

Results

Ara-CTP Production

Time course studies showed that Ara-CTP production was linear from 5–45 min. Ara-CTP production was linearly related to Ara-C concentrations in the medium up to 1 µ*M* (Fig. 1). Above 10 µ*M* Ara-C, Ara-CTP production was saturated or decreased. There was wide interpatient variation in Ara-CTP production, but the slopes of Ara-C concentration and Ara-CTP production were linear up to 1 µ*M* Ara-C. Lineweaver-Burk plots of 1/V (1/Ara-CTP produced), against 1/S (1/Ara-C concentration) for the intact cells, showed the Km for Ara-C phosphorylation ranged from 2.05–133 µ*M* (Fig. 2). The inhibition of Ara-CTP production at high Ara-C concentrations is clearly shown in these plots.

Effect of dC on Ara-CTP Production

Ara-C in concentrations of 10, 100, and 300 n*M* and 1 µ*M* was incubated with blasts and either 1 µ*M* dC or 1 m*M* THU (Fig. 3). THU completely inhibited deamination [14] but had little effect on Ara-CTP production, showing that interpatient variation in Ara-CTP production was not due to deamination of Ara-C (Fig. 3). dC greatly reduced Ara-CTP production in the blasts of some patients, but in others there was no effect. The higher the initial Ara-CTP production the greater the decrease produced by 1 µ*M* dC (Fig. 4). The least-squares fit was $y = 240.92\,x + 1.13$ (correlation = 0.86, $P < 0.01$).

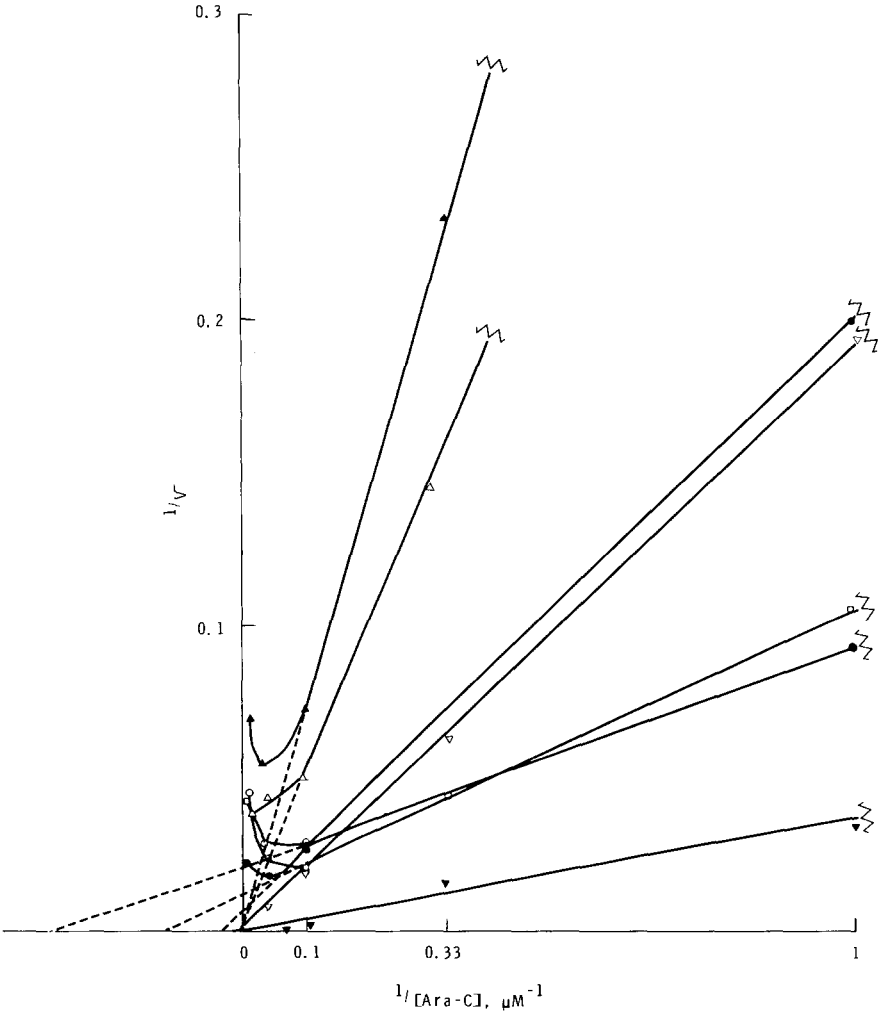

Fig. 2. Lineweaver-Burk plots of Ara-CTP production (I/v) against Ara-C concentration (I/s). Ara-CTP production was measured in intact marrow myeloblasts with [^{3}H]Ara-C in concentrations ranging from 3×10^{-8} to 10^{-4} mol/l. Ara-CTP production is plotted as I/v and Ara-C concentration as I/s. Each *point* is the mean of duplicates and each *symbol* represents a different patient

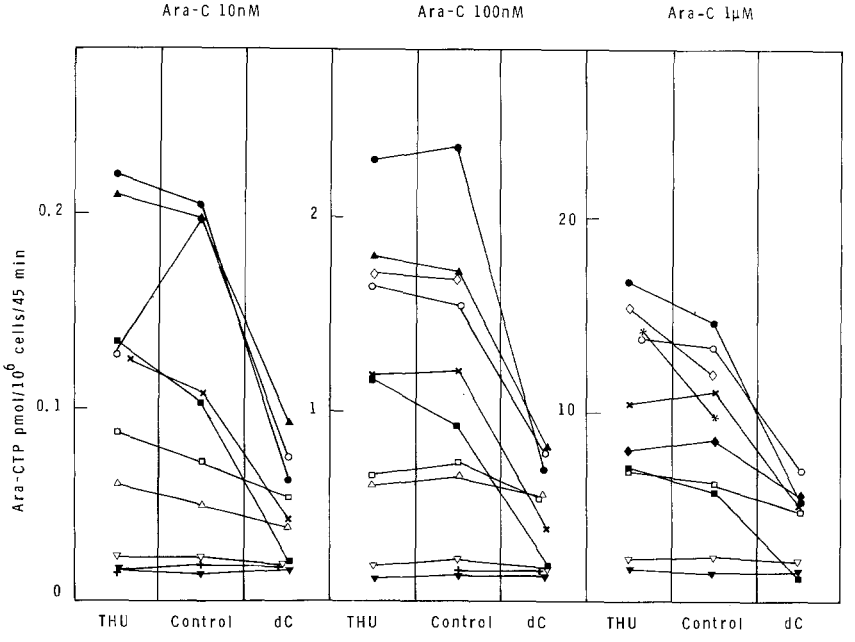

Fig. 3. The effects of deoxycytidine and THU on Ara-CTP production at different Ara-C concentrations. Ara-CTP production was measured in leukaemic marrow myeloblasts from 11 patients with the aid of [^{3}H]Ara-C, with and without THU or dC. Ara-C concentrations of 10 n*M*, 100 n*M*, and 1 μ*M* are shown, but 300 n*M* was also used. THU concentration was 1 m*M*, dC concentration 1 μ*M*. THU completely inhibited deamination and is included as a control to show that variation in Ara-CTP production is not due to deamination. dC reduced Ara-CTP production in blasts with high Ara-CTP production but not those with low levels. Results are means of duplicates or triplicates

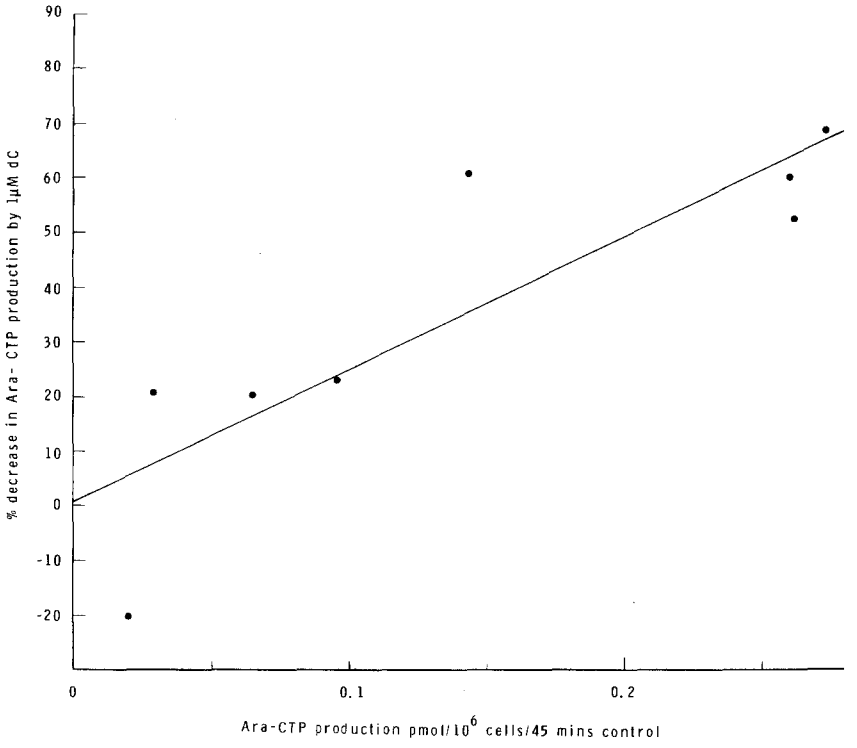

Fig. 4. The percentage decrease in Ara-CTP production caused by 1 μ*M* deoxycytidine compared with initial Ara-CTP production without deoxycytidine. Ara-CTP production was measured in duplicate, with and without 1 μ*M* dC at 10 n*M* Ara-C. Each *point* is the result from one patient. The Ara-CTP produced in the presence of dC was expressed as:

$$\frac{\text{Ara-CTP production without dC} - \text{Ara-CTP production with dC}}{\text{Ara-CTP production without dC}}$$

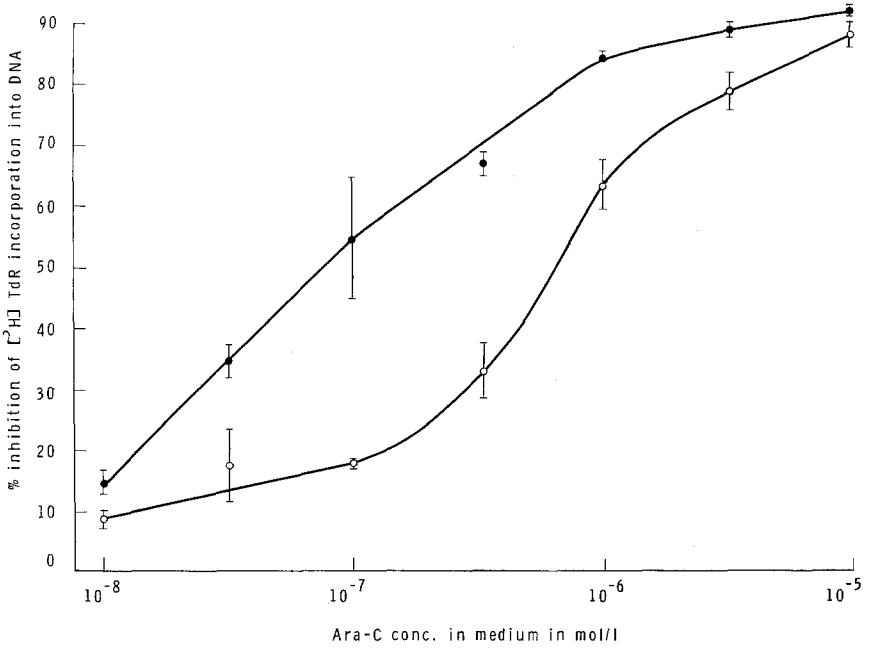

Fig. 5. Ara-C dose-response curves with and without dC. The results from one patient are shown. Inhibition of [^{3}H]TdR incorporation into DNA was measured with the aid of intact marrow myeloblasts and unlabelled Ara-C 10^{-8} to 10^{-5} mol/l. Each *point* is the mean of triplicates and *bars* represent standard deviation. ●, no dC; ○ with 1 μ*M* dC

The K_I for dC on Ara-CTP production ranged from 0.44–4.34 μ*M* dC.

Dose-Response Curves of Inhibition of DNA Synthesis by Ara-C

Dose-response curves for inhibition of DNA synthesis by Ara-C showed that 50% inhibition was produced by 30–100 n*M* Ara-C, and above 3 μ*M* Ara-C there was little further inhibition of DNA synthesis. Less than 10 n*M* Ara-C had no effect (Fig. 5). Incubation of myeloblasts with 1 μ*M* dC in addition to Ara-C produced a shift of the dose-response curve to the right. Ara-CTP production was measured at each Ara-C concentration, with and without 1 μ*M* dC, and the inhibition of DNA

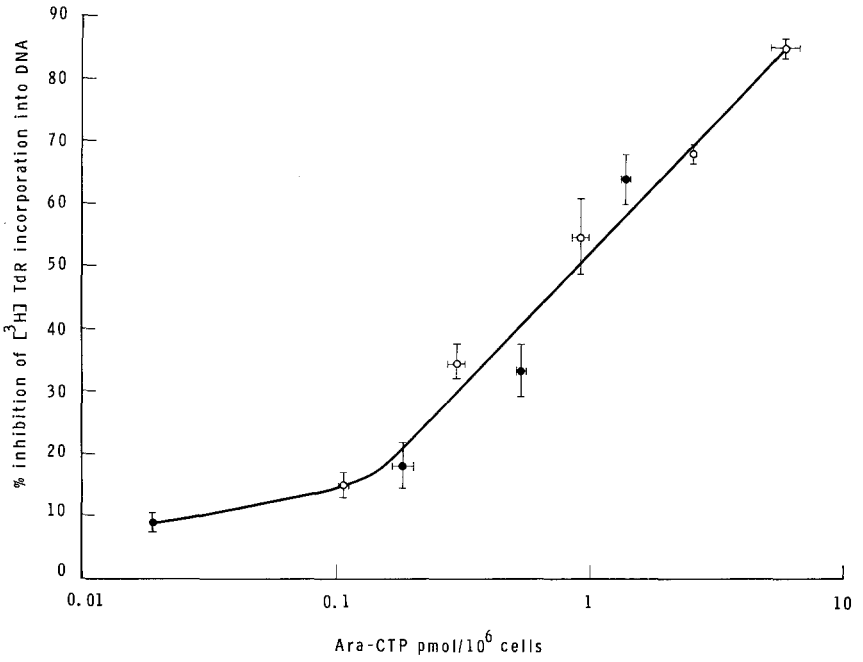

Fig. 6. Ara-CTP dose-response curves with and without dC. The results from the patient in Fig. 5 are shown. Ara-CTP production was measured with [^{3}H]Ara-C at 3×10^{-7}, 3×10^{-6}, 10^{-6}, 3×10^{-5}, 10^{-5} mol Ara-C/l with 1 μ*M* dC and at 3×10^{-8}, 10^{-8}, 3×10^{-7}, 3×10^{-6} mol Ara-C/l without dC. Ara-CTP production at each Ara-C concentration was plotted against inhibition of incorporation of [^{3}H]TdR into DNA at each Ara-C concentration. *Points* are means and *bars* are standard deviations of duplicates for Ara-CTP measurements and triplicates for [^{3}H]TdR measurements

synthesis was then plotted against Ara-CTP production (Fig. 6). The dose-response curves for inhibition of DNA synthesis by Ara-CTP in the presence or absence of dC were superimposable. Thus the reduction in inhibition of DNA synthesis produced by dC (Fig. 5) can be accounted for by the reduction in Ara-CTP production.

Dose-response curves with and without dC were measured in eight patients (Table 1). The results for 100 n*M* Ara-C are shown, since this concentration was on the steep part of the dose-response curve for each patient. In five of the eight patients a significant shift of the curve to the right was produced by dC.

dC Effect on Inhibition of DNA Synthesis by Ara-C

The effect of 1 μ*M* dC on Ara-CTP production at 100 n*M* Ara-C and the correlation with inhibition of DNA synthesis at 100 n*M* Ara-C were studied (Table 1). The blasts with the greatest reduction in Ara-CTP

Table 1. The effect of dC on Ara-CTP production and inhibition of DNA synthesis by Ara-C[a]

K_i of dC on Ara-CTP production (μ*M*)	Ara-CTP production at 100 m*M* Ara-C (pmol/10^6 cells/45 min)		Inhibition of DNA synthesis by 100 n*M* Ara-C (% SD)		
	Control	1 μ*M* dC	Control	1 μ*M* dC	Decrease
0.44	1.541	0.793	64.3 (1.2)	29.4 (3.4)	34.9
0.45	2.36	0.699	59.3 (1.2)	32.6 (1.2)	26.7
0.714	1.21	0.409	57.9 (5.3)	41.7 (1.8)	16.2
1.02	1.707	0.793	71.7 (2.4)	51.2 (1.8)	20.5
3.86	0.74	0.55	74.2 (2.8)	73.7 (3.7)	NS
4.34	0.65	0.556	73.2 (2.9)	63.5 (1.6)	9.7
–[a]	0.134	0.15	58.5 (7)	60 (2.5)	NS
–[a]	0.85	0.558	58.4 (5.1)	55.9 (6)	NS

[a] Bone marrow myeloblasts were incubated with unlabelled 100 n*M* Ara-C and [^{3}H]thymidine, with and without dC. Percentage inhibition of DNA synthesis is shown. Ara-CTP production with 100 n*M* [^{3}H]Ara-C in identical conditions was measured with and without 1 μ*M* dC. Only in blasts, in which dC produced a reduction of Ara-CTP by 50% or more, was there a decrease in the inhibition of DNA synthesis produced by dC

[b] K_i could not be calculated because only two concentrations of Ara-C were used with 1 μ*M* dC

production by dC (i.e., the lowest K_1) were the ones that showed the greatest decrease in inhibition of DNA synthesis by Ara-C. In blasts where only a small decrease, or no change in Ara-CTP production, occurred there was no significant effect of dC on inhibition of DNA synthesis by Ara-C.

The Ara-CTP concentrations associated with 50%–60% inhibition of DNA synthesis ranged from 0.134 pmol/10^6 cells (58.5% inhibition) to 2.36 pmol/10^6 cells (59.3% inhibition).

Correlation of Phosphorylation with Response

Seven of the 11 patients went into complete remission after treatment with Ara-C-containing regimens. Two of the four non-responders had the lowest two values for phosphorylation, but the difference in phosphorylation of responders and non-responders was not significant (Wilcoxon ranked sums). However, in a larger series of 25 patients phosphorylation was measured at 1 μM Ara-C and there was significantly lower phosphorylation in non-responders (A. L.Harris, unpublished results).

Discussion

Intact cells were used in this study because deoxycytidine kinase is very complex in its kinetic behaviour and studies with partially purified enzyme may not reflect its behaviour in vivo [15]. Deoxycytidine kinase is inhibited by dCTP (and also UDP, dTDP, dCMP, dCDP) and activated by dTTP, UTP and dUTP[1]. The Km for Ara-C varies from 9.3–175 μM and that for dC from 2.5–40 μM with partially purified enzyme from L1210 leukaemia [27], P815 tumour [19], human myeloblasts, and calf thymus [6, 8].

Although the assumptions behind the Lineweaver-Burk plots strictly apply to purified enzymes, the method has been applied to intact cells and membranes [23, 25]. Mammalian cells are freely permeable to Ara-C and the Km for entry is 400–450 μM [23, 25]. Deoxycytidine kinase is the rate-limiting enzyme in Ara-CTP production [18], the other two enzymes involved in the conversion of Ara-CMP to Ara-CDP (dCMP kinase) and Ara-CDP to Ara-CTP (nucleoside diphosphokinase) having Km values at least 1 log greater [18, 22]. Thus it is reasonable to obtain a Km for deoxycytidine kinase in vivo by plotting external Ara-C concentration as S and Ara-CTP produced as V. The range of 2.05–133 μM is similar to values obtained with partially purified enzyme. Ara-CTP production decreased at high Ara-C concentrations. Ara-C has not been reported to produce substrate inhibition with partially purified deoxycytidine kinase from human cells [5, 6]. However, with rat spleen cells [20] concentrations of Ara-C above 2 μM were associated with decreasing Ara-CTP production. In HeLa cell sonicates Ara-CTP concentrations of 20 μM inhibited phosphorylation of Ara-C by 46% [21]. This may be occurring in intact human myeloblasts. However, this phenomenon was not detected at the lower Ara-C concentrations that are found in vivo with current Ara-C dosage schedules (10 nM to 1 μM Ara-C) [13].

The interactions of deoxycytidine with Ara-C phosphorylation were studied at Ara-C concentrations of 10 nM to 1 μM. We used 1 μM dC because the range of K_1 of dC for Ara-C phosphorylation is 0.17–4.0 μM with partially purified enzyme [6, 16]. The K_1 in intact myeloblasts ranged from 0.44–4.34 μM. The greater the production of Ara-CTP in the absence of dC, the greater the effect of dC in decreasing Ara-CTP production. In cells where deoxycytidine kinase had an apparently low Km for Ara-C, a low K_1 for deoxycytidine was also found. Thus the range of Km and K_1 in intact cells seems similar to values reported for a variety of partially purified mammalian deoxycytidine kinases. However there is a wide interpatient variation. This may represent physical differences in the enzyme or, more probably, different states of activation or inhibition of deoxycytidine kinase by endogenous nucleosides and nucleotides.

The Ara-CTP measured in the cells is probably representative of the concentration in the S-phase cells, which are the target for Ara-CTP action [30], because reduction of the higher Ara-CTP concentrations by dC produced proportional changes in the inhibition of DNA synthesis. Furthermore, the dose-response curves for inhibition of DNA synthesis by Ara-CTP in the presence or absence of dC were superimposable (Fig. 6). This suggests that dC decreases the effects of Ara-C by inhibition of Ara-CTP production rather than by production of dCTP and competition with Ara-CTP.

Similar inhibition of DNA synthesis was found with widely differing Ara-CTP concentrations. Ara-CTP competes with endogenous dCTP to inhibit DNA synthesis and dCTP pool size in human leukaemia blast cells ranges from 0.6–3.1 pmol/10^6 cells [29], similar to the range of Ara-CTP found here to produce 50% inhibition of DNA synthesis

1 UDP, uridine diphosphate; UTP, uridine triphosphate; dUTP. deoxyuridine triphosphate; dTDP, thymidine diphosphate; dTTP, thymidine triphosphate; dCMP, dCDP, dCTP, deoxycytidine mono- di- tri-phosphates, respectively

(0.134–2.36 pmol/10^6 cells). Cells that produce higher Ara-CTP concentrations may be those with higher dCTP pools, and hence the greater Ara-CTP production does not necessarily produce greater inhibition of DNA synthesis. However, when dC is added the presence of high dCTP pools may result in a lower K_I for dC. These results show that the effect of Ara-C on inhibition of DNA synthesis can be reduced by dC in the blasts of patients with high Ara-CTP production but has no effect on those with low Ara-CTP production.

Low Ara-CTP production, or low deoxycytidine kinase activity in sonicates of blasts, is associated with poor clinical response to Ara-C [17, 29]. The use of dC in combination with Ara-C in L1210 leukaemia in mice had a protective effect on the normal marrow, and otherwise lethal doses of Ara-C could be used with good therapeutic results [3]. Such an approach would not be generally applicable in human AML because dC inhibits production of the active metabolite Ara-CTP in some patients' blasts. However, in those with low Ara-CTP production, dC did not inhibit Ara-CTP production or reduce the inhibition of DNA synthesis. Thus in Fig. 3, a ten-fold increase in Ara-C concentration (to 1 μM) produced Ara-CTP concentrations in the blasts of one patient that were similar to those produced at 100 nM in another. dC did not prevent the increase of Ara-CTP in the low-Ara-CTP blasts.

Further studies will be necessary to study the pharmacology and interaction of dC with normal tissues in man, but it is possible that 'protection' combined with high-dose Ara-C could offer therapeutic benefits to those patients not responding to conventional Ara-C-containing regimens.

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