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The Relationship of Ara-C Metabolism in vitro to Therapeutic Response in Acute Myeloid Leukaemia

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Summary. *Ara-C phosphorylation and Ara-C deamination was measured in vitro, using intact marrow myeloblasts from 25 patients with previously untreated acute myeloid leukaemia. At Ara-C concentrations above 10 μM there was no longer a linear relationship of phosphorylation to Ara-C concentration. Ara-U production was measured by sampling the incubation medium. This method showed greater Ara-U production than previous methods sampling the cell pellet alone. However, Ara-CTP/Ara-U ratios from intact myeloblasts were much higher than those recorded in studies using lysed myeloblasts. Using 1 μM Ara-C, a concentration representative of in vivo concentrations, deamination and phosphorylation were related to therapeutic response to Ara-C-containing drug regimens. There was no significant correlation of these variables with response, although 5/16 non-responders had low Ara-C phosphorylation (< 1.5 pmol/10⁶ cells/45 min/l pm Ara-C) compared with 0/9 responders. Measuring deaminase activity did not help in selecting non-responders. Even in patients with low phosphorylation increasing Ara-C concentration increased Ara-CTP levels proportionally, but up to 10 times conventional doses may be necessary to exceed endogenous dCTP levels.*

Introduction

Cytosine arabinoside (Ara-C) is a useful drug in adult acute myeloid leukaemia (AML), but when used alone it has given remission rates of 25%–47% with various schedules [1, 2]. The reasons for failure have been intensively investigated. Ara-C is converted to an inactive metabolic uracil arabinoside by deamination in leukaemic blasts [19] and human liver [3]. High deaminase activity in leukaemic blasts has been reported to correlate with poor response [19], but lysed cells were used and this increases deaminase activity up to 30-fold [13]. Furthermore, use of a deaminase inhibitor, tetrahydrouridine, has not increased the therapeutic ratio [15].

Ara-C is converted to its active metabolite cytosine arabinoside triphosphate (Ara-CTP) intracellularly in leukaemic blasts [4]. The rate-limiting enzyme is deoxycytidine kinase. Low Ara-C phosphorylation or low deoxycytidine kinase in marrow blasts have been associated with poor response, but other studies using peripheral blasts have not confirmed these observations [18]. All these studies have used Ara-C concentrations much greater than occur in vivo with current dose schedules [11].

We have therefore studied Ara-C phosphorylation and deamination in leukaemic marrow myeloblasts, using low Ara-C concentrations, and correlated these with response to Ara-C therapy.

Materials and Methods

Patients. Twenty-five patients with newly diagnosed untreated AML were studied. They were treated with three Ara-C-containing regimens. Sixteen patients received Ara-C infusions followed by Ara-C boluses 70 mg/m² daily/5 days, daunorubicin 55 mg/m² on day 1, repeated at 5-day intervals. Six patients were treated with Ara-C and daunorubicin boluses as described above. Three patients received Ara-C boluses 70 mg/m² daily/5 days plus oral thioguanine 100 mg/m² daily/5 days, daunorubicin 40 mg/m² day 1 plus prednisolone 30 mg/m² 5 days repeated at 5-day intervals. Complete remission was defined by the standard criteria of a normal peripheral blood count and normal marrow with less than 5% blasts.

Preparation of Marrow Myeloblasts. Marrow was obtained from adults with newly diagnosed untreated AML. Marrow aspirates from the posterior iliac crest were collected into 3 ml Eagle's minimal essential medium with Hank's salts (MEM; flow, Irving) plus 2 ml 10% Dextran 110/saline 0.9% (Fisons Ltd., Loughborough, Great Britain) with 200 units preservative-free heparin. The red cells were allowed to sediment, and the buffy coat was centrifuged at 200 *g* for 5 min and resuspended in heparin-free MEM. Cells were diluted to $0.5-10 \times 10^6$ cells/ml with MEM. Marrow infiltration was greater than 85% blasts. The cell concentration was chosen to limit deamination of Ara-C during incubations to less than 10%.

Ara-C Phosphorylation. Cell suspension (100 μl) was incubated with 3 n*M* to 100 μ*M* [5-³H] 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. Cells were centrifuged at 2,000 *g* at 0° C for 5 min and the supernatant removed to assay Ara-U. Cells pellets were sonicated with 25 μl 5% ice-cold TCA and 5 μl unlabelled Ara-CTP (1 μg/ml) using a soniprobe, tip 7530A. The suspension was neutralised with 8 μl Tris-acetate buffer pH 8.5, 1.5 *M* and cleared by

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centrifuging at 2,000 *g*. The supernatant was chromatographed on PEI cellulose plates 20 × 20, 0.1 mm MN 300/UV 254 (Camlab, Cambridge, Great Britain) in 0.5 *M* $(NH_4)_2SO_4$ by ascending chromatography [17]. Recovery of radioactivity was 99%. Ara-CTP was separated from all other labelled Ara-C metabolites (RF 0.245 ± 0.013 SD, n = 10).

After chromatography, the Ara-CTP band was visualised by UV light, the bands scraped into scintillation vials, and Ara-CTP eluted with 1 ml 0.7 Mg Cl_2 – 2.0 M Tris-HCl pH 7.4 (100 : 1 v/v) [17]. PCS (10 ml) (Searle, Amersham, Great Britain) 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. No Ara-CTP was detected in the incubation medium.

Ara-U Production. Because nucleosides rapidly efflux from intact cells Ara-U produced intracellularly may diffuse out into the medium. The time of Ara-U efflux was measured by incubating 5 × 10^6 myeloblasts with 1 μ*M* [5-^{3}H] Ara-C for 45 min at 37° C in a final volume of 500 μl MEM. At the end of incubation 10 ml MEM was added and the cells centrifuged at 200 *g* for 5 min at room temperature. Cells were resuspended in 5 ml MEM. Aliquots (250 μl) were placed in pairs of Eppendorf test tubes and incubated at 37° C. At various times the cells were spun down and Ara-U and Ara-C in the medium measured as described below. Trypan blue staining showed no change in viability during the procedure and no Ara-CTP was detected in the medium.

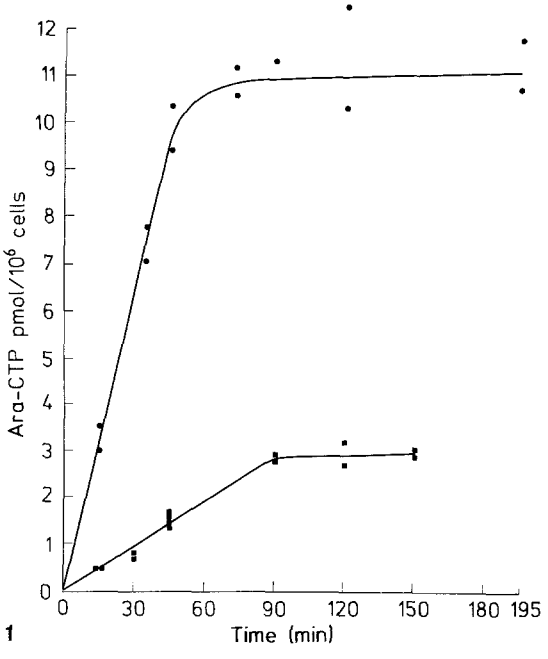
To measure Ara-U production 5–25 μl incubation medium at the end of 45 min incubation of cells with [5-^{3}H] Ara-C was added to Ag50WX4 200–400 mesh resin columns 5 mm × 2.5 cm. The columns were eluted with 10 ml 0.05 NHCl and Ara-U was eluted in the first 5 ml. Ara-C was eluted with 5 ml 4 *N* NH_4OH after washing the column with 5 ml distilled water. Recovery was greater than 95% from the columns, and the coefficient of variation of triplicates was 2%. The fractions containing Ara-C and Ara-U were counted in 10 ml PCS. No spontaneous deamination of Ara-C was detected in the absence of cells.

To measure Ara-U in the cell pellets, ice-cold 5% perchloric acid extracts were made and neutralised with 2*N* KOH. The extracts were then chromatographed as described above.

Results

Ara-CTP Production

Ara-CTP production was linear for 45–60 min (Fig. 1). Ara-CTP production was proportional to Ara-C concentration in the medium for any one patient's blasts, from 3 n*M* to 3 μ*M* Ara-C (Fig. 2). Above 10 μ*M* Ara-C Ara-CTP production became saturated or even decreased. There was a nearly 100-fold range in Ara-CTP production at 1 μ*M* Ara-C (Table 1). Ara-CTP intracellular concentrations exceeded Ara-C concentrations in the medium up to 10 μ*M* Ara-C (Fig. 2).



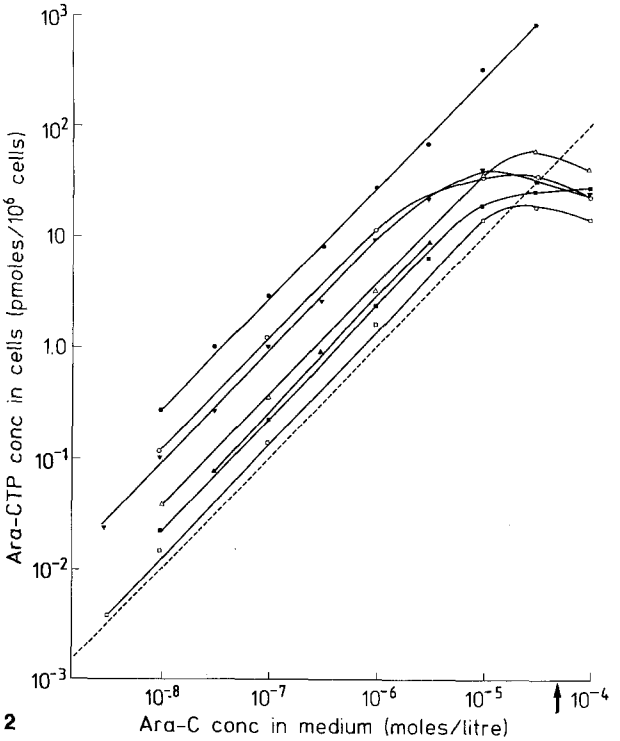


Fig. 1. Time course of intracellular Ara-CTP production in intact marrow myeloblasts. The different *symbols* represent different patients. Marrow myeloblasts were incubated with 1 μ*M* [5–^{3}H] Ara-C for 45 min at 37° C. Each *point* represents one measurement

Fig. 2. Ara-CTP production by bone marrow myeloblasts. Each *point* is the mean of duplicates, and different *symbols* represent different patients. Marrow myeloblasts were incubated with 3 × 10^{-9} to 1 × 10^{-4} mol/Ara-C/l for 45 min at 37° C. The *dashed line* represents an intracellular Ara-CTP concentration in mol/l equivalent to equimolar extracellular Ara-CTP concentrations, assuming 10^{12} cells = 1 l of cell water. The *arrow* indicates an Ara-C concentration of 50 μ*M*

Ara-U Efflux from Myeloblasts Preincubated with [5-^{3}H] Ara-C

Ara-U efflux was linear from 40 to 90 min after the final resuspension of cells in fresh medium. The Ara-C concentration in the medium ranged from 1 to 3 n*M* after resuspension. Ara-C content of the medium also increased but not linearly. In three of the four patients studied Ara-U efflux exceeded Ara-C efflux (Fig. 3).

Others have measured Ara-U production by intact myeloblasts, but only sampled the cell pellet. The efflux experiment shows that it is necessary to sample the medium to measure total Ara-U production.

When cell pellets were extracted after 45 min incubation with [5-^{3}H] Ara-C the amount of Ara-U in the pellet was less than 2% of that in the medium.

Ara-U Production

Ara-U production (as determined by sampling the medium) was linear for 2 h (Fig. 4) and was linearly related to Ara-C concentration in the medium from 3 n*M* to 100 μ*M* Ara-C (Fig. 5). There was a 3 log variation in Ara-U production at any Ara-C concentration.

Ara-CTP Production, Ara-C Deamination and the Response to Therapy

Since deamination and phosphorylation were both linearly related to Ara-C concentrations over the range 3 n*M* to 3 μ*M*, a single Ara-C concentration (1 μ*M*) was used to compare the two activities in 25 patients (Table 1). The ratio of Ara-CTP to Ara-U (kinase/deaminase ratio) ranged from 0.02 to 7.46, median 0.67.

Ara-C phosphorylation in the blasts of patients who went into remission was not significantly greater than in those that did not (median for remitters 9.4 pmol/10^6 cells; median for non-remitters 3.45 pmol/10^6 cells). However, none of the five patients with less than 1.5 pmol/10^6 cells responded and all nine remitters had higher levels.

Ara-C deamination was less in the blasts of patients who went into remission but this was not significant (Wilcoxon ranked sums, $P > 0.1$: median for remitters, 9.13 pmol/10^6 cells; median for non-remitters 16.60 pmol/10^6 cells).

The Ara-CTP/Ara-U ratio was not significantly higher in patients who went into remission; 4/9 responders had ratios greater than 1, and 1/11 non-responders had ratios greater than 1 (median for remitters 0.97; median for non-remitters 0.20). The overlap between remitters and non-remitters for the

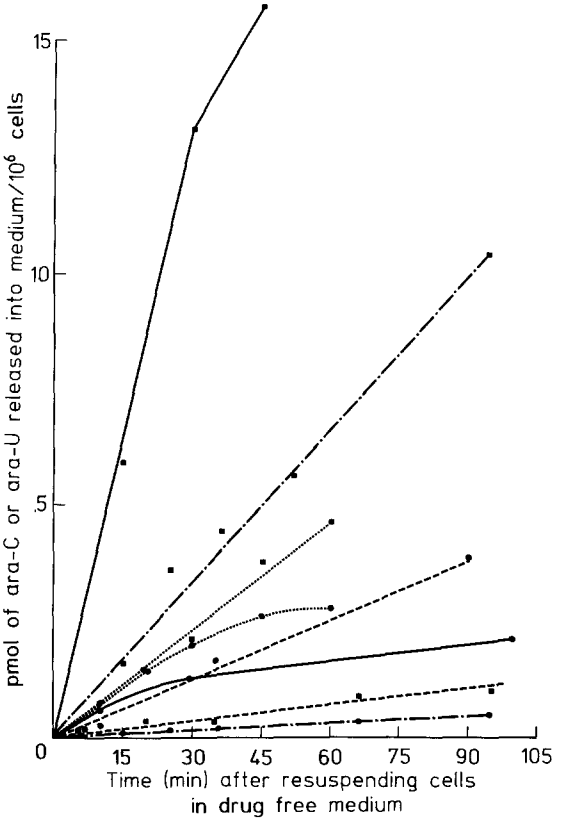


Fig. 3. Ara-U and Ara-C efflux from leukaemic myeloblasts preincubated with 1 μ*M* [5-^{3}H] Ara-C. Cells were preincubated with 1 μ*M* [5-^{3}H] Ara-C and then resuspended in fresh medium. Extracellular Ara-C concentration was 1–3 n*M* after resuspension. Each *point* is the mean of duplicates. (■) Ara-U efflux, (●) Ara-C efflux. (———) (· · · · ·) (– – –) (–·–·–) represents Ara-C or Ara-U efflux from 4 different patients

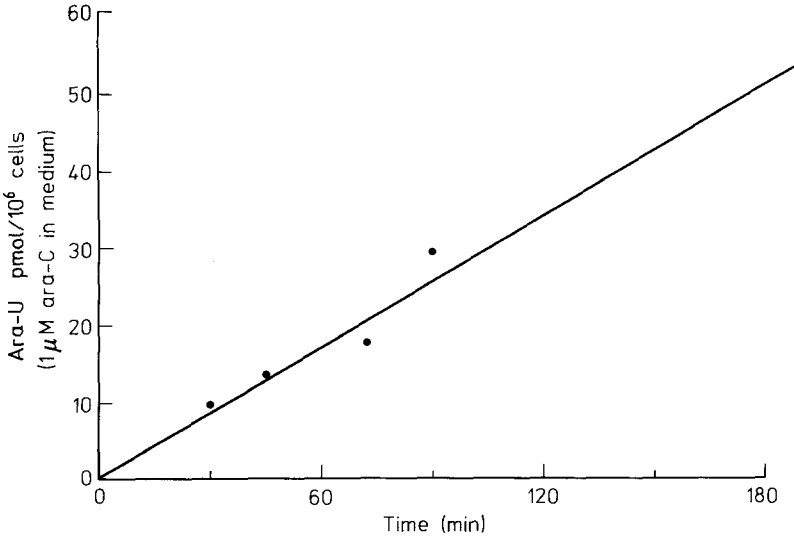


Fig. 4. Time course of Ara-C deamination. Each *point* is the mean of duplicates. Aliquots of leukaemic cells were incubated with 1 μ*M* [5-^{3}H] Ara-C for the times indicated and the Ara-U content of the medium was measured

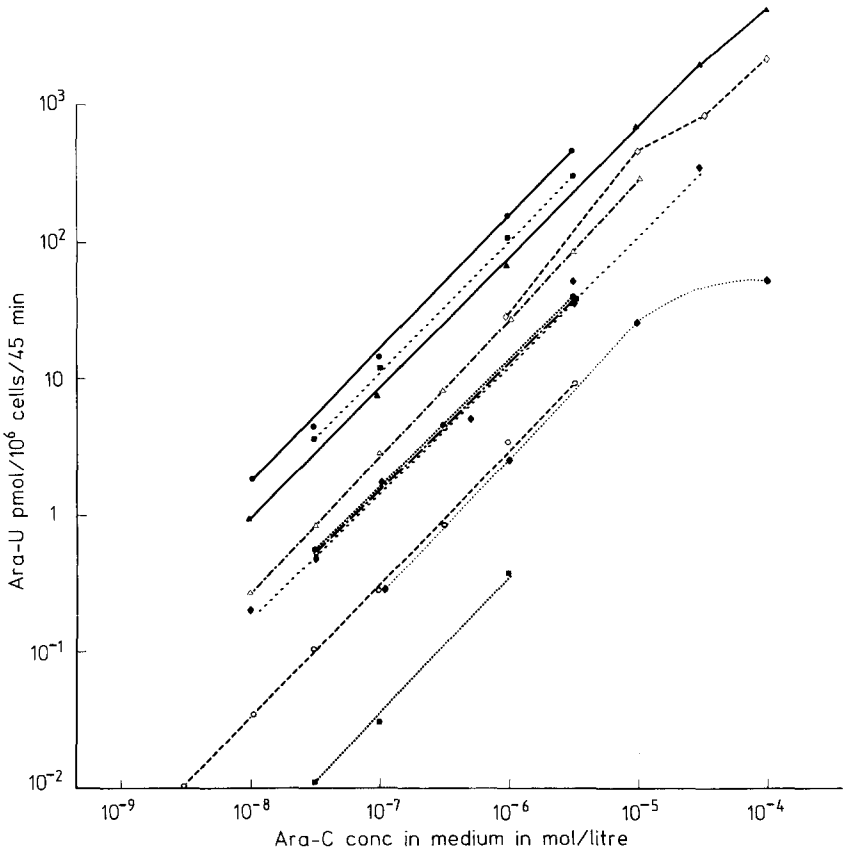


Fig. 5. Ara-C deaminated at different Ara-C concentrations in marrow myeloblasts. Each *point* is the mean of duplicates. Different *symbols* represent different patients. Marrow myeloblasts were incubated with 3×10^{-9} to 10^{-4} mol [5-^{3}H] Ara-C/l for 45 min at 37° C. Ara-U production was calculated from Ara-U content in the medium

Table 1. Relation of remission to Ara-C phosphorylation and deamination in myeloblasts

Patient	Remission			No remission		
	Ara-CTP pmol/10^6 cells (1 μ*M* Ara-C)	Ara-U pmol/10^6 cells (1 μ*M* Ara-C)	Ara-CTP/ Ara-U	Ara-CTP pmol/10^6 cells (1 μ*M* Ara-C)	Ara-U pmol/10^6 cells (1 μ*M* Ara-C)	Ara-CTP/ Ara-U
1	1.51	10.40	0.14			
2	1.55	2.61	0.67			
3	2.24	0.30	7.46			
4	6.49	66.80	0.1			
5	9.40	3.47	2.71			
6	10.90	67.50	0.16			
7	11.30	74.20	0.15			
8	13.25	4.18	3.16			
9	14.69	9.13	1.61			
10				0.23	104.8	0.002
11				0.66	3.68	0.18
12				0.72	–	
13				1.45	–	
14				1.49	2.83	0.53
15				2.82	1.52	1.85
16				2.83	12.67	0.22
17				3.45	16.60	0.21
18				4.07	–	–
19				4.45	12.00	0.37
20				6.09	26.30	0.23
21				9.44	23.50	0.40
22				12.15	43.98	0.28
23				14.36	–	–
24				17.04	–	
25				26.47	28.00	0.95
Mean	7.92	26.51	1.80	6.79	25.08	0.47
SD	5.15	32.46	2.43	7.45	29.37	0.52
Median	9.40	9.13	0.67	3.45	16.60	0.28

deaminase activity was such that in any individual patient remission could not be predicted.

Discussion

Much interest has focussed on the two main pathways of Ara-C metabolism because the conversion to an inactive or active metabolite may determine the in vivo response to the drug [12, 19, 20].

In this study low Ara-CTP production (less than 1.5 pmol/10^6 cells at 1 μM Ara-C) was found in non-responders and higher Ara-CTP production was found in both responders and non-responders. Ara-C concentrations that were representative of in vivo levels 30 min after 2 mg/kg boluses were used [11]. At higher Ara-C concentrations, particularly 30 μM and above, Ara-CTP production was saturated and intracellular Ara-CTP concentration no longer exceeded the Ara-C concentration in the medium. At these concentrations, however, deaminase activity was still linearly related to Ara-C concentration. Deamination was not the source of variation in Ara-CTP production, since incubation conditions were chosen to ensure that less than 10% of the Ara-C in the medium was converted to Ara-U.

These results may be compared with those of Smyth et al. [18], whose study is the only other one to measure Ara-CTP and Ara-U production by intact blasts. They used 50 μM Ara-C and found no correlation of phosphorylation or deamination with response. Since they did not measure the Ara-U in the medium they found less median Ara-U production using 50 μM Ara-C than we found using 1 μM Ara-C. The results showing Ara-U efflux explain their underestimation of Ara-U production. Peripheral blasts were used which tend to be more differentiated than marrow blasts, and kinase activity falls and deaminase activity rises with cell maturity [20]. The labelling index of peripheral leukocytes is also less than that in marrow [16]. Thus their negative results did not exclude the importance of Ara-C metabolism for response.

Chou et al. [4] found very similar phosphorylation to that described here. The range was equivalent to 2.48–10.7 pmol/10^6cells/45 min/l μm Ara-C in 12 responders and 0.8–7 in 12 non-responders. Levels were below the lower limit of the responders in 6/12 non-responders. These results were statistically significant. Deamination was not measured and the patients were treated with an Ara-C derivative whose pharmacology is poorly understood (AAFC 2,2^1-anhydro-1-β-D-arabinofuranosyl-5-fluorocytosine).

Kessel et al. [14] found that high phosphorylation of Ara-C correlated with good clinical response. It was not clear whether they used peripheral or marrow blasts, and the detailed discussion of their results referred to 5 grades of response and 3 categories of prediction for 15 patients [9]. Ara-C concentrations of 50 μM were used.

Tattersall et al. [21] found low deoxycytidine kinase in marrow sonicates correlated with poor response to Ara-C in combination therapy, but this was not a prospective study and the patients had already been treated at the time of study. Deoxycytidine kinase is extremely complex in its allosteric behaviour [7, 8], and whether its modulation in vitro is similar to that in the intact cell is unknown. Lysing human cells does increase deoxycytidine kinase activity [13].

The present study and the other two studies using marrow myeloblasts [4, 12], in spite of the problems discussed, show that low Ara-CTP production or deoxycytidine kinase activity is associated with poor response to Ara-C or its derivatives in combination therapy.

This study also shows that measuring deaminase activity is of no additional value. Stuart and Burke [19] showed high deaminase activity in lysed cells correlated with poor response to Ara-C. However, lysing human leucocytes [13] or freeze-thawing them [18] increases deaminase activity 20–30 fold. The kinase/deaminase ratios in lysed leukaemic cells (Coleman et al. [5], mean 0.197, median 0.05) are nearly 10 times lower than those found here (mean 1.24, median 0.67), probably because of the greatly increased deaminase activity. The deaminase activity of lysed cells and activity in intact cells does not correlate with poor response in individual patients.

The reasons for high Ara-CTP production failing to discriminate between remitters and non-remitters may be related to endogenous deoxycytidine triphosphate levels (dCTP). dCTP concentrations range from 0.3 to 3.1 pmol/10^6 leukaemia cells [21]. The Km for dCTP with human leukaemic DNA polymerase ranges from 2.5 to 3.1 μM (equivalent to 2.5–3.1 pmol/10^6 cells, if the volume of one myeloblast is assumed to be 100 fl) [22]. The K_1 for Ara-CTP ranges from 1.4 to 2.9 μM (equivalent to 1.4–2.9 pmol/10^6 cells). In another study [10], the intracellular Ara-CTP concentration associated with 50% inhibition of DNA synthesis in intact marrow myeloblasts, incubated under the conditions described here, ranged from 0.134 pmol/10^6 cells to 2.36 pmol/10^6 cells.

The Ara-CTP production shown in Table 1, if measured at 100 nM Ara-C (Fig. 1), will be 0.023–2.7 pmol/10^6 cells; 100 nM Ara-C is a concentration that can be expected with constant infusions of Ara-C of 150 mg/24 h or within 1 h of a 2 mg/kg bolous [11]. Thus even high Ara-CTP production may not exceed endogenous dCTP concentrations if the latter are in the upper range. However, low Ara-CTP production would be associated with poor response, whether dCTP concentrations were high or low.

These results also show that in patients' blasts with low Ara-CTP production, increasing the Ara-C concentration in the medium produces a proportional increase in Ara-CTP intracellularly, at least up to 3 μM Ara-C. Hence in patients with low Ara-CTP production higher doses of Ara-C could be tried, but they may need up to 10 times conventional doses to increase Ara-CTP concentrations above dCTP concentrations.

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