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Cytosine Arabinoside Transport by Human Leukaemic Cells*

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Abstract—*The membrane transport of cytosine arabinoside (araC) has been studied in blasts freshly isolated from a variety of acute leukaemias. The major fraction of araC influx was facilitated and this fraction was* 80–87% *at* 1 μM *araC and* 68–80% *at* 200 μM *araC. Competitive kinetics were observed between araC and deoxycytidine for entry into leukaemic blasts and, moreover, araC influx was blocked by phloretin, a broad-spectrum inhibitor of facilitated transport systems. Kinetic analysis of facilitated araC influx gave* K_m*s which varied over a* 10*-fold range between patients and which were positively correlated to the* V_{max}*. Nucleoside influx* V_{max} *also varied over an* 80*-fold range between individuals, although the mean araC transport was* 4*-fold greater in myeloblasts than in lymphoblasts. Larger transport of araC may explain the greater sensitivity of acute myeloid leukaemia to this drug.*

INTRODUCTION

CYTOSINE arabinoside (araC) is used widely in the therapy of acute non-lymphoblastic leukaemia, and in combination with daunorubicin gives complete remission rates of 60–70% in adult acute myeloid leukaemia [1]. Even as a single agent araC has been reported to yield remissions in up to 50% of such patients [2]. The optimal schedule for administration of araC is by constant intravenous infusion of 100–150 mg/m²/day, a dosage which gives plasma levels of 0.4–1.0 μM [3–5]. In contrast, the results of treatment of acute lymphoblastic leukaemia with araC have generally been disappointing whether the drug is given by daily intravenous injection or 5-day continuous infusion [6, 7]. Despite this, araC is often included in combination therapy for acute lymphoblastic leukaemia, both in initial treatment and at first relapse, although few patients respond favourably to this drug. More recently, high-dose therapy producing plasma levels of 50–200 μM araC has been used with some success in patients who do not respond to standard low-dose treatment [8, 9].

The basis for the variable response of acute leukaemia to araC may reside in differences in its conversion within the cell to araCTP since the latter is the active metabolite of the drug [10]. Approximately 15% of patients with drug-resistant acute non-lymphoblastic leukaemia have blasts which fail to convert extracellular araC to intracellular araCTP [11]. One limiting factor for the effect of araC may be the rate of membrane transport and, indeed, blasts from some patients who fail to respond to chemotherapy show an extremely low influx of araC [12]. Evidence from tissue culture experiments supports the concept that membrane transport of araC may be rate-limiting for its effect. Cultured S 49 lymphoid cells in which nucleoside transport is impaired by mutation can survive exposure to lethal doses of araC [13]. Moreover, cultured leukaemic cells can be 'protected' against the cytotoxic actions of araC by inhibitors of membrane transport such as nitrobenzylmercaptopurine riboside (NBMPR) or dipyridamole [14]. In cultured cells and erythrocytes the entry of araC is largely by a facilitated diffusion mechanism, which shows a broad specificity for

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Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; araCTP, the 5'-triphosphate of araC; CdR, deoxycytidine; araU, 1-β-D-arabinofuranosyluracil; NBMPR, nitrobenzylthioinosine or 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosyl purine; AML, acute myeloblastic leukaemia; AMML, acute myelomonocytic leukaemia; ALL, acute lymphoblastic leukaemia; AUL, acute undifferentiated leukaemia; PMN, polymorpholeucocyte neutrophils.

both purine and pyrimidine nucleosides and their analogues [for review see ref. 15].

The present study confirms that fresh leukaemic blasts transport araC predominantly by a facilitated diffusion mechanism both at low- and high-dose therapeutic levels. Moreover, the transport of araC into leukaemic blasts is competitive with its natural analogue, deoxycytidine (CdR), while important differences in the kinetics of araC entry are evident between different leukaemias. Lymphoblasts showed lower transport rates than myeloblasts, and this difference parallels the lower responsiveness of acute lymphoblastic leukaemia to araC therapy.

MATERIALS AND METHODS

Materials

Imidazole-buffered saline (145 mM NaCl, 5.0 mM KCl, 5.0 mM imidazole-Cl, 1.0 mM $MgCl_2$, 5.0 mM glucose, pH 7.4) was ultrafiltered through Millipore Millex 0.22-μm filter units (Millipore Corp., Bedford, MA) before use. All washing and incubation media contained deoxyribonuclease (10 μg/ml, Sigma Chemical Co., St. Louis, MO) to reduce the tendency of white cells to clump. Dextran T500 and Ficoll-Isopaque (density 1.077) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Di-*n*-butyl phthalate (British Drug Houses, Poole, U.K.) and di-*n*-octyl phthalate (Ajax Chemicals Ltd., Australia) of densities 1.044 and 0.986 respectively were blended 4:1 (v/v), yielding an oil mixture of density 1.032. Nitrobenzylmercaptopurine riboside (NBMPR), synthesised as described by Paul *et al.* [16], was stirred vigorously with imidazole-buffered saline for 4 hr at 18–20°C and allowed to settle, yielding a saturated solution of approximately 20 μM. This solution was prepared on the day of each experiment. [5-^{3}H]-Cytosine β-D-arabinoside, deoxy[5-^{3}H]-cytidine and [U-^{14}C]-sucrose in sterile 3% aqueous ethanol were from the Radiochemical Centre, Amersham, U.K. Solutions of 5.0 mM cytosine arabinoside (Upjohn Co., MI) and deoxycytidine (Sigma) in isotonic saline were mixed with 0.1 vol. of the appropriate tritiated stock solution, the exact concentrations of the final solutions (4.1–4.5 mM) being confirmed spectrophotometrically at pH 1 using a molar extinction coefficient of 1.32×10^4 at 280 nm.

Patient data

Patients with acute leukaemia had peripheral white cell counts of 13,500–300,000/μl (60–99% blasts) and were diagnosed from morphology and cytochemistry of blood and bone marrow specimens. All samples were collected prior to the initiation of any chemotherapy. Patients ranged in age from 18 months to 70 yr. Acute lymphoblastic leukaemia was only diagnosed when the periodic acid-Schiffs (PAS) stain was positive for blasts of lymphoid morphology. Two out of 5 patients with ALL had blasts which were immunologically typed as T cells. Acute myeloblastic leukaemia was diagnosed when 3% or more of blasts gave a positive peroxidase reaction or Auer rods were seen [17]. Acute myelomonocytic leukaemia or monocytic leukaemia were diagnosed when the proportion of promonocytes and monocytes in blood or marrow exceeded 20%, and these diagnoses were confirmed by a positive non-specific esterase (NSE) reaction or elevation of serum lysozyme above 10 μg/ml. Because AML and AMML are derived from a common stem cell, these two leukaemias were analysed in the one category. Acute undifferentiated leukaemia was diagnosed when no blast differentiation was apparent and the peroxidase, PAS and NSE reactions were all negative.

White cell and blast isolation

Venous blood from healthy subjects (120–180 ml) and leukaemic patients (10–30 ml) was defibrinated and lymphocytes and blasts separated by density centrifugation on a Ficoll-Isopaque gradient as previously described [12]. Polymorphonuclear leucocytes were prepared from defibrinated venous blood (60–180 ml) from normal donors by initial sedimentation in 0.4% Dextran T500, after which the supernate (containing mainly leukocytes) was centrifuged over a Ficoll-Isopaque density gradient.

Cell suspensions

Cells were suspended at $0.5–2.5 \times 10^7$ cells/ml in imidazole-buffered saline. Cell counts were performed on a Coulter Model DN electronic particle counter with a 100-μm orifice (Coulter Electronics Ltd., U.K.). Homogeneity and cell morphology were checked for each preparation on a cytocentrifuge preparation. Normal lymphocyte preparations contained 95 ± 2% (S.D.) lymphocytes, 3 ± 1% monocytes and 2 ± 1% polymorphs, while acute leukaemic preparations always contained 95% blasts.

Measurement of [^{3}H]-*araC and* [^{3}H]-*CdR influx*

In general influx was calculated from the uptake of radioactivity by cells incubated briefly in media containing different concentrations of [^{3}H]-nucleoside. The specific activity of [^{3}H]-araC in the media was always measured to allow the calculation of [^{3}H]-araC uptake in pmol/10^7 cells. Specifically, the cell suspension (1.2 ml) was preincubated for 5 min in stoppered plastic tubes at 20.0°C, [^{3}H]-araC (1–380 μM) added at zero time and the suspension gently agitated. In most

experiments samples (1.0 ml) were removed, quickly layered over oil (0.3 ml) in an Eppendorf microcentrifuge tube and at 30 sec the uptake was terminated by centrifuging the cells at 8000 *g* for 4 min through the phthalate–oil mixture ('oil-stop' technique) [15]. The supernatant above the oil interface was sampled and counted to determine the concentration of araC. The remainder of the supernatant was aspirated, the walls of the microfuge tubes washed thrice with H_2O and most of the oil removed with the final washing. Cell pellets were solubilized in 0.5 ml of 0.5 N NaOH, scintillation fluid added and the vials acidified and counted. The non-facilitated uptake of [^{3}H]-araC was always measured in parallel incubations of cell suspensions containing 3 μM NBMPR. Facilitated influx was taken as the difference between the uptake of araC in the absence and the presence of NBMPR. All uptake values were corrected for araC in the trapped extracellular space of the cell pellet (0.15–0.30 μl/10^7 cells), the latter being measured in each experiment using a [^{14}C]-sucrose marker. This correction accounted for 10–50% of the total [^{3}H]-araC present in the cell pellets.

Kinetic analyses

The kinetics of facilitated influx of araC and CdR into white cells and blasts was analysed by two kinetic treatments: the Woolf–Augustinsson–Hofstee plot (V vs V/S) and the Hanes-Woolf plot (S/V vs S). Concordance was observed between estimates of the kinetic parameters from the two methods. Mean values ± S.D. are shown, and differences between means were analysed by Student's t test. All regression lines were fitted by the method of least squares. The inhibition of CdR influx by varying concentrations (73–365 μM) of araC was analysed according to a double reciprocal plot, with the inhibition constant being obtained from a secondary plot of slopes vs araC concentration.

RESULTS

Uptake characteristics

The time-course of uptake of araC into cells incubated at 20°C was measured so that the initial velocity of transport (i.e. influx) could be determined. Incubations were terminated at intervals of up to 60 sec by the addition of a high-affinity inhibitor NBMPR followed by a rapid separation of cells from extracellular label by centrifugation through phthalate oil. Figure 1 shows that nucleoside uptake into leukaemic blasts is linear up to 40 sec after the addition of [^{3}H]-araC. The non-facilitated influx, determined as the uptake of [^{3}H]-araC in the presence of 3 μM NBMPR, represents approximately 14% of the total uptake. The subsequent kinetic analyses refer only to the facilitated influx, i.e. the difference between the total and non-facilitated uptake of nucleoside after 30 sec incubation.

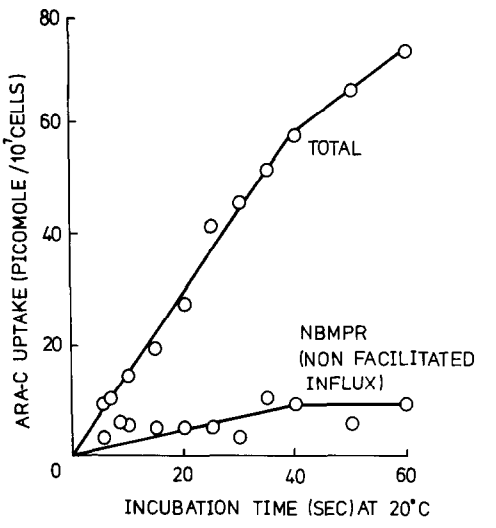

Fig. 1. Time-course of araC uptake by leukaemic myeloblasts. AraC was added to give 75 μM final concentration in a cell suspension preincubated at 20°C in the absence or presence of 3 μM NBMPR.

Kinetic analysis of araC influx

The initial uptake rate (influx) of [^{3}H]-araC was determined on blast preparations from a variety of leukaemias for araC concentrations usually from 1–380 μM. Figure 2 shows that the facilitated [^{3}H]-araC influx increased in a hyperbolic or saturating dependence on araC concentration when at each concentration the facilitated component was defined as the difference between total and non-facilitated influx. The linear plot of S/V vs S (bottom, Fig. 2) derived from the influx-concentration curve yields a V_{max} of 227 pmol/10^7 cells/min and a K_m of 383 μM for blasts from a patient with acute undifferentiated leukaemia.

AraC and its natural analogue deoxycytidine (CdR) showed similar kinetics for facilitated influx into blasts. The kinetic parameters for the two substrates were measured in parallel on the same preparation of blasts from 4 different leukaemic patients, and Table 1 shows that the V_{max} for araC and CdR did not differ significantly. However, the mean K_m for CdR influx was significantly lower than for araC influx ($P < 0.01$).

Competition between deoxycytidine and araC for entry

The effect of araC on the uptake of its natural analogue deoxycytidine was studied. Double reciprocal plots of the influx of [^{3}H]-deoxycytidine into leukaemic myeloblasts (H.F.) at four

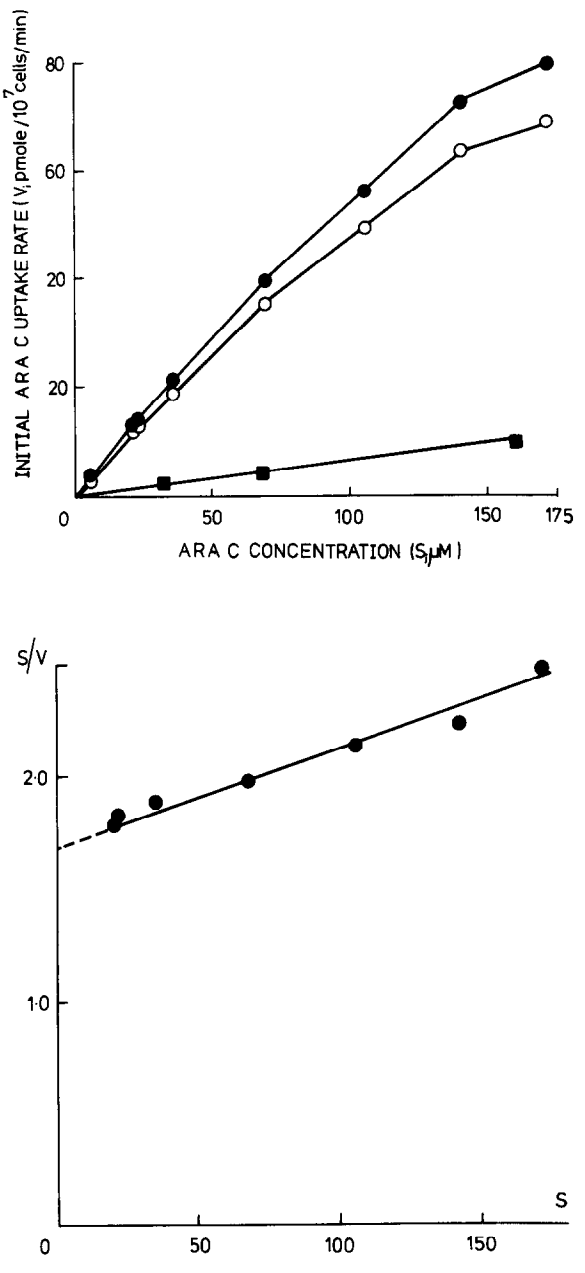

Fig. 2. Dependence of araC influx on araC concentration. A suspension of leukaemic blasts was incubated with 5–160 μM araC either in the absence ● or presence ■ of 3 μM NBMPR. The difference between the two influxes represents facilitated influx ○ and was analysed kinetically by plotting S/V vs S (bottom).

different concentrations of araC (0, 73, 182 and 365 μM) are shown in Fig. 3. Plots were linear and intersected on the ordinate axis, suggesting that araC was a competitive inhibitor of deoxycytidine influx. The slopes of these plots were proportional to the araC concentration (inset Fig. 3) and a value of 584 μM was obtained for the inhibitor constant (K_1). In blasts from the same patient the influx of [^{3}H]-CdR added alone yielded kinetic parameters of V_{max} 340 pmol/10^7 cells/min and K_m 169 μM.

During the infusion of araC to patients with leukaemia a high plasma level of araU results from hepatic deamination of the drug [3]. AraU inhibits the influx of araC into blasts and competitive kinetics is observed. In one experiment an inhibitor constant of 616 μM was observed in a preparation of undifferentiated blasts (R.G.) which transported [^{3}H]-araC with K_m = 340 μM and V_{max} = 74 pmol/10^7/min in the absence of araU.

Effect of phloretin on araC influx

The plant sterol phloretin is a powerful inhibitor of many facilitated diffusion systems. Phloretin inhibited araC influx into myeloblasts and analysis yielded an inhibitor constant of 7 μM (Fig. 4). The glycoside derivative phloridzin was several fold less effective as an inhibitor of araC influx.

Facilitated AraC influx into blasts of the different leukaemias

The kinetics of araC influx into blasts from 24 patients with acute leukaemia were measured and compared to normal lymphocyte and polymorph preparations (Table 2). Maximal araC influx (V_{max}) into blasts was 9- to 12-fold greater than for the corresponding mature cells of the same series ($P < 0.01$). AraC influx into myeloblast was 3.9-fold greater than into lymphoblasts ($P < 0.02$). Blasts from acute undifferentiated leukaemia transported araC with a V_{max} intermediate between myeloblasts and lymphoblasts. Table 2 shows that the conclusions above also held true

Table 1. Comparison of kinetic parameters for cytosine arabinoside and deoxycytidine influx in leukaemic blasts

Patient	Facilitated influx rate (pmol/10^7 cells/min) at V_{max} AraC	CdR	at 1 μM AraC	Cdr	K_m (μM) AraC	CdR
K.C.(AML)	513	502	1.49	1.89	344	265
A.B.(AUL)	270	262	1.45	2.37	185	109
H.M.(AMML)	324	316	1.11	1.66	292	190
V.P.(AMML)	433	331	1.17	2.32	368	142

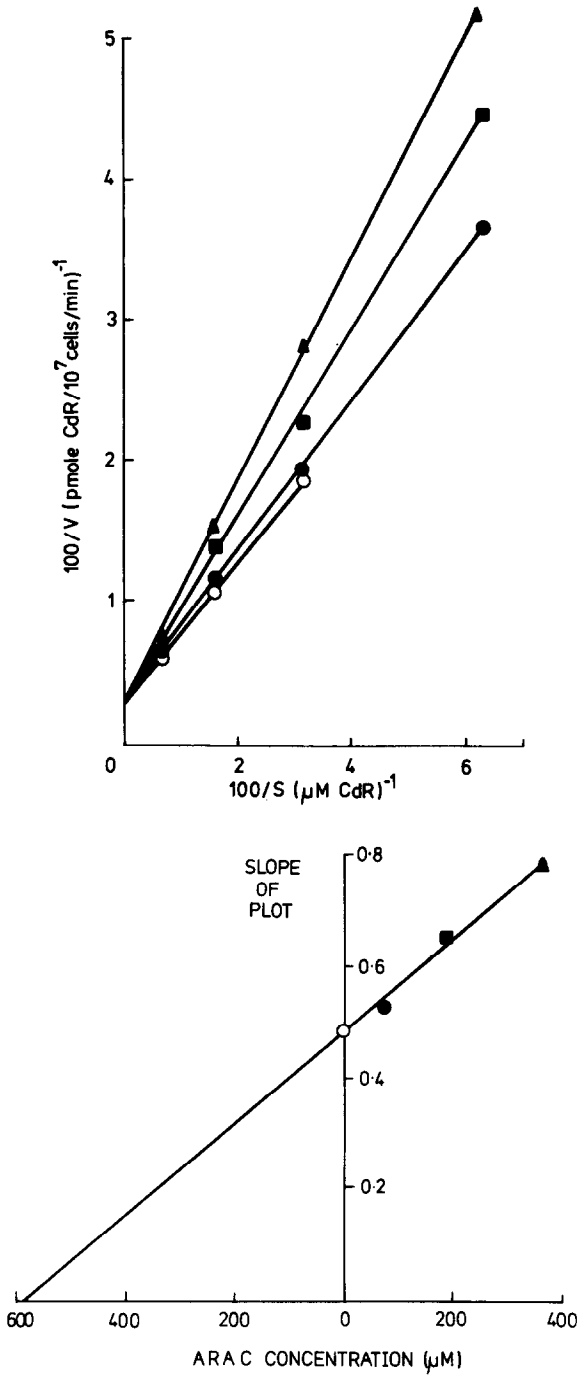

Fig. 3. Double reciprocal plot of deoxycytidine influx into leukaemic blasts and its inhibition by araC. Cell suspensions were preincubated at 20°C and araC added to give final concentrations of zero ○, 73 μM ●, 182 ■ and 365 μM ▲ immediately prior to commencing [³H]-deoxycytidine influx. The linear relation between araC concentration and the slope of the double reciprocal plots (bottom) extrapolates to yield a K_i of 584 μM araC.

for araC influx at 1 μM concentration. The K_m for the transport of araC varied considerably between individuals, although the lowest values were observed in normal lymphocytes and the highest in blasts (Table 2). Myeloblasts and lymphoblasts did not differ significantly in their mean K_m values for araC influx (255 and 214 μM respectively). Comparison of the two parameters of nucleoside transport showed that the two appeared to be related since normal white cells with low V_{max} showed low K_m, while blasts with high V_{max} had high K_m (Fig. 5).

Relation of facilitated to total influx in different leukaemias

Although the total araC influx was much larger for myeloblasts than lymphoblasts, a constant proportion of the total flux appeared to be facilitated. Thus 80–87% of the total flux was facilitated at 1 μM araC and at 200 μM araC the facilitated fraction was 68–80% in leukaemic preparations of all types (Table 3). Although none of the leukaemias showed significant differences in the ratio of facilitated to non-facilitated influx, lymphocytes demonstrated a lower facilitated fraction (11–37% of total) than the other white cells studied (Table 3).

DISCUSSION

Facilitated diffusion of araC has been demonstrated in a variety of malignant cells such as hepatoma, ascites tumour cells and L1210 murine leukaemic blasts [18, 19]. The present study reports the kinetics of araC influx into fresh leukaemic blasts obtained from patients on no therapy or prior to initiating treatment. The major part of araC influx could be inhibited by NBMPR, a nucleoside analog which binds with high affinity to the facilitated transport mechanism [20]. While V_{max} for araC influx varied over an 80-fold range (10.9–898 pmol/10^7 cells/min) in blasts from different leukaemic individuals, the proportion of the total influx which could be inhibited by NBMPR was constant (80–87%) at a fixed therapeutic concentration of 1 μM araC. At araC concentrations corresponding to those attained by high-dose therapy (50–200 μM), it is

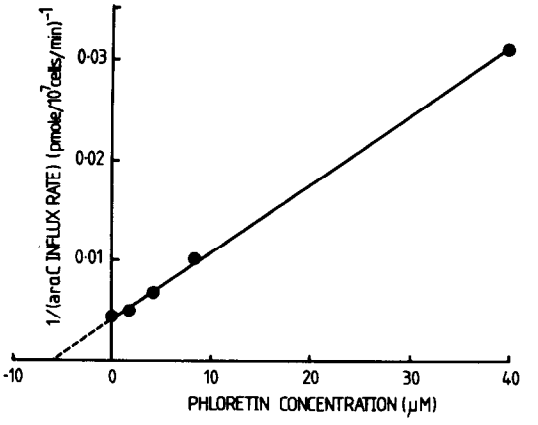

Fig. 4. A kinetic plot of the inhibition of araC influx by phloretin. The linear relation between the reciprocal of araC influx and inhibitor concentration extrapolates to yield a K_i of 7 μM for phloretin.

Table 2. Kinetic parameters for cytosine arabinoside influx into normal white cells and leukaemic blasts

Cell type	n	(pmol/10^7 cells/min) V_{max}*	V at 1 μM*	K_m* (μM)
Normal lymphocytes	5	10 ± 8	0.23 ± 0.22	51 ± 25
Normal polymorphs	3	54 ± 44	0.33 ± 0.15	142 ± 89
A.M.L./A.M.M.L.	12	503 ± 265	2.04 ± 0.98	255 ± 111
A.U.L.	7	155 ± 111	1.13 ± 0.76	197 ± 132
A.L.L.	5	128 ± 123	0.52 ± 0.40	214 ± 80

*All values show the mean ± 1 S.D.

Table 3. Relative amount of facilitated to total araC influx in different leukemias

	Facilitated influx/total araC influx × 100 (% of total) AML	AUL	ALL	PMN	LYMPHS
at 1 μM araC	87	88	80	77	37
at 100 μM araC	84	80	73	66	18
at 200 μM araC	80	75	68	62	11

The above percentages are the mean of between 4 and 13 different preparations of normal or leukemic white cells. Both polymorph and lymphocyte preparations were subjected to hypotonic shock step to reduce red cell contamination <10% while all leukemic preparations contained >95% blasts. Facilitated influx was always defined as the difference between total influx and the influx in the presence of 3 μM NBMPR measured in parallel incubations.

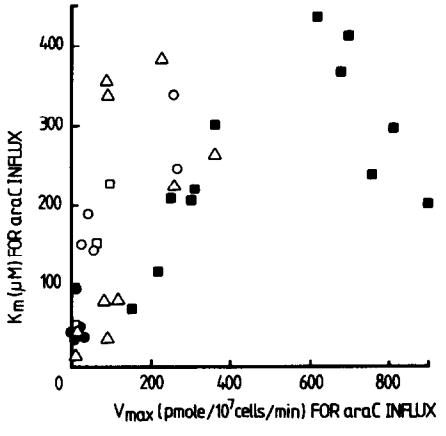

Fig. 5. Kinetic parameters V_{max} and K_m for araC influx in normal and leukaemic white cell preparations. The 35 different preparations were for AML or AMML ■, AUL △ and ALL ○ blasts, as well as normal lymphocyte ● and polymorph □ preparations. The positive correlation between K_m and V_{max} is r = 0.63.

evident from Table 3 that the bulk of araC entry into blasts (68–84%) is still by facilitated influx. However, the non-facilitated or leak pathway becomes relatively more important for araC entry at higher concentrations. In general, leukaemic and normal white cells from peripheral blood resemble other cells in which the predominant route of nucleoside influx is by a facilitated diffusion mechanism [15].

The influx of araC and its natural analog deoxycytidine into leukaemic blasts has similar kinetic characteristics. Values of the V_{max} are similar but deoxycytidine appears to be bound more strongly to the carrier, as evidenced by the lower K_m value (Table 1). Double reciprocal plots of kinetic data (Fig. 3) show that araC acts as a competitive inhibitor of deoxycytidine uptake, although in the patients studied the inhibitor constant (K_i = 584 μM) was 2-fold larger than the K_m for the araC uptake alone ($K_m \simeq 300$ μM). A similar discrepancy between apparent K_i and K_m has been found in cultured fibroblasts in which various nucleosides such as adenosine, uridine, guanosine, thymidine and cytidine all compete with araC for entry during 10-sec incubations [21]. However, the kinetic significance of a half maximal inhibitor concentration is obscure since two permeating nucleosides will not only compete for entry, but also produce accelerative exchange diffusion with nucleoside which has entered the cell [22]. The collective evidence suggests that araC and deoxycytidine are transported into the cell on the same carrier molecule. Certainly, the competition between these two nucleosides for transport may explain in part why araCTP formation in leukaemic myeloblasts incubated with araC is usually decreased by the addition of equimolar deoxycytidine [23]. The inhibitory effect of phloretin on araC influx provides further evidence for the facilitated nature of nucleoside permeation. Phloretin is a well-known inhibitor of glucose transfer in human red cells, as well as leucine transport and Na^+–Li^+ counter-transport [24–26].

The concentration of phloretin required to half maximally inhibit these transport processes lies in the range 6–12 μM, similar to the value of 7 μM obtained for inhibition of nucleoside fluxes. This broad inhibitory action of phloretin on many facilitated transport processes must arise from its action either on the hydrophobic portion of the transporter or by some pertubation of the membrane bilayer itself.

The wide differences in K_m for araC transport in leukaemic and normal white cells was unexpected but is supported by one kinetic study of araC influx into cultured cells *in vitro*. Murine L1210 leukaemic cells with low V_{max} for araC influx showed a K_m of 30 μM, while in contrast, Yoshida ascites tumour cells with high araC influx showed a K_m of 400 μM [18]. The present data show that blasts with high araC influx have a K_m between 200 and 250 μM, while normal white cells generally demonstrate lower K_m values. The same phenomenon has also been shown for the facilitated transport of sugars, where raising carrier mobility (V_{max}) with insulin leads to a parallel increase in K_m [27]. The positive correlation between V_{max} and K_m depicted in Fig. 5 suggests that the rate of the transport process (k_2) makes a significant contribution to the K_m value. If we accept that the facilitated transport occurs via a Michaelian mechanism, then both V_{max} and K_m are controlled by the rate of the transport process:

$$T + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TS \xrightarrow{k_2} T + S$$

$$K_m = (k_{-1} + k_2)/k_1$$
$$V_{max} = k_2(T)_0,$$

where $(T)_0$ is the total concentration of transport sites and k_2 is the rate of translocation of the substrate across the membrane (i.e. turnover rate of the carrier). Thus in this system the K_m value cannot be approximated to the dissociation constant for the binding of the transported compound to its carrier protein. We have shown previously that the maximum rate of transport of araC in a variety of leukaemic cells is directly proportional to the number of transport sites on the cell membrane [12], and this remains the main determinant of the differences in V_{max} amongst the various types of leukaemia (Table 2).

The first important conclusion from this study is that araC enters leukaemic blasts predominantly by a facilitated diffusion pathway both at conventional therapeutic levels (0.4–1.0 μM) and at concentrations attained by the new high-dose araC protocols. Table 3 shows that uptake of araC by myeloblasts is 87% facilitated at 1 μM, 84% facilitated at 100 μM and 80% facilitated at 200 μM, the latter corresponding to peak plasma levels observed with high-dose araC (2 or 3 g/m^2 given every 12 hr as a short infusion). Lymphoblasts also transport araC predominantly by the facilitated pathway at concentrations between 1 and 200 μM (80 and 68% respectively). The second major conclusion is that the 4-fold greater facilitated araC transport in myeloblasts than in lymphoblasts (Table 2) also holds for total uptake rates at 1, 100 and 200 μM araC. Does the greater transport rate of araC into myeloblasts explain the relative responsiveness of this leukaemia to araC? Certainly, no differences in deoxycytidine kinase activity can be demonstrated between patients who are responders or non-responders to the drug [28, 29]. However, membrane transport is a major rate-limiting step for araCTP formation from 1 μM araC [Wiley, Taupin and Sawyer, unpublished data], and the use of high-dose araC should generate more araCTP in acute leukaemias with proven low capacity for membrane transport of this drug. Pilot studies of high-dose araC have given promising results in the therapy of refractory acute lymphoblastic leukaemia and lymphomas for which conventional dose araC would be ineffective [8, 9].

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