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^{19}F NMR Study of the Uptake of 2′-Fluoro-5-methyl-β-L-arabinofuranosyluracil in Erythrocytes

EVIDENCE OF TRANSPORT BY FACILITATED AND NONFACILITATED PATHWAYS

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ABSTRACT. The ^{19}F NMR resonances of intra- and extracellular 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU) in suspensions of human erythrocytes are well resolved. This phenomenon allows its transport behavior to be monitored in a ^{19}F NMR time-course experiment. The rate of L-FMAU uptake at 25° in a suspension containing L-FMAU at an initial extracellular concentration of 4 mM was $7.6 \pm 1.0 \times 10^{-7}$ pmol cell^{-1} sec^{-1} ($N = 5$). Concentration-dependent uptake studies of L-FMAU indicate the existence of both saturable and nonsaturable transport mechanisms, with a K_m for the saturable uptake of approximately 1 mM. Although the transport of L-FMAU at 25° was inhibited significantly (54–65%) by nitrobenzylthioinosine (NBTI) and dipyridamole, consistent with the participation of the nucleoside transporter, these inhibitors did not achieve complete blockage of L-FMAU uptake. The participation of the nucleobase transporter in L-FMAU uptake was ruled out by the absence of competition with uracil uptake, and by the lack of inhibition by papaverine. In addition, the NBTI-insensitive uptake of L-FMAU was not affected by pretreatment of the cells with the sulfhydryl reagent, *p*-chloromercuriphenylsulfonic acid (pCMBS). However, the NBTI- and dipyridamole-insensitive transport of L-FMAU was found to increase upon treatment of the erythrocytes with butanol, an agent that affects membrane fluidity. The partition coefficient of L-FMAU in octanol/phosphate-buffered saline determined by absorption spectrophotometry was 0.31. These data indicate that under the conditions of the studies, L-FMAU uptake by erythrocytes proceeds by both the nucleoside transporter and nonfacilitated membrane diffusion. BIOCHEM PHARMACOL **55**;10:1611–1619, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; nucleoside analogs; membrane transport; erythrocytes; ^{19}F NMR

Nucleoside analogs represent one of the most useful classes of pharmacological agents for the treatment of cancer, AIDS, hepatitis, and other viral diseases [1, 2]. Indeed, most of the antiviral agents currently in clinical use are nucleoside analogs. The biochemical mechanism of the antiviral activity of analogs such as AZT§ (or Zidovudine) is based largely on the inhibition of HIV reverse transcriptase [3, 4], whereas analogs such as 3TC (or Lamivudine) inhibit hepatitis B virus by terminating the viral DNA replication catalyzed by DNA polymerase [5].

The ability of a drug to function at the cellular level is a function of many parameters, including its transport into cells [6–8]. The pathways involved in the transmembrane uptake of nucleoside analogs vary widely as a function of molecular structure and cell type [9, 10]. Several dideoxynucleosides (e.g. AZT) that inhibit HIV and hepatitis B virus are known to be transported across human erythrocyte membranes by nonfacilitated diffusion [6–8, 11]. The substantial transport of AZT by simple nonfacilitated diffusion is thought to reflect the much greater octanol/water partition coefficient (1.26) compared with that of thymidine (0.085), which results from substitution of the 3′-OH of thymidine with an azido group [6, 12]. As a result of the transport by membrane diffusion, the uptake of AZT at high concentrations (>2 mM) by erythrocytes becomes more efficient than that of naturally occurring nucleosides such as thymidine and uridine that are transported exclusively by the erythrocyte nucleoside transporter [6, 13, 14]. This uptake mechanism for AZT is also thought to explain its increased effectiveness in inhibiting the virus when used in conjunction with dipyridamole [6]. The preferential inhibition of thymidine uptake by dipyridamole may decrease the competition of thymidine for phosphorylation by cellular thymidine kinase (EC 2.7.1.21), thus

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§ *Abbreviations:* AZT, 3′-azido-3′-deoxythymidine; L-FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; NBTI, nitrobenzylthioinosine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; and pCMBS, *p*-chloromercuriphenylsulfonic acid.

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increasing the cellular level of the active, triphosphate form of AZT.

L-FMAU is a fluorinated L-arabinoside analog of thymidine. It has been shown to inhibit the growth of hepatitis and Epstein–Barr virus under *in vitro* conditions [15–17]. In contrast to the D-enantiomer, L-FMAU is a potent inhibitor (IC_{50} = 0.1 μM) of viral DNA replication in human HepG2 cells transfected with the HBV genome (2.2.15 cells) [16]. In addition, Pai *et al.* [16] have reported that L-FMAU does not show significant cytotoxicity at concentrations as high as 200 μM. Similarly, L-FMAU shows a potent inhibitory effect on Epstein–Barr viral replication (EC_{90} = 5 μM) in H1 cells [17]. The high antiviral potency and selectivity of L-FMAU have prompted us to investigate the pathways involved in the uptake of this compound. We report here the characterization of the pathways of L-FMAU transport across human erythrocyte membranes based on ^{19}F NMR spectroscopic measurements of its uptake.

MATERIALS AND METHODS

L-FMAU (purity > 99%) was synthesized as described previously [15]. L-FMAU was dissolved in PBS (10 mM of Na_2HPO_4/NaH_2PO_4, 145 mM of NaCl, 10 mM of glucose, pH ~7), or HEPES-buffered saline (10 mM of HEPES, 145 mM of NaCl, 10 mM of glucose, pH 7.4) before being added to the erythrocyte suspensions to achieve the required concentration for the transport measurements. NBTI, dipyridamole, papaverine, pCMBS, and uracil were purchased from the Sigma Chemical Co. All other reagents are of analytical grade.

The partition coefficient of L-FMAU in 1-octanol/PBS was determined using a shaker-flask procedure [12]. Three samples of PBS containing 0.1 mM of L-FMAU were mixed with an equal volume of 1-octanol. The suspensions were then mixed vigorously using a vortex mixer for ~5 min at room temperature. The PBS phase and octanol phase were then separated by centrifugation (2984 *g*, 10 min, ~4°). The relative concentration of L-FMAU in each phase was then measured spectrophotometrically at 270 nm on an HP-8451A spectrophotometer. The partition coefficient is given as the ratio of $[\text{L-FMAU}]_{octanol}/[\text{L-FMAU}]_{PBS}$.

Human erythrocytes derived from healthy volunteers were obtained from the American Red Cross. Routinely, the red cells were re-suspended in isotonic PBS or 10 mM HEPES-buffered saline, and the Blood Bank preserving medium was removed by aspiration after centrifugation of the suspensions. Then the cell suspensions were saturated continuously with CO for ~10 min to convert oxy- and deoxyhemoglobin to diamagnetic carbonmonoxyhemoglobin, which helps to decrease the linewidth of the NMR resonances of L-FMAU in the erythrocyte suspensions.

The uptake of L-FMAU was initiated by the addition of an aliquot of the stock L-FMAU solution (10 mM in isotonic PBS or HEPES-buffered saline) to the erythrocyte suspensions for a final total concentration in suspension of 2.5 mM (i.e. an initial extracellular L-FMAU concentration of ~4 mM) at 25°, unless otherwise indicated. At this sub-physiological temperature, the uptake of L-FMAU was sufficiently slow to allow it to be monitored by acquiring a series of ^{19}F NMR spectra as a function of time. Typically, the first NMR spectrum was acquired ~2 min after initiation of L-FMAU uptake. Proton decoupled-^{19}F ($^{19}F\{^1H\}$) NMR spectra were acquired on a GN-500 or a Varian Unity Plus 500 MHz NMR spectrometer, both equipped with 5-mm $^1H/^{19}F$ dual probes. Typically, the data from 32, 56, or 64 scans were acquired in blocks of 4 K data points. The spectra were acquired with either a 68° or 90° pulse and a duty cycle of 1.28 or 6.18 sec (T_1 = 1.1 sec for L-FMAU in PBS at 22°), where indicated, respectively. To correct for the partial NMR signal saturation when a 68° pulse and 1.28 sec duty cycle were used for spectral acquisition, a fully relaxed spectrum of the sample was acquired with a 90° pulse and a duty cycle of 6.18 sec subsequent to transmembrane equilibration of L-FMAU. A comparison of the intensities of the spectra acquired with and without full relaxation allows the correction of the resonance intensities for the effects of overpulsing. The midpoint of the acquisition of each spectrum was taken as the time of sampling of L-FMAU uptake and used in the subsequent data analysis. The relative distribution of L-FMAU between the cytosol and the extracellular solution was derived from the resonance intensities determined by either spectral integration or fitting a Lorentzian function to the spectral peak and corrected by hematocrit and intracellular solvent fractional volume, as discussed below. The typical errors in spectral intensity estimates are approximately 10–15%. The NMR samples (typical hematocrit ~45%) were not spun to minimize cell sedimentation during spectral acquisition.

The intracellular and extracellular L-FMAU resonance intensities were corrected for overpulsing and normalized using the following expressions:

$$S_i = \frac{I_i f_i^{sat}}{I_i f_i^{sat} + I_e f_e^{sat}}, \qquad S_e = \frac{I_e f_e^{sat}}{I_i f_i^{sat} + I_e f_e^{sat}} \tag{1}$$

where I_i and I_e are the intracellular and extracellular resonance intensities, and f_i^{sat} and f_e^{sat} are the corresponding saturation factors. The signal intensities were converted to the ratio between the concentration of intracellular L-FMAU ($[\text{L-FMAU}]_i$) and that of L-FMAU of the suspension ($[\text{L-FMAU}]_{susp}$) according to:

$$\frac{[\text{L-FMAU}]_i}{[\text{L- FMAU}]_{susp}} = \frac{S_i}{\alpha H_t} \tag{2}$$

and similarly:

$$\frac{[\text{L-FMAU}]_e}{[\text{L- FMAU}]_{susp}} = \frac{S_e}{1 - H_t} \tag{3}$$

for extracellular L-FMAU, where H_t is the hematocrit of the NMR sample. The parameter α is the fractional intracellular volume accessible to L-FMAU, which was estimated from the mean cell volume (MCV) of the suspensions and the $MCV_{physiological}$ and $\alpha_{physiological}$ of erythrocytes under physiological conditions ($MCV_{physiological} = 8.3 \times 10^{-11}$ cm^3 [18]; $\alpha_{physiological} = 0.717$ [19]). MCV was determined from mean cell count and H_t. $[L\text{-FMAU}]_{susp}$ is the concentration calculated based on L-FMAU/V_o, where V_o is the total volume of the NMR sample. We note that since all of the L-FMAU is initially in the extracellular compartment, thus, $[L\text{-FMA}]_e$ ($t = 0$) is greater than $[L\text{-FMAU}]_{susp}$. A linear function was then fitted to the initial portion of the time-dependent concentration ratio data to obtain the influx rate constant k_i (see Fig. 1B), and the corresponding membrane permeability coefficients (P_i) were obtained using the relation:

$$P_i = k_i \frac{MCV(1 - H_t)}{A_{cell}H_t} \quad (4)$$

A_{cell} is the mean cell surface area (1.43×10^{-6} cm^2) [20]. The initial rate of L-FMAU influx (V_i) was then calculated from the rate constant by:

$$V_i = [L\text{-FMAU}]_{extracellular}(1 - H_t)k_i \frac{MCV}{H_t} \quad (5)$$

where $[L\text{-FMAU}]_{extracellular}$ is the extracellular L-FMAU concentration at $t = 0$.

In the present work, the inhibition of L-FMAU uptake by NBTI of various concentrations was measured at 15° except where otherwise indicated. All other transport measurements were performed at 25°. The permeability coefficients and influx determined in the presence of transport inhibition were compared with the respective controls determined at the corresponding temperature in order to correct for the temperature dependence of L-FMAU uptake. Inhibition of L-FMAU uptake by nucleoside transport inhibitors was described by the function:

$$P_i = P_o - C_1 \frac{[Inhibitor]}{[Inhibitor] + K_i} \quad (6)$$

where P_o denotes the permeability coefficient in the absence of inhibitors; K_i is the inhibition constant, and C_1 is a constant that reflects the transport by the inhibitor-sensitive carrier. An analogous equation can be written for V_i. The L-FMAU concentration-dependent uptake in the absence of inhibitor was analyzed by fitting the following modified Michaelis–Menten equation [21]:

$$V_i = \frac{V_{max}[L\text{-FMAU}]_{extracellular}}{[L\text{-FMAU}]_{extracellular} + K_m} + C_2[L\text{-FMAU}]_{extracellular} \quad (7)$$

were V_{max}, K_m and C_2 denote the maximum rate of uptake, the apparent affinity of L-FMAU to the transporter, and a proportionality constant that reflects the transport by inhibitor-insensitive pathways, respectively.

RESULTS

L-FMAU Uptake by Erythrocytes with and without the Presence of Various Inhibitors

Figure 1A shows a series of sequential proton decoupled ^{19}F NMR spectra acquired as a function of time after the addition of L-FMAU to an erythrocyte suspension (H_t = 41.2%) at 25°. Note that the intracellular L-FMAU resonated at 0.38 ppm up-frequency from the extracellular L-FMAU. The uptake of L-FMAU by the erythrocytes was apparent from the time-dependent increase in the intracellular L-FMAU resonance and the simultaneous decrease in the respective extracellular resonance. The intracellular L-FMAU linewidth (~47 Hz) was typically broader than the extracellular value (~19 Hz). The S/N ratios for the intra- and extracellular L-FMAU were 3 and 68, and 9 and 36 for the spectra of the first and last time point, respectively. The ratios between both the time-dependent intra- and extracellular L-FMAU concentrations and that of L-FMAU in the suspension (Equations 2 and 3) are shown in Fig. 1B. Note the calculated intra- and extracellular L-FMAU concentration ratios became nearly equal after reaching equilibrium (Fig. 1B). The influx rate constant of the uptake was 0.095 ± 0.008 sec^{-1}; the corresponding permeability and influx rate were 17.0 ± 1.4 × 10^{-8} cm sec^{-1} and 10.2 ± 0.9 × 10^{-7} pmol cell^{-1} sec^{-1}, where the errors are derived from the standard deviations of the respective rate constants. The uptake of L-FMAU by erythrocytes from 8 donors was measured at both 15° and 25° under analogous conditions (Table 1). It was evident that an increase in temperature from 15° to 25° resulted in an ~3-fold increase in the L-FMAU permeability and influx rate.

Treatment of the red cells with the nucleoside transport inhibitor NBTI [22] resulted in an NBTI concentration-dependent decrease in L-FMAU influx (Fig. 2). At 15° and low NBTI concentrations (<0.5 μM), there was an initial, pronounced drop in L-FMAU influx. However, further increases in the NBTI concentration had no significant effect on the uptake, which leveled off at higher NBTI concentrations to 35.3 ± 3.5% of the uninhibited rate of influx (Fig. 2). Similarly, at 25°, L-FMAU transport was significantly, but incompletely, inhibited by NBTI (Tables 1 and 2). The fractional influx that was insensitive to the inhibitor at this temperature was 34.7 ± 7.2%.

Inhibition studies at 25° using dipyridamole, also a potent inhibitor of erythrocyte nucleoside transporter [10], yielded results that were similar to those obtained using NBTI. The influx rate decreased with an increase in dipyridamole concentration (Fig. 3). Similar to the findings with NBTI, apparent saturation of the nucleoside transporter with dipyridamole did not completely block L-FMAU uptake. Analysis of the data using Equation 6 yielded the fractional influx insensitive to dipyridamole as

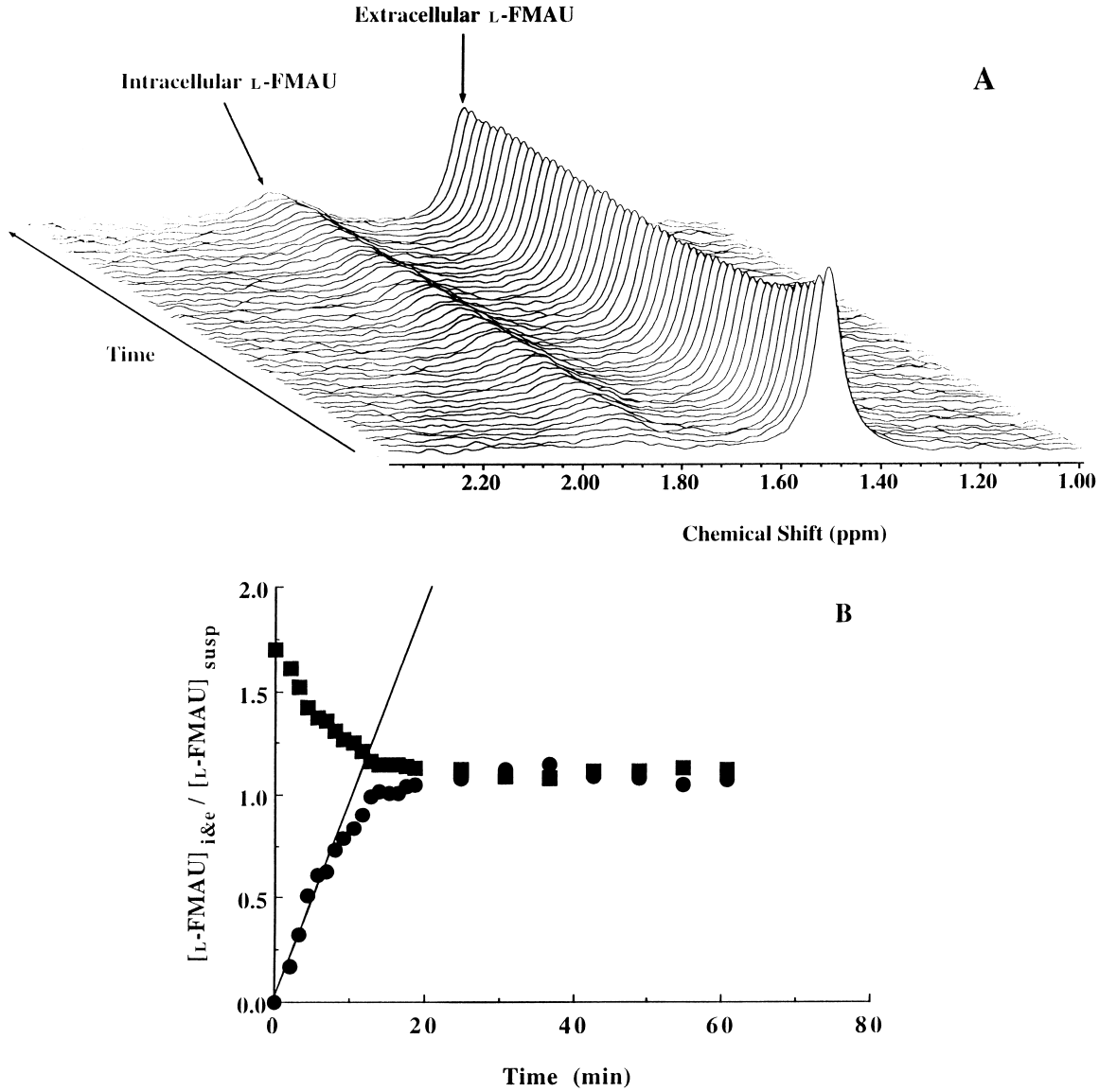

FIG. 1. (A) Proton decoupled-^{19}F NMR spectra of L-FMAU in a suspension of human erythrocytes (H_t = 41.2%) obtained as a function of time upon addition of L-FMAU to give an initial extracellular concentration of 4.2 mM (total suspension concentration of 2.5 mM) at 25°. A 10-Hz exponential apodization was applied to the FID prior to Fourier transformation. The carrier frequency of 470.48 MHz was arbitrarily set to 0.0 ppm as the chemical shift reference. Each spectrum is the sum of 56 acquisitions acquired over 1.2 min, and the total time course shown corresponds to 61 min. The midpoint of each acquisition, referenced to the addition of L-FMAU at t = 0, corresponds to the time of L-FMAU uptake. (B) Plot of the concentration ratio of intracellular L-FMAU (●) or extracellular L-FMAU (■) relative to $[L\text{-FMAU}]_{susp}$ at various time points after the addition of L-FMAU to the sample. The ratios were estimated using Equations 2 and 3. After 19 min when the sample had reached transmembrane distribution equilibrium, only every fifth data point is shown to simplify the presentation. The initial apparent rate constant for L-FMAU uptake determined from a linear regression analysis of the data shown was ~0.095 sec^{-1}.

45.7 ± 2.1% of the control determined in the uninhibited cells. Under the present conditions, the K_i and IC_{50} of dipyridamole on L-FMAU uptake were 81 ± 35 nM and ~27 nM, respectively.

To investigate the possible involvement of the nucleobase transporter in L-FMAU transport, L-FMAU permeability coefficients were measured in the NBTI-treated cells that were incubated subsequently with papaverine (25° for 10 min), a reported inhibitor of the nucleobase transporter [23, 24], at concentrations ranging from 0 to 1000 μM. No consistent change in the L-FMAU permeability coefficient was correlated with papaverine concentration. The mean permeability measured at seven different papaverine concentrations was $5.7 \pm 0.6 \times 10^{-8}$ cm sec^{-1} (N = 7), which is similar to the mean value of $4.5 \pm 0.8 \times 10^{-8}$ cm sec^{-1} (N = 7) reported in Table 2, which was determined in the presence of NBTI only. Similarly, the influx rates of L-FMAU were not affected by the papaverine incubation (data not shown here).

The transport of L-FMAU was also measured in the

TABLE 1. L-FMAU uptake by human erythrocytes in the absence of transport inhibitors*

Donor	15° P_i ($\times 10^8$ cm sec^{-1})	15° V_i ($\times 10^7$ pmol cell^{-1} sec^{-1})	Donor	25° P_i ($\times 10^8$ cm sec^{-1})	25° V_i ($\times 10^7$ pmol cell^{-1} sec^{-1})
1	4.6 ± 0.4	2.9 ± 0.3	4	10.2 ± 0.4	5.7 ± 0.2
2	2.6 ± 0.1	2.1 ± 0.1	5	12.8 ± 1.1	7.2 ± 0.6
3	5.7 ± 1.5	2.8 ± 0.7	6	9.7 ± 0.4	6.1 ± 2.6
			7	16.9 ± 1.4	10.7 ± 0.4
			8	12.9 ± 1.6	8.6 ± 1.1
Mean ± SD	4.3 ± 1.3 ($N = 3$)	2.6 ± 0.4 ($N = 3$)	Mean ± SD	12.5 ± 2.6 ($N = 5$)	7.6 ± 1.0 ($N = 5$)

*The mean permeability coefficients (P_i) and influx rates (V_i) for the uptake of L-FMAU at an initial extracellular concentration of 4 mM were determined as described in Materials and Methods. The SDs of the permeability coefficient and influx rate of the individual donors were derived from a linear regression analysis of duplicate measurements for the respective time-course data.

presence of uracil, a substrate for the nucleobase transporter [10, 25]. As in the above studies, the erythrocytes were preincubated with NBTI, and L-FMAU uptake was monitored at initial extracellular uracil concentrations ranging from 0.9 to 8.7 mM. As in the papaverine studies, the measured L-FMAU permeability was found to be independent of the presence of extracellular uracil. A mean L-FMAU permeability of 4.3 ± 1.0 cm sec^{-1} ($N = 4$) was obtained, which again is in good agreement with the data of Table 2 for cells treated with NBTI. Additionally, no evidence of uracil dependence of the influx rates of L-FMAU was found in erythrocytes not pretreated with NBTI (data not shown).

To further estimate the effects of other transporters containing reactive sulfhydryl groups, the uptake of L-FMAU was also measured in cells treated with both NBTI and pCMBS, a mercurial sulfhydryl reagent. The results for the doubly treated cells gave a mean permeability of 5.8 ± 2.0 cm sec^{-1} ($N = 4$) at pCMBS concentrations ranging from 0.1 to 4 mM. This is again consistent with the data given in Table 2, suggesting that no other sulfhydryl-containing transporters contribute significantly to L-FMAU uptake in erythrocytes. We note, however, that there was significant hemolysis of the cells upon pCMBS incubation, which may have contributed to the significant error noted above for the measured permeability values.

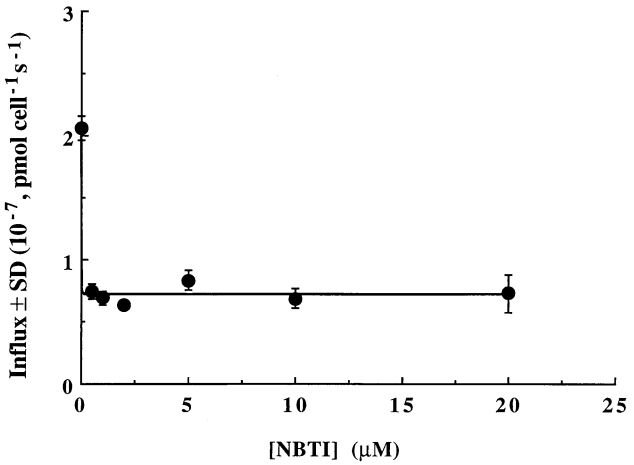

FIG. 2. L-FMAU influx rate across the membranes of erythrocytes of a single donor as a function of NBTI concentration. The erythrocytes were pretreated with the indicated concentrations of NBTI prior to the addition of L-FMAU to give an initial extracellular concentration of ~4 mM, and the subsequent spectra were acquired at 15°. The mean influx rate measured at [NBTI] > 1 μM, 7.2 ± 0.1 × 10^{-8} pmol cell^{-1} sec^{-1} (N = 6), represents the influx insensitive to the nucleoside transporter inhibitor under these conditions of concentration and temperature. The error bars denote the SDs derived from a linear regression of the uptake data derived from duplicate measurements.

Concentration Dependence of L-FMAU Uptake

L-FMAU uptake was monitored as a function of its concentration. At low substrate concentrations (<10 mM), L-FMAU uptake was markedly non-linear, and followed a curve predicted by Equation 7 (Fig. 4). As is apparent from the figure, L-FMAU uptake was found to be nonsaturable, suggesting L-FMAU uptake independent of a transporter-

TABLE 2. L-FMAU uptake at 25° by human erythrocytes in the presence of maximum inhibition of nucleoside transporter*

Donor	P_i ($\times 10^8$ cm sec^{-1})	V_i ($\times 10^7$ pmol cell^{-1} sec^{-1})	Inhibitor
4	4.9 ± 0.8	2.7 ± 0.2	1 μM Dipyridamole
4	4.4 ± 0.2	2.5 ± 0.1	5 μM Dipyridamole
5	6.2 ± 0.4	3.4 ± 0.2	5 μM NBTI
7	4.7 ± 0.3	2.7 ± 0.2	5 μM NBTI
10	3.5 ± 0.2	2.2 ± 0.1	5 μM NBTI
11	3.6 ± 0.2	2.3 ± 0.1	5 μM NBTI
12	4.3 ± 0.6	2.6 ± 0.4	5 μM NBTI
Mean ± SD	4.5 ± 0.8 ($N = 7$)	2.6 ± 0.4 ($N = 7$)	

*The mean permeability coefficients (P_i) and influx rates (V_i) for the uptake of L-FMAU at an initial extracellular concentration of 4 mM are shown; the erythrocytes were suspended in PBS with preincubation with the inhibitors of nucleoside transporter indicated. The permeabilities and influx rates were determined as described in Materials and Methods. The SDs of the permeability coefficient and influx rate of the individual donors were derived from a linear regression analysis of duplicate measurements for the respective time-course data.

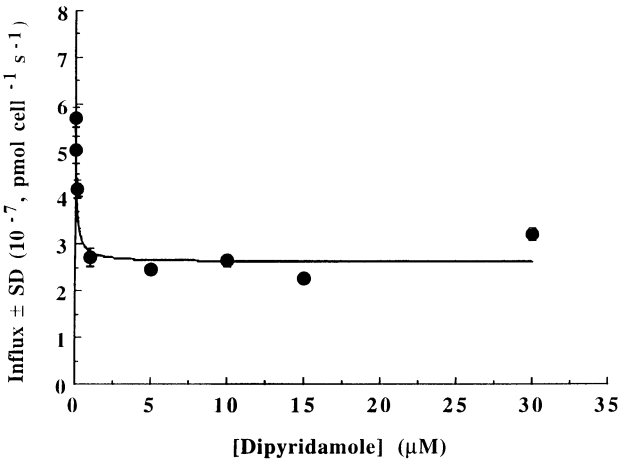

FIG. 3. Dipyridamole inhibition of L-FMAU uptake. The suspensions of erythrocytes in PBS were pretreated with dipyridamole at the concentrations indicated at 25° for ~20 min. The uptake was then monitored at 25° subsequent to the addition of L-FMAU to the dipyridamole-treated cells to achieve an initial extracellular concentration of 4 mM. The time-dependent uptake data were analyzed as described in Materials and Methods. Fitting of Equation 6 ($V_i = 5.7 - 3.1$ [Inhibitor]/([Inhibitor] + 8.1×10^{-2}) to the data by nonlinear least squares regression yields the fractions of transport by the dipyridamole-sensitive and -insensitive pathways, respectively. The error bars denote the SDs derived from a linear regression of the uptake data derived from duplicate measurements on the erythrocytes of a single donor.

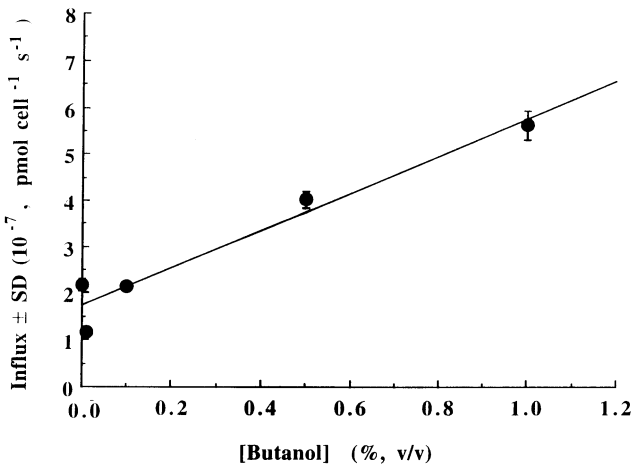

FIG. 5. Effect of 1-butanol on the L-FMAU influx rates determined from samples derived from a single donor. The erythrocyte suspensions in PBS ($H_t \sim 50\%$) were first incubated with a saturating level (~5 μM) of NBTI at 37° for 5 min. Aliquots of 1-butanol were then added gently to the suspensions to achieve the desired final concentration. This was followed by a further incubation at 25° for ~25 min prior to the addition of L-FMAU to initiate the uptake at 25°. The error bars denote the SDs derived from a linear regression of the uptake data corresponding to duplicate measurements on the erythrocytes from the same donor. A linear regression gave: $V_i = 1.7 + 4.0$ [butanol] ($R^2 = 0.95$).

mediated pathway. Nonlinear least squares regression of Equation 7 to the data yielded K_m and V_{max} values for L-FMAU uptake of 1 ± 0.8 mM and 7.1 ± 1.5 × 10^{-7} pmol $cell^{-1}$ sec^{-1}, respectively. This K_m value is typical of those found for non-natural nucleoside analogs [26]. Thus, at 4.7 mM of initial extracellular L-FMAU (or 2.5 mM of L-FMAU in the suspensions), the transport mediated by the nucleoside transporter was ~77.2% of the overall uptake. This is similar to that determined from the NBTI inhibition study (65.3 ± 13.2%), but slightly greater than that obtained under dipyridamole inhibition (54.3 ± 2.8%). The smaller fraction of transporter-mediated uptake shown in the dipyridamole study may be attributed to the lower (5.7 ± 0.2 × 10^{-7} pmol $cell^{-1}$ sec^{-1}) than average (7.6 ± 1.0 × 10^{-7} pmol $cell^{-1}$ sec^{-1}, Table 1) rate of uptake in the erythrocytes of this donor in the absence of inhibitor.

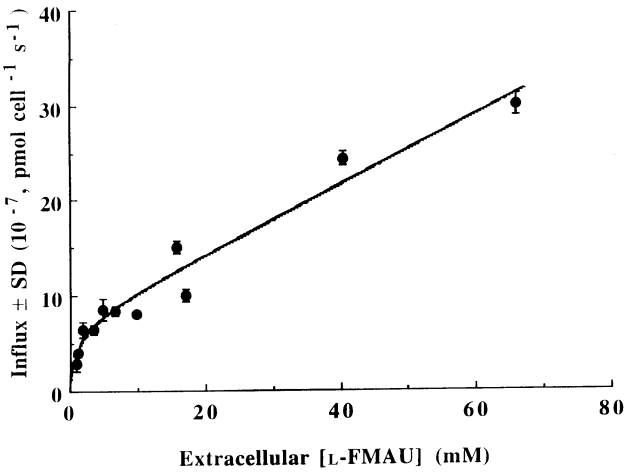

FIG. 4. Concentration dependence of L-FMAU uptake by erythrocytes in PBS (H_t = 35–47%). The uptake of L-FMAU at 25° was monitored after addition of aliquots of L-FMAU to the suspensions to achieve extracellular concentrations ranging from 0.8 to 65.8 mM. The data were fit using a nonlinear least squares regression to give the relation: $V_i = V_{max}$ $[L\text{-FMAU}]_{extra}/([L\text{-FMAU}]_{extra} + K_m) + C_2$ $[L\text{-FMAU}]_{extra}$, where K_m = 1.0 ± 0.8 mM, $C_2 = 4 \times 10^{-8}$ pmol $cell^{-1}$ sec^{-1} mM^{-1}, and $V_{max} = 7.1 \pm 1.5 \times 10^{-7}$ pmol $cell^{-1}$ sec^{-1}. SDs were derived from the non-linear regression of Equation 7 to the influx data derived from duplicate estimates on the red cells from a single donor.

1-Butanol Acceleration of NBTI-Insensitive Uptake and L-FMAU Partition Coefficient

To further test the hypothesis that the NBTI-insensitive transport of L-FMAU is due to nonfacilitated membrane diffusion, an agent capable of altering membrane properties was used to alter the membrane fluidity. When the NBTI-treated cells were incubated with 1-butanol at concentrations ranging from 0.01% (v/v, 1.7 mM) to 1% (v/v, 167 mM), a linear increase in the influx of L-FMAU as a function of butanol concentration was observed (Fig. 5). A butanol concentration of 1.0% was sufficient to compensate effectively for the inhibition resulting from NBTI treatment. Using the approach discussed in Materials and Methods, the octanol/PBS partition coefficient of L-FMAU was determined to be 0.31 ± 0.04 (N = 3) at 25°.

DISCUSSION

The advantages and limitations of NMR approaches to study the membrane transport of various compounds have been reviewed extensively (e.g. [27, 28]). An important advantage of this technique for studying fluorinated compounds is the ability to resolve resonances arising from intra- and extracellular compounds [29–32]. Hence, no isotopically labeled forms of the compounds need to be synthesized. Although the physical basis for this shift separation is not completely clear at present, available data point to the disruption of R–F . . . H–O hydrogen bonding resulting from the hydration of intracellular hemoglobin as the possible principal cause of the transmembrane chemical shift differences [33]. The major limitation on the technique is the low sensitivity of the NMR method, which can limit the time resolution attainable. In studies of non-naturally occurring fluorinated nucleosides, the cellular uptake rates are typically reduced due to poorer interaction with the transporter [26, 32]. Hence, spectral averaging of many transients can be achieved, and the NMR method provides ample sensitivity. This is apparent for the case of L-FMAU uptake by erythrocytes, as seen in Fig. 1. Alternatively, for rapid transmembrane exchange occurring within the sub-minute time domain, the rates of equilibrium exchange can be monitored using magnetization transfer techniques, as was done, for example, in studies of trifluoroacetamide transport [34].

As noted above, the uptake of L-FMAU by human erythrocytes is readily determined by ^{19}F NMR methods. Conservation of total resonance intensity over the course of the experiment indicates that no significant metabolism is occurring in this system, nor is there significant binding to macromolecules, which would lead to severe broadening of the intracellular resonance and/or an apparent loss of signal intensity. Further evidence against significant binding of the intracellular L-FMAU pool arises from the equality of the intra- and extracellular L-FMAU concentrations attained after the system reaches transmembrane distribution equilibrium.

Human erythrocytes only express the NBTI-sensitive nucleoside transporter ($\sim 1.2 \times 10^4$ nucleoside transporters/cell, reviewed in [10]), which has a K_D of $\sim$1 nM [35]. However, even at NBTI concentrations well in excess of those required to saturate the available transporters, i.e. NBTI:nucleoside transporter ratios ranging from 4:1 to 169:1, significant residual transport of L-FMAU was observed. This was demonstrated most clearly by the observation of approximately constant influx rates measured at [NBTI] > 0.5 μM (Fig. 2). The 35.3 ± 3.5% and 34.7 ± 7.2% residual influx values determined at 15° and 25°, respectively, indicate transport via pathways other than the nucleoside transporter. Dipyridamole not only binds to its high-affinity binding sites ($K_D = \sim 10$ nM, $\sim 5 \times 10^5$ sites/cell in human erythrocytes), but possibly to other membrane proteins of erythrocytes as well [10, 36]. The minimum dipyridamole concentration required to produce the maximum inhibition was $\sim$1 μM, which corresponds to a dipyridamole:nucleoside transporter ratio of 8:1. The K_i estimate (81 ± 35 nM) is comparable with the reported K_D (4–20 nM) for the binding of dipyridamole to erythrocyte membrane proteins at 22° [36]. The IC_{50} ($\sim$27 nM) of dipyridamole on L-FMAU uptake was similar to that (20–50 nM) reported for inhibition of nucleoside transport in human erythrocytes [37]. Thus, results from the studies using dipyridamole are consistent with the conclusions derived from the studies using NBTI.

The nucleobase transporter is also capable of mediating the transport of certain nucleosides [38, 39]. Papaverine is a non-competitive inhibitor of nucleobase transport with a $K_I \cong 12$ μM for hypoxanthine transport in human erythrocytes [23, 24]. The absence of papaverine inhibition of L-FMAU transport thus suggests the lack of participation of the nucleobase transporter in L-FMAU uptake. Uracil is transported by the nucleobase transporter with a low affinity ($K_m = 5.5$ mM) [25]. Because of its limited solubility, the maximum initial extracellular uracil concentration used in these studies was 8.7 mM. Nevertheless, the lack of inhibition of L-FMAU transport by uracil at concentrations up to 8.7 mM is consistent with a lack of participation of the nucleobase transporter in L-FMAU uptake. The possibility of significant contributions due to other transporters containing reactive sulfhydryl reagents was probed with the use of pCMBS, a nonspecific sulfhydryl reagent that reacts with a broad range of membrane proteins. The lack of a consistent pCMBS dependence of the membrane permeability coefficients and influx rates (data not shown) in the cells inhibited with both NBTI and pCMBS supports the conclusion that no additional membrane transporter-mediated pathway(s) contributes significantly to L-FMAU uptake.

Butanol diffuses across erythrocyte membranes rapidly and increases membrane basal permeability, and therefore nonspecific diffusion, by membrane fluidization [40]. It has been used to characterize the membrane diffusion pathways involved in the transport of Cd^{2+} [41], H^+ [42], and, more recently, dipeptides [43]. The increase in L-FMAU permeability values, measured as a function of butanol concentration, is consistent with a nonfacilitated diffusion uptake pathway. In addition to its effects on membrane fluidity, butanol could also exert more complex effects on L-FMAU uptake due to structural perturbations of membrane proteins [40]. However, a structural perturbation of the nucleoside transporter would be more likely to reduce than to increase permeability, in contrast to the observations.

Chemical modification of nucleosides often results in a significant change of their uptake pathways [6, 20, 44]. Increased transport of nucleosides by nonfacilitated diffusion has been correlated with larger octanol/water partition coefficients [6, 7]. The partition coefficient that we determined for L-FMAU (0.31 ± 0.04, $N = 3$) is greater than the values for uridine (0.017 [12]) and thymidine (0.082 [12]), but comparable to the value for 2′,3′-dideoxythymidine (0.2 [7]). Because the former are reported to be trans-

ported via the nucleoside transporter and the latter primarily by nonfacilitated diffusion [7], the intermediate value obtained for L-FMAU supports the conclusion that both pathways contribute to L-FMAU uptake.

In summary, the ^{19}F NMR studies presented here provide a clear indication of the contribution of two different mechanisms to L-FMAU uptake in human erythrocytes: facilitated transport via the nucleoside transporter, and nonfacilitated diffusion. The former pathway was found to be dominant under the conditions of the study, and is also predicted to predominate at pharmacological concentrations of the drug. The uptake of L-FMAU by the nucleoside transporter was characterized by a Michaelis constant of approximately 1 mM. This value is typical of values reported for non-natural nucleoside drugs [26]. We note also that although the transport was temperature dependent, the consistent fractional contribution by the nucleoside transporter to the uptake at both 15° and 25° supports the relevance of the present findings to L-FMAU uptake at 37°.

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