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GANCICLOVIR PERMEATION OF THE HUMAN ERYTHROCYTE MEMBRANE

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Abstract—The membrane permeation of ganciclovir (DHPG)—a structural analogue of acyclovir (ACV) with activity against cytomegalovirus—was investigated in human erythrocytes at 37° with an "inhibitor-stop" assay. DHPG influx was nonconcentrative, occurred without permeant metabolism, and was rate-saturable. While substantial inhibition of the influx of 13 μM DHPG occurred only in the presence of permeants of the purine nucleobase carrier, nucleosides and inhibitors of nucleoside transport markedly inhibited DHPG influx at higher DHPG concentrations (≥200 μM). Adenine and dilazep (a potent inhibitor of the nucleoside carrier) each inhibited the influx of DHPG only partially; when present together, however, they inhibited DHPG permeation completely. DHPG permeation via the purine nucleobase carrier (K_m = 0.89 mM) was characterized by assessing influx in the presence of 1.0 μM dilazep. Adenine and ACV were shown to competitively inhibit this process, while DHPG (K_i = 0.90 mM) was found to competitively inhibit adenine influx. DHPG influx via the nucleoside carrier (K_m = 14 mM) was characterized by assessing influx in the presence of 2 mM adenine. DHPG (K_i = 10 mM) also appeared to competitively inhibit the influx of 5-iodo-2′-deoxyuridine. These results indicate that DHPG permeates the human erythrocyte membrane primarily by the purine nucleobase carrier and secondarily by the nucleoside transporter.

DHPG†, a structural analogue of the antiherpetic drug ACV, exhibits activity against all of the herpesviruses and has been shown to be useful in the management of HCMV infections in immunocompromised patients [1–3]. DHPG is converted to its triphosphate form in HCMV-infected cells 10–100 times more efficiently than in uninfected cells and with a much greater efficiency than is ACV [4–6]. DHPG triphosphate is then able to inhibit HCMV-induced DNA polymerase [6–8].

Since DHPG must first permeate the cell membrane before it can be phosphorylated, it was of interest to characterize the mechanism of cellular influx of this agent. Although ACV was shown to traverse the erythrocyte membrane virtually exclusively via the purine nucleobase carrier [9], DHPG has an extra hydroxymethyl group on its side chain that gives this molecule a more nucleoside-like structure. Nucleoside permeation into human erythrocytes occurs by a single, nonconcentrative, and NBMPR-sensitive transport system [10–12] that is present in almost all animal cells [12]. However, as summarized in recent reviews [11–13], at least two other types of nucleoside transporter have been reported recently to occur in nonhuman mammalian cells: an NBMPR-insensitive, nonconcentrative transporter; and a Na^+-dependent, NBMPR-insensitive, concentrative transporter. The relevance of these latter transporter systems to human cells remains under investigation. Since the influx of ACV [9], nucleosides [10–13] and nucleobases [14] has been well characterized using human erythrocytes, these primary human cells provided a useful model for evaluating DHPG influx relative to the influx of related compounds. In this report, DHPG influx into human erythrocytes is shown to be a composite of nucleobase (≥67%) and nucleoside (≤33%) transport at all achievable DHPG concentrations.

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† The trivial names and abbreviations used are: DHPG, ganciclovir (9-([2-hydroxy-1-hydroxymethylethoxy]methyl)guanine); ACV, acyclovir (9-[2-hydroxyethoxymethyl]guanine); NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; PBS, (Dulbecco's) phosphate-buffered saline; IdUrd, 5-iodo-2′-deoxyuridine; [^{125}I]IdUrd, 5-[^{125}I]iodo-2′-deoxyuridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; desciclovir, 2-amino-9-(2-hydroxyethoxymethyl)purine; and HCMV, human cytomegalovirus.

EXPERIMENTAL PROCEDURES

Materials. [8-^{14}C]DHPG (53.2 Ci/mol) was synthesized in these laboratories according to an unpublished procedure and was purified to >99% using reversed-phase high performance liquid chromatography; [^{14}C]guanine contamination was ≤0.03%, as determined by reversed-phase high performance liquid chromatography [15]. [Side chain 2-^{3}H]ACV (18.6 Ci/mmol) and [U-^{14}C]sucrose (4 Ci/mol) were obtained from DuPont-New England Nuclear; [8-^{3}H]adenine (29 Ci/mmol) and [^{125}I]IdUrd (5 Ci/mg) were from the Amersham Corp. Nonradioactive nucleosides and nucleobases, papaverine hydrochloride, dipyridamole and NBMPR were purchased from Sigma. DHPG and ACV [16] were synthesized in these laboratories and, as determined by reversed-phase high performance

liquid chromatography, were both shown to be >99% pure with ≤0.2% contamination by guanine. Dilazep was provided by Hoffmann-La Roche. PBS and Hepes were products of GIBCO.

Preparation of human erythrocytes. Erythrocytes were isolated from blood collected from healthy human volunteers, washed three times with Buffer A (10 mM Hepes–saline, pH 7.3), and resuspended in this buffer to a final hematocrit of 20–40% [14].

Kinetics of DHPG and adenine influx. Assays of [^{14}C]DHPG (0.05 to 1.8 μCi/assay) and [^{3}H]adenine (0.2 to 2.2 μCi/assay) influx into human erythrocytes were performed at 37° with Buffer A using the "papaverine-stop" method described previously for measuring nucleobase influx [14]. Influx assays were initiated by the rapid addition of permeant (60–80 μL) to the erythrocyte suspension (20–40 μL of 20–40% hematocrit) so that a total assay volume of 100 μL, containing either 5 or 10 μL of packed cells, was maintained. Influx velocities (expressed in terms of moles of permeant taken up per 5 or 10 microliters of packed cells per second) were determined by linear regression analysis of the plots of cell-associated radiolabeled permeant versus assay time, using data obtained during the linear phase of influx. In the case of adenine permeation, influx rates were corrected so as to exclude the contribution of nonfacilitated diffusion [14]. The amount of extracellular medium present in the cell pellet was determined with [^{14}C]sucrose [17]. DHPG influx was also measured using an "oil-stop" method [14] which was essentially identical to the method described above except that the papaverine addition was omitted and assay termination was redefined as the starting time of the microcentrifuge.

Kinetics of IdUrd influx. Assays of [^{125}I]IdUrd (0.01 to 0.15 μCi/assay) influx into human erythrocytes were performed at 37° with PBS as buffer using a "dilazep-stop" method [15]. The assay system was modified so that a 100-μL assay containing 5 μL of packed cells was terminated by the addition of 400 μL of 670 μM dilazep. Initial velocities were determined by linear regression analysis of the plots of cell-associated [^{125}I]IdUrd versus assay time, using data consisting of duplicate assay values obtained during the linear phase of influx: 0, 0.4 and 0.8 sec.

Kinetics of ACV influx. Assays of [^{3}H]ACV (0.5 μCi/assay) influx into human erythrocytes were performed at 37° with PBS as buffer using a "papaverine-stop" method [9].

DHPG metabolism studies. Human erythrocytes were incubated for 30 min at 37° with 15 μM and 7.5 mM [^{14}C]DHPG. Each incubation was terminated with 700 μL of an ice-cold, saturated solution (≈20 mM) of papaverine. The cell-associated radioactivity was extracted with cold trichloroacetic acid as described previously [14] and analyzed by reversed-phase high performance liquid chromatography [18].

Kinetic analysis. Kinetic constants were determined by fitting the data to a hyperbola according to the method of Wilkinson [19] and the computer program of Cleland [20]. These data were analyzed further for conformity to the competitive model by the method of Spector and Hajian [21].

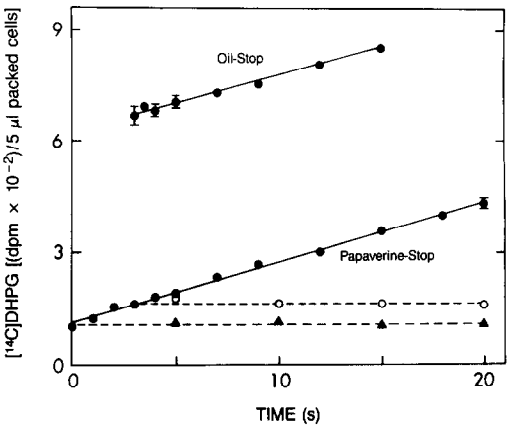

Fig. 1. Initial velocity of DHPG influx as determined by "oil-stop" and "papaverine-stop" transport assays. Human erythrocytes (5 μL packed cells) were incubated in Buffer A, for the indicated times at 37°, with 7.9 mM [^{14}C]DHPG (0.17 Ci/mol) in a total volume of 100 μL. The "oil-stop" method used the initiation of centrifugation as the assay-terminating time, without correction for the time required for the cells to sediment into the oil phase. The "papaverine-stop" method used the addition of 700 μL of an ice-cold, saturated solution of papaverine to each transport assay as the assay-terminating time; centrifugation of the cells through oil was initiated 10 sec after the papaverine addition. In the "papaverine-stop + delay" condition (dashed lines), longer delay times prior to activation of the microcentrifuge, defined as 10 sec *plus* the additional time specified, are shown for assay times of 0 (▲) and 3.0 (○) sec respectively. Each time point represents the mean ± SEM of triplicate values. Error bars were omitted where they did not extend beyond the boundary of the symbol.

Statistics. Statistical comparisons of influx rates were determined with the PROC GLM computer program from the SAS Institute, Inc. (Cary, NC).

RESULTS

"Papaverine-stop" assay of DHPG influx. The "papaverine-stop" method, previously described as useful for measuring initial rates of purine nucleobase influx into human erythrocytes [14], was used in this study to measure DHPG influx (Fig. 1). The effectiveness of papaverine as a "chemical stopper" in this assay system was demonstrated using several criteria. First, the cell-associated [^{14}C]DHPG at zero time (0.044 ± 0.006% of the total radioactivity present in each assay mixture) was similar to the [^{14}C]sucrose space (0.038 ± 0.002%) of the erythrocyte cell pellet, indicating that cell-associated permeant at zero time was virtually all extracellular. Second, as shown for assay times of 0 and 3.0 sec, varying the time between the papaverine addition and the centrifugation of the cells through oil from 10 to 30 sec had no effect upon the DHPG content of the resultant cell pellet (Fig. 1, dashed lines). Third, the same rate of influx of 7.9 mM DHPG resulted from measurements with the "papaverine-stop" (43 ± 1 pmol/sec/5 μL packed cells) and "oil-stop" (40 ± 3 pmol/sec/5 μL packed cells) methods

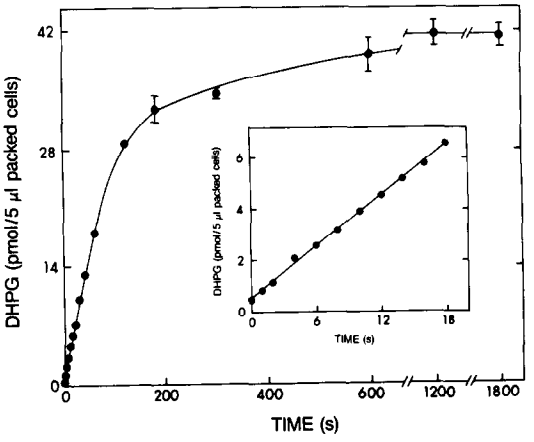

Fig. 2. Time dependence of DHPG influx. Human erythrocytes (5 μL packed cells) were incubated in Buffer A for the indicated times at 37° with 10 μM [^{14}C]DHPG (53 Ci/mol). Time points represent the means ± SEM of triplicate values. Error bars were omitted where they did not extend beyond the symbol boundaries.

(Fig. 1, solid lines). The influx of 7.9 mM [^{14}C]DHPG into human erythrocytes at 37° was linear for at least 20 sec, at which time these cells contained 900 pmol DHPG/5 μL packed cells (Fig. 1).

Time dependence of DHPG influx. The influx of 10 μM DHPG into human erythrocytes appeared linear for at least 18 sec and proceeded to equilibrium by 20 min (Fig. 2). At equilibrium, the intracellular DHPG concentration (42 pmol/5 μL packed cells) was approximately equal to that present in the assay medium.

Metabolic inertness of DHPG. Metabolism of [^{14}C]DHPG by human erythrocytes was assessed after incubations for 30 min at high (7.5 mM) and low (15 μM) concentrations of permeant. By HPLC analysis, greater than 96% of the cell-associated radioactivity was eluted from the reversed-phase column at the retention time of authentic DHPG standard.

Effects of various classes of compounds on DHPG influx. Inhibitors of nucleoside transport and various nucleosides, nucleobases and related "acyclic nucleosides" were tested for their abilities to inhibit DHPG influx into human erythrocytes (Table 1). The influx of 13 μM DHPG was inhibited markedly (56–93%) by adenine, hypoxanthine, guanine and ACV, all permeants of the purine nucleobase carrier [9, 14]. When the concentration of DHPG was increased to 3.0 mM, these inhibitors retained their relative inhibitory potencies, but to a diminished extent (20–71%). Although inhibitors of nucleoside transport (NBMPR, dilazep and dipyridamole) and permeants of the nucleoside transporter (thymidine and uridine) had little or no effect upon the influx of 13 μM DHPG, these same agents caused significant inhibition (18–29%) of the influx of 3.0 mM DHPG. Thymine and desciclovir also inhibited DHPG influx.

Concentration dependence of DHPG influx in the absence and presence of adenine and dilazep. Since both the purine nucleobase and the nucleoside carriers were implicated in the influx of DHPG (Table 1), the permeant concentration dependency

Table 1. Effects of nucleoside transport inhibitors, nucleosides, nucleobases, ACV and desciclovir on the influx of DHPG into human erythrocytes

Inhibitor	Percent inhibition of influx: DHPG permeant concentration 13 μM	3.0 mM
Adenine (1.0 mM)	93*	71*
Hypoxanthine (1.0 mM)	79*	40*
Guanine (56 μM)	56*	20*
Cytosine (1.0 mM)	0	0
Thymine (1.0 mM)	35*	14†
Uracil (1.0 mM)	10†	5
ACV (1.0 mM)	76*	34*
Desciclovir (1.0 mM)	41*	16†
NBMPR (1.0 μM)	8†	28*
Dilazep (1.0 μM)	4	28*
Dipyridamole (1.0 μM)	11*	29*
Thymidine (1.0 mM)	11*	18*
Uridine (1.0 mM)	10*	24*

The inhibition of DHPG influx into human erythrocytes was determined at 37° by comparing initial velocities of DHPG influx in the absence and presence of inhibitor. Initial velocities were derived by linear regression analysis of data obtained during the linear phase of influx (0, 4, 8, 12 and 16 sec for 13 μM DHPG; and 0, 5, 10, 15 and 20 sec for 3.0 mM DHPG). At permeant concentrations of 13 μM and 3.0 mM, control rates for DHPG influx of 0.90 and 56 pmol/sec/10 μL of packed cells, respectively, were measured.

* Inhibited rate significantly different from control rate, $P < 0.001$.

† Inhibited rate significantly different from control rate, $P \leq 0.01$.

of DHPG influx was assessed in the absence and presence of inhibitors of these two carriers. For this study, dilazep was used to inhibit the nucleoside transporter [15, 22], and adenine (K_i = 12 μM) was used to inhibit the purine nucleobase transporter [9, 14].

In the absence of inhibitors, DHPG influx into human erythrocytes appeared to be a single saturable process (Fig. 3, open circles). However, Eadie–Scatchard plots of these data (Fig. 4, open circles) and similar data obtained over the wider DHPG concentration range of 0.060 to 7.8 mM (data not shown) were nonlinear, a result diagnostic of more than one transport process [23].

In the presence of 1.0 μM dilazep (Fig. 3, closed circles), DHPG influx was progressively inhibited from 12 to 33%, as the permeant concentration was increased from 0.23 to 4.8 mM. This residual influx of DHPG in the presence of dilazep was also saturable and, based on the linear relationship between V/[DHPG] and V in the presence of dilazep (Fig. 4, closed circles), appeared to occur via a single transport system [23]. On the basis of the inhibition results shown in Table 1, this latter transport system was tentatively identified as the purine nucleobase carrier. From these and other data obtained under similar conditions, a K_m of 890 ± 20 μM (N = 3) was calculated for DHPG influx into human erythrocytes via the nucleobase carrier.

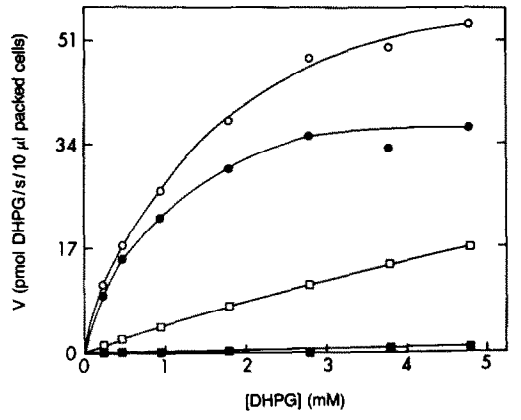

Fig. 3. Concentration dependence of DHPG influx rates in the absence and presence of adenine and dilazep. [^{14}C]DHPG (2.6 Ci/mol) influx assays were performed with human erythrocytes (10 µL packed cells/assay) in the absence of inhibitors (○), in the presence of 1.0 µM dilazep (●) or 2.0 mM adenine (□), or in the presence of both inhibitors (■), as described under Experimental Procedures. Initial velocities of influx were derived from linear regression analysis of data obtained during the linear phase of DHPG influx: 0, 5, 10 and 15 sec; or, 0, 20, 40 and 60 sec whenever 2.0 mM adenine was present.

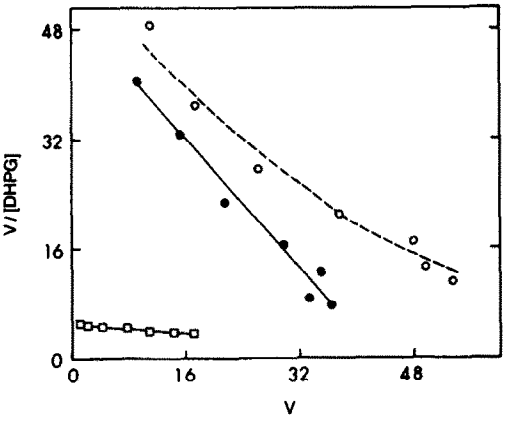

Fig. 4. Eadie–Scatchard plots of DHPG influx rates. The same data presented in Fig. 3 are represented in Eadie–Scatchard plots. For DHPG influx velocities obtained in the presence of 1.0 µM dilazep (●) and 2.0 mM adenine (□), lines were drawn using linear regression analysis (solid lines). For DHPG influx velocities in the absence of inhibitors (○), a theoretical curve was constructed (dashed line), where V is defined as the sum of the predicted DHPG influx velocities mediated by the nucleoside transporter and the nucleobase carrier for each DHPG concentration; velocities associated with each carrier were calculated using the Michaelis–Menten equation and the respective kinetic parameters derived from each of the two linear plots presented in this figure [23].

DHPG influx into human erythrocytes was also examined in the presence of 2.0 mM adenine in order to completely inhibit* transport occurring via the purine nucleobase carrier. Under these conditions, a significant residual component (≤33%) of total DHPG influx was observed (Fig. 3, open squares). The apparently linear Eadie–Scatchard plot of these data (Fig. 4, open squares) was consistent with the suggestion of a discrete second transport system which, based on the inhibition results presented in Table 1, was tentatively identified as the nucleoside transport system. From these and other data obtained under similar conditions, a K_m for DHPG influx via the nucleoside transporter was estimated as 14 ± 3 mM (N = 3).

Inhibition of DHPG influx by dilazep and adenine appeared to be complementary and complete: at each DHPG permeant concentration shown in Fig. 3, the sum of the partial inhibitions caused by each of these two inhibitors (closed circles and open squares) was 101 ± 2% (N = 7) of total influx, while inhibition by these agents in combination was 98–100% of total influx (closed squares).

The specificity and potency of dilazep and adenine as inhibitors of the nucleoside and purine nucleobase carriers, respectively, were also assessed by determining the concentration dependence of their inhibition of DHPG (4.0 mM) influx into human erythrocytes. Dilazep, which was tested at concentrations as high as 5.0 µM, inhibited DHPG influx maximally (18 ± 2% [N = 7]) at concentrations ≥20 nM (Fig. 5B, open circles). When 2.0 mM adenine was also present, DHPG influx was inhibited completely (>99%) by similar concentrations of dilazep (Fig. 5B, closed circles). By comparison, inhibition of DHPG influx caused by adenine (≤3.1 mM) alone approached a maximum value of approximately 80% (Fig. 5A, open circles). However, in the presence of dilazep (1.0 µM), DHPG influx was inhibited 96 and >99% by 1.1 and 3.1 mM adenine, respectively (Fig. 5A, closed circles).

Kinetic analysis of DHPG as permeant for, and inhibitor of, purine nucleobase influx. Since the influx of DHPG into human erythrocytes appeared to occur via two carriers, the kinetics of purine nucleobase carrier-mediated DHPG influx were characterized in the presence of dilazep (1.0 µM) in order to eliminate any contribution by the nucleoside carrier to the influx process. Under these conditions, ACV (K_i = 260 µM) and adenine (K_i = 7.5 µM) were found to be competitive inhibitors of DHPG influx (Fig. 6). These K_i values were similar to influx K_m values for these compounds (Table 2). Conversely, DHPG was found to competitively inhibit adenine transport into human erythrocytes (Fig. 7A) with a K_i value of 900 µM, which was similar to its K_m value for influx across this carrier (Table 2). In a previous report [9], DHPG, although present at less than optimal concentrations (≤0.90 mM), was shown to competitively inhibit the influx of ACV with a K_i value of 1500 µM. A summary of kinetic parameters derived from these and related experiments is presented in Table 2.

Kinetic analysis of DHPG as an inhibitor of IdUrd influx. Since the nucleoside carrier was implicated as a secondary transport pathway for DHPG permeation of the erythrocyte membrane, the effects of DHPG on the influx of IdUrd, a permeant of the nucleoside transport system [15], were examined.

* Based upon the Michaelis–Menten equation for competitive inhibition and the kinetic parameters presented in Table 2, the nucleobase carrier-mediated influx of ≤5.0 mM DHPG should be inhibited ≥97% by 2.0 mM adenine.

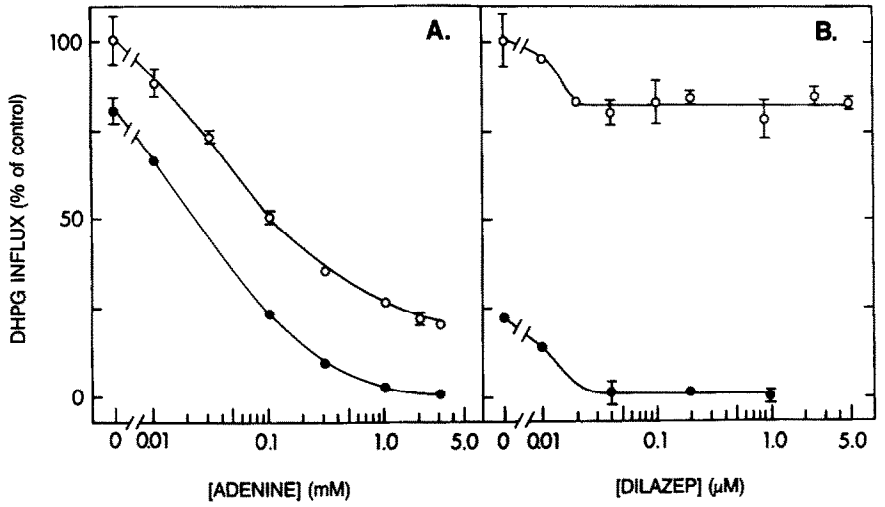

Fig. 5. Effects of adenine and dilazep upon DHPG influx. The influx of 4.0 mM [^{14}C]DHPG (0.5 Ci/mol) into human erythrocytes (10 μL packed cells/assay) was assayed at 37°, as described under Experimental Procedures. DHPG influx is expressed as a percentage relating the inhibited influx rate to the control DHPG influx rate (35 pmol/sec/10 μL cells) measured in the absence of both adenine and dilazep. Inhibitors present were (A) adenine (○) or adenine + 1.0 μM dilazep (●) or (B) dilazep (○) or dilazep + 2.0 mM adenine (●). Initial velocities ± SE were derived from linear regression analysis of data obtained during the linear phase of DHPG influx: 0, 6, 12 and 18 sec in the absence and presence of dilazep; 0, 12, 24 and 36 sec in the presence of 0.1 to 0.3 mM adenine; 0, 15, 30 and 45 sec in the presence of 0.1 to 0.3 mM adenine + 1.0 μM dilazep; and 0, 20, 40 and 60 sec for all assay conditions that included ≥1.0 mM adenine.

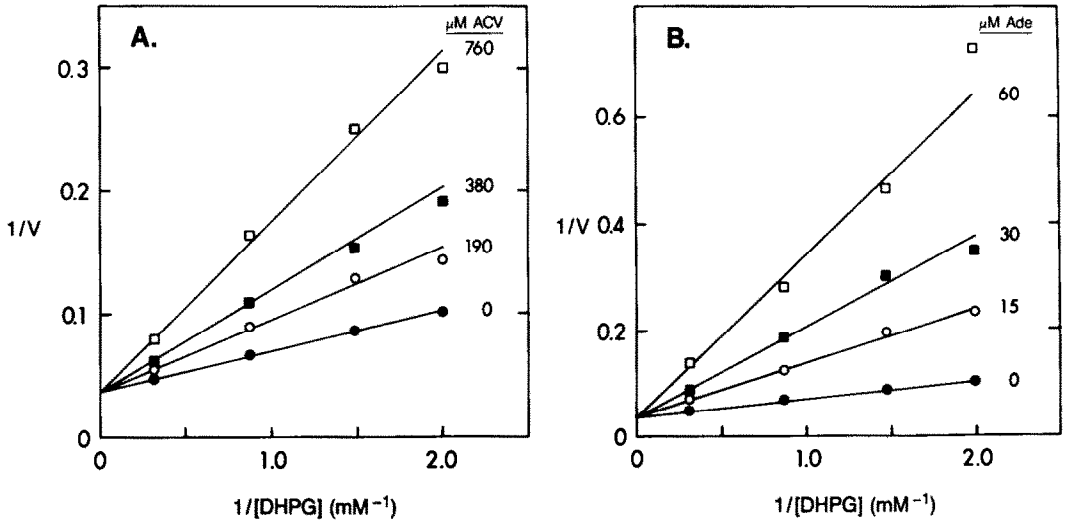

Fig. 6. Double-reciprocal plots for the inhibition of purine nucleobase carrier-mediated DHPG influx velocities by (A) ACV and (B) adenine. The influx of [^{14}C]DHPG (2.8 Ci/mol) into human erythrocytes (5 μL packed cells/assay) was assayed at 37° in the presence of 1.0 μM dilazep, as described under Experimental Procedures. Initial velocities of influx (pmol of DHPG/sec/5 μL packed cells) were derived from linear regression analysis of data obtained during the linear phase of influx: 0, 10, 15 and 20 sec.

As shown in Fig. 7B, DHPG (K_i = 10 mM) appeared to be a competitive inhibitor of IdUrd transport. The nucleoside transporter-related kinetic parameters are summarized in Table 3.

Evidence of ACV influx via the nucleoside transporter. The influx of ACV (≤1.0 mM) was reported previously to be a single process, dependent only upon the purine nucleobase carrier [9]. Since DHPG influx appears to be mediated by two carriers, and because DHPG and ACV are structurally similar, ACV transport into human erythrocytes was reinvestigated at the highest permeant concentration

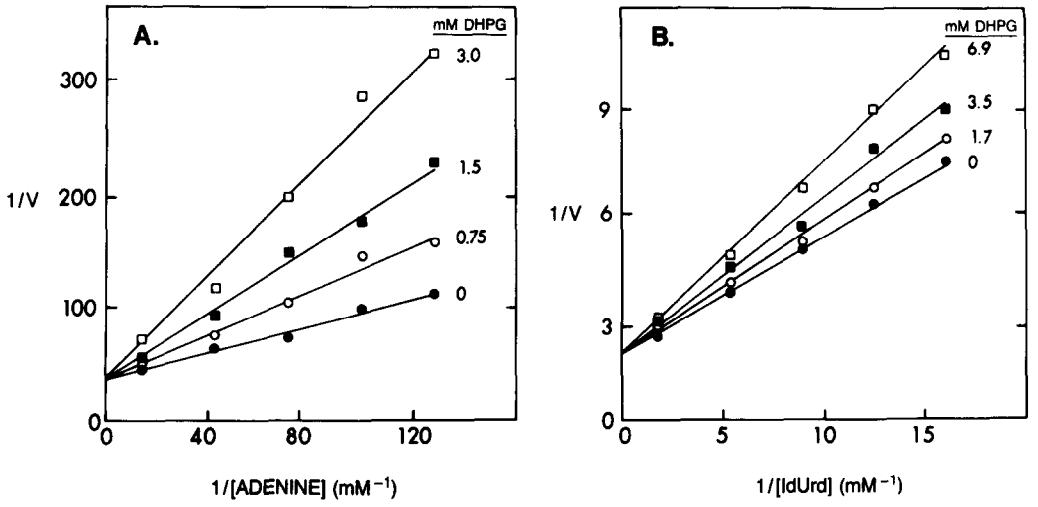

Fig. 7. Double-reciprocal plots for DHPG inhibition of the influx of (A) adenine and (B) IdUrd. The influx of (A) [^{3}H]adenine (310 Ci/mol) and (B) [^{125}I]IdUrd (4.9 Ci/mol) into human erythrocytes (5 μL packed cells/assay) was assayed at 37° as described under Experimental Procedures. Initial velocities of influx (nmol of permeant/sec/5 μL packed cells) were derived from linear regression analysis of data obtained during the linear phase of influx: 0, 0.5, 1.0 and 1.5 sec for adenine influx; and 0, 0.4, 0.4, 0.8 and 0.8 sec for IdUrd influx.

Table 2. Kinetic parameters for the zero-trans influx of DHPG, ACV and adenine into human erythrocytes via the purine nucleobase carrier

	Kinetic parameters for permeant influx*		Inhibitor K_i (μM)		
Permeant	V_{max} (pmol/sec/5 μL cells)	K_m (μM)	DHPG	Adenine	ACV
DHPG	25 ± 3	890 ± 20	—	7.5 ± 0.3†	260 ± 10†
Adenine	21 ± 2‡	13 ± 1‡	900 ± 90†	—	280 ± 10§
ACV	33 ± 6§	260 ± 20§	1500 ± 200§	9.5 ± 0.5§	—

* Values are means ± SEM; N = 3 for DHPG values and N = 8 for the adenine values.
† The K_i value ± SE of that value for the fit of twenty data points (from a single preparation of human erythrocytes) to the competitive inhibition model [21] is presented.
‡ Includes data reported previously [14].
§ Reported previously in Ref 9.

Table 3. Kinetic parameters for the zero-trans influx of DHPG and IdUrd into human erythrocytes via the nucleoside transporter

	Kinetic parameters for permeant influx		Inhibitor K_i (μM)
Permeant	V_{max} (pmol/sec/5 μL cells)	K_m (μM)	DHPG
DHPG	31 ± 2	14,000 ± 3000*	—
IdUrd	540 ± 40	150 ± 10†	10,000 ± 1000†

* Mean ± SEM of three values is given.
† Mean ± average deviation of two values is given.

achievable in the assay (1.4 mM) (Table 4). Under these conditions, ACV influx was inhibited strongly (87–88%) by 2.0 mM adenine, but was not detectably affected by either IdUrd or potent inhibitors of nucleoside transport (NBMPR and dilazep). However, when adenine and any one of these inhibitors of nucleoside transport were present together, inhibition of ACV influx was virtually

Table 4. Effects of inhibitors of nucleoside transport on the influx of ACV (1.4 mM) into human erythrocytes

Inhibitor(s)	Percent inhibition of influx
Experiment I	
Adenine (2.0 mM)	87*
Dilazep (1.0 μM)	7
NBMPR (1.0 μM)	12
Dilazep (1.0 μM) + adenine (2.0 mM)	98*†
NBMPR (1.0 μM) + adenine (2.0 mM)	99*†
Experiment II	
Adenine (2.0 mM)	88*
IdUrd (1.0 mM)	9
IdUrd (1.0 mM) + adenine (2.0 mM)	95*†

The inhibition of ACV (1.4 mM) influx into human erythrocytes was determined at 37° by comparing the initial velocity of ACV influx in the absence and presence of inhibitor(s). Initial velocities were derived by linear regression analysis of data obtained during the linear phase of ACV influx (0, 2.0, 4.0, 6.0 and 8.0 sec, except where 2.0 mM adenine was present and assay times of 0, 10, 20, 30 and 40 sec were used). The control rates for Experiments I and II were 23 and 25 pmol/sec/5 μL of packed cells respectively.

* Inhibited rate significantly different from control rate, $P < 0.001$.

† Inhibited rate significantly different from rate determined in the presence of adenine alone, $P < 0.001$.

complete (adenine + NBMPR or dilazep: ~99%; adenine + IdUrd: 95%) and statistically different ($P < 0.001$) from the inhibition caused by adenine alone.

DISCUSSION

The mechanism of DHPG permeation into human erythrocytes was characterized by means of the "papaverine-stop" assay used previously to measure purine nucleobase influx [14]. Based upon several criteria, the addition of seven assay volumes of an ice-cold, saturated (≈20 mM) solution of papaverine was shown to stop completely and instantaneously the influx of DHPG at the highest permeant concentration (7.9 mM) achievable. As discussed below, these results represent the first example of the usefulness of this inhibitor-stop method for measuring nucleoside transporter-mediated influx.

DHPG was found to be metabolically inert in human erythrocytes. Thus, the kinetics of influx of this permeant were not complicated by the cellular metabolism of DHPG that might have occurred on either side of the cell membrane. The nonconcentrative equilibration of radioisotope in erythrocytes during incubation with [^{14}C]DHPG (10 μM) (Fig. 2) was consistent with this finding.

Several lines of evidence indicated that the mechanism of DHPG influx is a composite of two transport processes. First, the nonlinear Eadie–Scatchard plot (Fig. 4, open circles) suggested the presence of more than one permeation process [23]. Second, the influx of 3.0 mM DHPG was inhibited by inhibitors and permeants of the nucleoside transporter as well as by permeants of the purine nucleobase transporter (Table 1). Third, DHPG influx was inhibited completely when dilazep, an inhibitor of the nucleoside transporter, and adenine, a permeant of the purine nucleobase carrier, were present together in the erythrocyte suspension (Fig. 3, closed squares, and Fig. 5, closed circles); this complete inhibition, observed even at high DHPG concentrations, also indicates the inability of DHPG to permeate the membrane by nonfacilitated diffusion.

Through the use of adenine and dilazep as transporter-specific inhibitors, DHPG influx occurring across each carrier was measured separately. At the purine nucleobase carrier, adenine and ACV were found to be competitive inhibitors of DHPG influx (Fig. 6), and DHPG was a competitive inhibitor of the influx of adenine (Fig. 7A) and ACV [9]. DHPG was shown to be transported to a lesser extent by the nucleoside transporter (Fig. 3) and was found to competitively inhibit the influx of IdUrd (Fig. 7B). The kinetic parameters derived from these and related studies (Tables 2 and 3) provide quantitative support and definition for the two-carrier model for DHPG influx. For example, in human erythrocytes, these parameters predict that the purine nucleobase carrier mediates approximately 90% of all DHPG influx at DHPG concentrations below 200 μM.* For the sake of comparison, patients receiving DHPG (10–20 mg/kg) orally exhibit steady-state peak plasma levels of 2–3 μM [24].

While DHPG permeation has been shown to be primarily dependent upon the purine nucleobase carrier, DHPG is, nevertheless, a relatively poor permeant for this transporter. While the maximum velocity of DHPG influx via this transport system is similar to that of guanine and acyclovir [9, 14], DHPG has a much diminished affinity for this carrier: 3.4- and 24-fold less than that of ACV [9]

* This is based upon estimates of DHPG influx rates occurring at the respective carriers, as predicted by the Michaelis–Menten equation and the kinetic parameters presented in Tables 2 and 3.

and guanine [14] respectively. These results offer an explanation for the relatively poor oral bioavailability of DHPG compared to that of ACV [24, 25].

ACV permeation into human erythrocytes, found previously (using permeant concentrations $\leq$1.0 mM) to occur only via the purine nucleobase carrier [9], was reexamined. At the highest concentration (1.4 mM) of ACV achievable in these transport assays, evidence for only a minor ($\approx$10%) contribution of nucleoside transporter-mediated ACV influx was obtained (Table 4). This, however, appears to reflect at least a qualitative similarity between the membrane permeation characteristics of ACV and DHPG. The importance of the 3′-hydroxyl moiety of nucleosides to their ability to interact with the nucleoside transporter has been described previously [26–28]. Both DHPG and ACV can exist in solution in a conformation wherein a side chain hydroxyl moiety is positioned similarly to the 3′-hydroxyl moiety of a ribose-containing nucleoside, and this circumstance may allow these "acyclic nucleosides" to serve as low-affinity permeants of the nucleoside transporter. Nevertheless, the minor role of this transporter in the influx of DHPG and ACV supports the conclusion of Gati *et al.* [27] that, while the 3′-hydroxyl moiety is important, optimal interactions with the nucleoside carrier depend more broadly on the combined effects of all sugar substituents in determining the desirable conformation of the nucleoside permeant.

Previous reports suggest that carrier-mediated permeation of purine and pyrimidine nucleobases occurs by different carriers [12]. Yet, in human erythrocytes, inhibition of DHPG influx by thymine (Table 1) is consistent with other observations that thymine and uracil inhibit purine nucleobase influx [14]. Also, 5-fluorouracil has been shown recently to be transported into human erythrocytes by the same carrier that transports adenine and hypoxanthine [29]. Thus, at least in human erythrocytes, purine and pyrimidine nucleobases appear to share a single common carrier.

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