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DESCICLOVIR PERMEATION OF THE HUMAN ERYTHROCYTE MEMBRANE BY NONFACILITATED DIFFUSION

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Abstract—The mechanism of transport of desciclovir (DCV)—a structural analogue and prodrug of acyclovir (ACV) which provides an improved oral bioavailability of ACV—was investigated in human erythrocytes with a "papaverine-stop" assay. DCV influx was nonconcentrative, linearly dependent on DCV concentration (0.9 μM to 15 mM), insensitive (≤20% inhibition) to nucleobases, nucleosides, or potent inhibitors of nucleoside transport, and occurred without permeant metabolism. However, DCV was a weak competitive inhibitor of the influx of adenine (K_i = 1.3 mM) and of 5-iodo-2′-deoxyuridine (K_i = 2.9 mM), permeants of the erythrocyte nucleobase and nucleoside carriers, respectively. This indicates that DCV has an affinity for both of these transporters, even though it appears not to be an effective permeant. We conclude that, in contrast to ACV which enters human erythrocytes primarily via the nucleobase carrier, DCV permeates these cells chiefly (≥80%) by nonfacilitated diffusion. This mechanistic difference in transport between ACV and DCV is attributed to differences in their desolvation energies and suggests an explanation for the differences in the oral bioavailability of ACV which is observed after the administration of these two "acyclic nucleosides."

Desciclovir (DCV†) is metabolized extensively *in vivo* by the enzyme xanthine oxidase [1–3] and thus serves as a prodrug of the antiherpetic agent acyclovir (ACV) (Zovirax®). Whereas ACV is approximately 20% orally bioavailable in humans [4], DCV was found to be rapidly absorbed and metabolized, resulting in greater than 70% oral ACV bioavailability [1, 5–12].

This difference in the absorption of DCV and ACV is especially interesting since (1) the molecules differ only by the presence of the 6-oxo group in ACV (Fig. 1) and (2) ACV, which in human erythrocytes is transported primarily by the purine nucleobase carrier [13, 14], was found to have a 6.5-fold greater affinity than DCV for this carrier [13]. Therefore, we have characterized the mechanism of influx of DCV into human erythrocytes and have found major differences in the mechanisms by which DCV and ACV permeate the cell membrane. These differences suggest an explanation for the observed differences in the oral absorption of these agents.

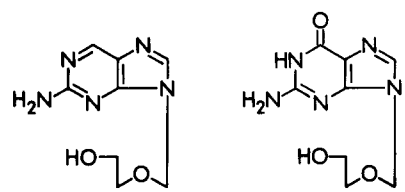

Fig. 1. Structures of DCV and ACV.

METHODS

Materials. [Side-chain 1-^{3}H]DCV (2-[(2-amino-9*H*-purin-9-yl)-methoxyl]-1-[^{3}H]ethanol, 8.2 Ci/mmol) was synthesized in the Wellcome Research Laboratories by Allan R. Moorman and John A. Hill [15]. [U-^{14}C]Sucrose (4 mCi/mmol) and [^{3}H]water (1 mCi/g) were obtained from Dupont-New England Nuclear. [8-^{3}H]Adenine (29 Ci/mmol) and [^{125}I]IdUrd (26 Ci/mmol) were from the Amersham Corp. Nonradioactive DCV and ACV (Fig. 1) were synthesized in these laboratories according to published procedures [1, 16]. Unlabeled nucleobases and nucleosides, papaverine hydrochloride, dipyridamole, and 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) were purchased from Sigma. Dilazep was provided by Hoffman-La Roche. Phosphate-buffered saline (PBS) and Hepes were obtained from GIBCO, and SepPak® C_{18} cartridges were obtained from Waters Associates.

Purity of radioisotopes. The [^{3}H]DCV was ≥98% radiochemically pure as determined by reversed-phase HPLC [17] and was used without further purification. [^{3}H]Adenine [14] and [^{125}I]IdUrd [18] were purified (≥96%) through the use of Sep-Pak® C_{18} cartridges.

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† Abbreviations: ACV, acyclovir [9-(2-hydroxyethoxymethyl)guanine]; DCV, desciclovir (2-[(2-amino-9*H*-purin-9-yl)methoxy]ethanol); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IdUrd, 5-iodo-2′-deoxyuridine; [^{125}I]IdUrd, 5-[^{125}I]iodo-2′-deoxyuridine; NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; and PBS, (Dulbecco's) phosphate-buffered saline.

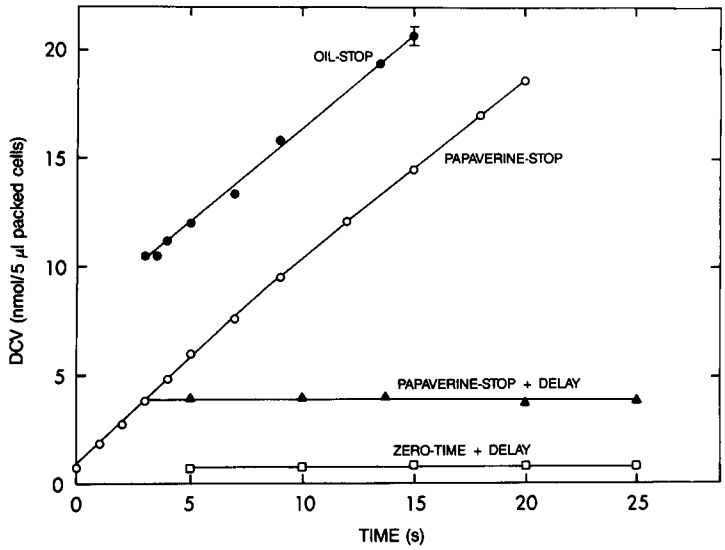

Fig. 2. Efficacy of a cold papaverine solution as a "stopper" for DCV influx. Human erythrocytes (5 μL packed cells) were incubated in Hepes–saline buffer, pH 7.3, for the indicated times at 37° with 15 mM [^{3}H]DCV (0.25 mCi/mmol) in a total volume of 100 μL. The "oil-stop" method used the initiation of centrifugation as the assay termination time, with no attempt to correct for the time required for the cells to sediment into the oil phase. The "papaverine-stop" method used the addition of 700 μL of ice-cold 19 mM papaverine to each transport assay as the termination time, and centrifugation of the cells through oil was initiated 10 sec after the papaverine addition. In the "papaverine-stop + delay" condition, papaverine was added to each assay at 3.0 sec, and the samples were kept at room temperature for 10 sec *plus* the indicated times before centrifugation. In the "zero-time + delay" condition, papaverine was added before the [^{3}H]DCV, and the samples were kept at room temperature for the indicated times before centrifugation. Each time point is the mean ± SEM of triplicate values. Error bars were omitted when they did not extend beyond the symbol boundaries.

Preparation of human erythrocytes. Human erythrocytes were collected from healthy volunteers and prepared as described previously [14, 19].

Kinetics of DCV and adenine influx. "Papaverine-stop" assays of DCV or adenine influx were performed at 37° in 10 mM Hepes–saline buffer, pH 7.3, as described previously for nucleobase influx [14]. Briefly, the total assay volume was 100 μL : 25 μL of cell suspension (20% hematocrit) plus 75 μL of radiolabeled permeant (0.01 to 2 μCi). Unless specified otherwise, inhibitors were added to the assay in the permeant solution. Assays were terminated by the addition of 700 μL of an ice-cold 19 mM papaverine solution followed by the addition of 240 μL of ice-cold *n*-butylphthalate and centrifugation of the sample within 10 sec. Cell pellets were treated as described previously [19]. Initial velocities of influx were determined by linear regression analysis of the slopes of plots of cell-associated radioisotope versus assay time during the linear phase of influx.

"Oil-stop" assays [20] of DCV influx were performed with the modifications described previously [14]. The assay time was defined as the interval between the addition of the permeant to the cells and the starting of the microfuge.

The amount of extracellular radioactivity in the cell pellet was determined using [^{14}C]sucrose [19].

Kinetics of IdUrd influx. Initial velocities of [^{125}I]IdUrd influx were measured as described previously [18] with several modifications. Assays were performed at 37° using a 100-μL assay volume: 20 μL of the cell suspension (25% hematocrit) plus 80 μL of radioactive permeant (25,000–230,000 cpm/assay). Inhibitors were present in the permeant solution. Assays were terminated by the addition of 400 μL of 670 μM dilazep followed by the addition of 240 μL of *n*-butylphthalate and centrifugation of the cells.

Metabolism studies. Cells were incubated for 15 min with 1 μM or 18 mM [^{3}H]DCV under the same conditions used for DCV influx measurements. The incubations were terminated by the addition of 700 μL of ice-cold papaverine, and the resulting 800-μL mixture was centrifuged through a layer of ice-cold 1-bromododecane (600 μL) into cold 20% trichloroacetic acid (100 μL). The acidic extracts were treated with trioctylamine as described previously [14], and these neutralized extracts were analyzed by reversed-phase HPLC [17].

Data analysis. The data from kinetic analyses were directly fitted to an hyperbola according to the method of Wilkinson [21] and the Cleland computer program [22]. Inhibition data were analyzed for conformity to the competitive model by the method of Spector and Hajian [23].

RESULTS

Use of a cold papaverine solution as a "stopper"

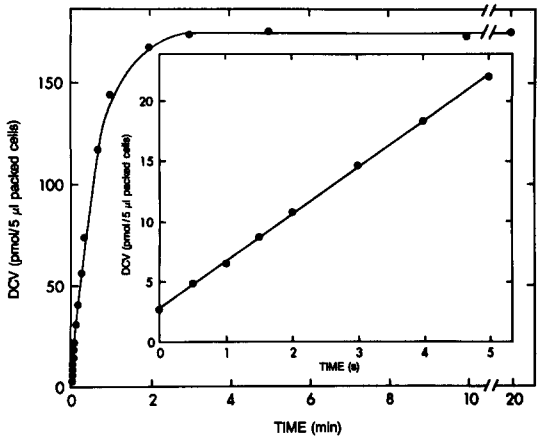

Fig. 3. Time dependence of DCV influx. Human erythrocytes (5 μL packed cells) were incubated in Hepes–saline buffer, pH 7.3, for the indicated times at 37° with 50 μM [^{3}H]DCV (90 mCi/mmol). Each time point is the mean of duplicate assays, and the deviation from the mean did not exceed 9%.

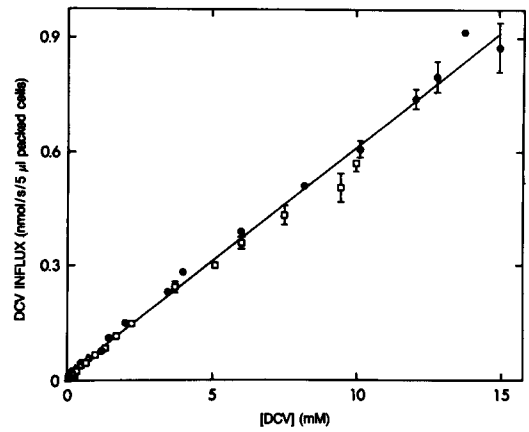

Fig. 4. Concentration dependence of DCV influx. Influx assays were performed at 37° as described under Methods with 6.5 μM to 15 mM [^{3}H]DCV (0.92 to 20.3 mCi/mmol). The different symbols represent values from three experiments in which the velocities of DCV influx were determined with assay times of 0, 1.0, 2.0, 4.0, 6.0 sec; 0, 1.0, 2.0, 3.0 sec; and 0, 1.0, 2.0, 4.0 sec. Initial velocities of influx were derived by linear regression analysis of the data obtained during this linear phase of DCV influx. Error bars represent the standard errors of the slopes obtained with this analysis, and these were omitted where they did not extend beyond the symbol boundaries.

for DCV influx. The influx of 15 mM DCV into human erythrocytes was measured with the "papaverine-stop" assay [14], which consisted of 7 vol. of ice-cold 19 mM papaverine. This solution was shown previously to be effective in stopping both the facilitated and nonfacilitated diffusion of purine nucleobases. The traditional "oil-stop" assay [20] was also evaluated for comparison (Fig. 2). Although both procedures appeared to yield linear initial velocities for DCV influx, the influx of DCV determined by the "papaverine-stop" method was linear only to approximately 10 sec with an initial rate that was 10% higher ($P < 0.01$) than that obtained with the oil-stop method (970 ± 17 vs 870 ± 21 pmol/sec/5 μL packed cells). The effectiveness of the "papaverine-stop" assay for instantaneous and complete stoppage of DCV influx is demonstrated by several criteria (Fig. 2). First, no significant changes in cell-associated DCV with increasing delay times before centrifugation of the cells through oil were detected in either a 3-sec assay ("papaverine-stop + delay" curve) or a zero-time assay ("zero-time + delay" curve). Second, the cell-associated radioactivity observed at zero-time (0.05%) was similar to the extracellular space as determined with [^{14}C]sucrose (0.04%).

Time dependence of DCV influx. The influx of 50 μM DCV was linear for at least 5 sec (Fig. 3, inset) and permeant equilibration across the erythrocyte membrane was reached within 3 min (Fig. 3). At equilibrium, the intraerythrocytic concentration of DCV (172 pmol DCV/5 μL packed cells, approximately 46 μM) was equal to the extracellular concentration.

Metabolism. Metabolism of 1.0 μM or 18 mM DCV was examined after 15-min incubations with human erythrocytes at 37°. In each case, ≥97% of the cell-associated radioactivity was eluted from the reversed-phase HPLC column with the same retention time as that of the authentic DCV standard.

Concentration dependence of DCV influx. The rate of DCV influx appeared to be linearly dependent on the concentration of DCV over the entire concentration range studied (6.5 μM to 15 mM, Fig. 4), with a rate constant of 60 ± 0.8 fmol/sec/μM DCV/5 μL packed cells.

Effects of various compounds on DCV influx. Purine nucleobases, ACV, nucleosides, or inhibitors of nucleoside transport were examined for their abilities to inhibit 0.9 μM DCV influx into human erythrocytes (Table 1). DCV influx appeared to be no more than marginally inhibited by a variety of compounds: 1.0 mM adenine or hypoxanthine (≤10% inhibition); 1.0 mM ACV (≤9% inhibition); 1.0 mM thymidine or uridine (≤6% inhibition); or 1.0 μM NBMPR, dipyridamole, or dilazep (≤18% inhibition). Combinations of adenine (1.0 mM) and inhibitors of nucleoside transport (1.0 μM) also resulted in only marginal (≤22%) inhibition of DCV influx.

Kinetic analysis for DCV as an inhibitor of purine nucleobase or nucleoside transport. DCV was found to be a linear competitive inhibitor of adenine influx ($K_m = 12$ μM) into human erythrocytes via the nucleobase transporter and of IdUrd influx ($K_m = 150$ μM) via the nucleoside transporter (Fig. 5), with K_i values of 1.3 and 2.9 mM, respectively.

DISCUSSION

The mechanism of transport of DCV into human erythrocytes was characterized by means of the "papaverine-stop" assay. This assay procedure has been used previously to measure the influx of

Table 1. Effects of ACV, nucleobases, nucleosides, and inhibitors of nucleoside transport on the influx of 0.9 μM DCV into human erythrocytes

Additive	DCV influx (% inhibition)
Adenine (1.0 mM)	10 ± 3 (5)
Hypoxanthine (1.0 mM)	7 ± 4 (4)
ACV (1.0 mM)	9 ± 3*(2)
Thymidine (1.0 mM)	4 ± 4 (3)
Uridine (1.0 mM)	6 ± 2 (3)
NBMPR (1.0 μM)	13 ± 3 (5)
Dipyramidole (1.0 μM)	18 ± 2 (5)
Dilazep (1.0 μM)	14 ± 3 (4)
Adenine + NBMPR	19 ± 2 (3)
Adenine + dipyramidole	19 ± 3 (3)
Adenine + dialzep	22 ± 2 (4)

The initial velocity of influx of 0.9 μM [^{3}H]DCV (1.9 to 5.1 mCi/μmol) was determined at 37° in the absence or presence of inhibitors as described in Methods. ACV, nucleobases, or nucleosides were added simultaneously with the [^{3}H]DCV. Inhibitors of nucleoside transport were preincubated with the cells for 20 min at 37° prior to the addition of [^{3}H]DCV. Initial velocities were derived by linear regression of data obtained during the linear phase of influx (0–6 sec) with at least five different time points per rate curve. Values are means ± SEM of multiple determinations, and the number of experiments is given in parentheses. Control rate was 0.076 ± 0.006 pmol/sec/5 μL packed cells.

* Value is the mean of two determinants ± the average deviation from the mean.

permeants of both the nucleobase [14, 24] and nucleoside carriers [24, 25], as well as of nucleobases which permeate human erythrocytes by nonfacilitated diffusion [14, 26]. The addition of seven assay volumes of ice-cold 19 mM papaverine was shown by several criteria to stop instantaneously and completely the influx of 15 mM DCV, the highest DCV concentration used in this study. The uptake of [^{3}H]DCV reflected true influx since DCV was not metabolized during 15-min incubations with human erythrocytes under the experimental conditions used in this study. Consistent with this absence of metabolism, DCV uptake was nonconcentrative in human erythrocytes.

The rate of influx of DCV was linearly dependent on the DCV concentration from 6.5 μM to 15 mM, with no evidence of saturation (Fig. 4). This linear relationship is characteristic of cell permeation by nonfacilitated diffusion. The influx of 0.9 μM DCV was only marginally inhibited (≤20%) by purine nucleobases, ACV, nucleosides, or inhibitors of nucleoside transport (Table 1), suggesting that the nucleoside and nucleobase carriers play little or no role in DCV influx. We therefore conclude that DCV enters human erythrocytes primarily (≥80%) by nonfacilitated diffusion with an influx rate constant of 60 ± 0.8 fmol/sec/μM DCV/5 μL packed cells at 37°. DCV was found to be a weak competitive inhibitor of the influx of both adenine (a permeant of the purine nucleobase tranporter) and IdUrd (a permeant of the nucleoside transporter), with K_i values of 1.3 and 2.9 mM, respectively. Although these results indicate that DCV has some affinity for each of these carriers, direct evidence supporting DCV permeation via these transporters could not be obtained due to the large

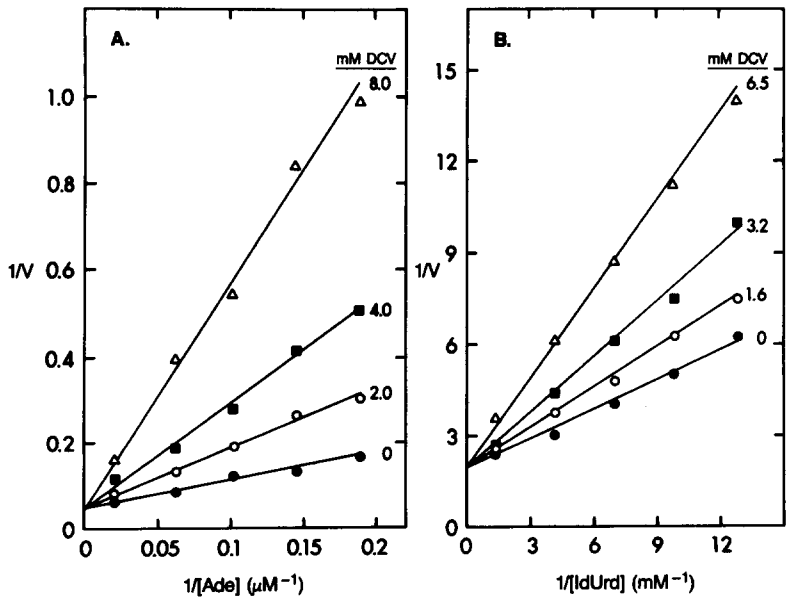

Fig. 5. Double-reciprocal plots for DCV inhibition of the influx of (A) adenine and (B) IdUrd. Influx assays were performed at 37° as described in Methods, and initial velocities were derived from linear regression analysis of data obtained during the linear phase of influx: (A) 0, 0.5, 1.0, 1.5 sec and (B) 0, 0.4, 0.8, 0.4, 0.8 sec. Initial velocities for [^{3}H]adenine (123 mCi/mmol) influx are in terms of pmol/sec/5 μL packed cells and are corrected for nonfacilitated diffusion of adenine as described previously [14]. Initial velocities for [^{125}I]IdUrd (1.5 mCi/mmol) influx are in terms of nmol/sec/5 μL packed cells.

degree of nonfacilitated diffusion of this permeant (≥80% of the total influx at 0.9 μM DCV).

DCV differs structurally from ACV only by the absence of the 6-oxo group (Fig. 1), and this single modification results in striking differences in two biological properties of these analogues: transport and gastrointestinal absorption. Whereas ACV membrane permeation appears to occur exclusively by facilitated diffusion [13, 24], DCV permeation of the human erythrocyte membrane occurs primarily by nonfacilitated diffusion. The partition coefficient (K_p) has been traditionally used as a measure of a compound's lipophilicity and ability to diffuse nonspecifically across a membrane. However, the K_p values for both DCV and ACV are low (≤0.1) [27], and the small differences do not seem adequate to provide an explanation for the remarkable difference in how these two analogues relate to the membrane. An alternative explanation for these results may be based on a difference in the desolvation energies of DCV and ACV. This latter parameter has been identified recently as a kinetic determinant for the passive diffusion of other compounds [28]. The presence of the 6-oxo group on ACV would predict a >2-fold increase in the desolvation energy which would adversely affect nonfacilitated diffusion by >30-fold [29, 30]. Thus, the desolvation energies for DCV and ACV may be the most important physiochemical parameter that accounts for the membrane permeability differences of these analogues.

Oral bioavailability is an important factor in drug efficacy. Attempts to identify physiochemical properties of compounds which directly influence their gastrointestinal absorption have led to the suggestions that lowered melting points, increased lipid solubilities, and increased K_p values are all associated with enhanced oral bioavailability [31, 32]. Since these reports failed to recognize or take into account the mechanisms of transport of the model compounds, a clear relationship between these parameters and the absorption process (i.e. facilitated vs nonfacilitated diffusion) was not apparent [1, 5–12]. Compared to ACV administration, administration of DCV to humans results in a 3.5-fold increase in the oral bioavailability of ACV. DCV is 18 times more water soluble than ACV and permeates human erythrocytes by the strikingly different mechanism of nonfacilitated diffusion. We suggest that the increased water solubility, in combination with permeation via a nonsaturable process, could confer an absorption advantage to DCV relative to ACV and that this accounts for the enhanced ACV oral bioavailability associated with DCV administration.

A recent *in vitro* study with rat jejunum [33] attributes low absorption of ACV to a low rate of nonfacilitated diffusion rather than to limitations associated with a saturable process. However, *in vivo* studies of oral ACV absorption have shown saturable pharmacokinetics in mice, rats, and dogs [34–36]. Similar studies in humans have also shown ACV absorption to be poor and variable with the suggestion of saturability [4, 9, 37–41, *]. By contrast, oral administration of DCV [8, 12] or Retrovir® [42], both of which permeate human erythrocytes by nonfacilitated diffusion, resulted in good absorption that was nonsaturable. Thus, the *in vivo* absorption profiles of these agents correlate well with their transport characteristics in human erythrocytes.

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