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# MEMBRANE PERMEATION MECHANISMS OF 2′,3′-DIDEOXYNUCLEOSIDES

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**Abstract**—The mechanism of membrane permeation of several 2′,3′-dideoxynucleosides was investigated at 37° with human erythrocytes using an "inhibitor-stop" assay. Transport (per 5 μL cells) via the nucleoside and nucleobase carriers was assessed by inhibition of influx with dilazep and adenine, respectively. Mechanisms of cellular entry were highly individualized: 2′,3′-dideoxyadenosine and 3′-deoxythymidin-2′-ene via nonfacilitated diffusion, with high rates; 2′,3′-dideoxyguanosine mainly via the nucleobase carrier ($K_m$ = 390 μM, $V_{max}$ = 32 pmol/sec); 2′,3′-dideoxyinosine by both nucleobase ($K_m$ = 850 μM, $V_{max}$ = 2.7 pmol/sec) and nucleoside ($K_m$ = 7.4 mM, $V_{max}$ = 16 pmol/sec) carriers, with a low rate of nonfacilitated diffusion; and 2′,3′-dideoxycytidine, equally by the nucleoside carrier ($K_m$ = 23 mM, $V_{max}$ = 65 pmol/sec) and by nonfacilitated diffusion, with a low rate. These results demonstrate that the nucleobase carrier plays an important role in the influx of two of these dideoxynucleosides and that nonfacilitated diffusion is not necessarily the chief mode of membrane permeation of this class of drugs.

Antiviral 2′,3′-dideoxynucleosides (ddNs)† have been reported to enter cells either chiefly [1–7] or partially [8–13] by nonfacilitated diffusion. These conclusions have been based primarily on the degree of inhibition of cellular influx by inhibitors of nucleoside transport. We reported recently that (−)-9-[4α-(hydroxymethyl)cyclopent-2-ene-1α-yl]guanine (carbovir), which can be viewed as a ddN, permeates human erythrocytes primarily via the nucleobase carrier [14]. The possible role of a nucleobase carrier in the transport of other ddNs has only been addressed in a few studies where nucleobases were tested as inhibitors of ddN influx: 2′,3′-dideoxycytidine (ddC) in P388 mouse leukemia cells and human erythrocytes [13]; and 3′-deoxythymidin-2′-ene (d4T) [7] and 3′-deoxythymidine (3′-dThd) [2] in human erythrocytes. We therefore reinvestigated the characteristics of ddN transport in human erythrocytes, cells that have separate and distinct carriers for nucleosides and nucleobases [15]. The results of this comparative study reveal the highly individualized characteristics of ddN influx and demonstrate an important role for the nucleobase carrier in the membrane permeation of some ddNs.

## MATERIALS AND METHODS

*Materials.* [2′,3′-$^3$H]ddNs [Ci/mmol: 2′,3′-dideoxyadenosine (ddA), 29; ddC, 44; 2′,3′-dideoxyguanosine (ddG), 23; 2′,3′-dideoxyinosine (ddI), 35; and d4T, 63] were from Moravek Biochemicals, Inc. [Side chain 2-$^3$H]acylovir (ACV; 28.3 Ci/mmol) and [U-$^{14}$C]sucrose (4 mCi/mmol) were from DuPont-New England Nuclear. All radioisotopes were purified on Sep-Pak® $C_{18}$ cartridges (Waters Associates) to ≥97% as assessed by reversed-phase HPLC [16]. Nonradioactive ddA was from the Fairfield Chemical Co. 2′-Deoxycoformycin was from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. ddG, ddI, d4T, and ACV (9-[2-hydroxyethoxymethyl]guanine) were synthesized in these laboratories. Dilazep was provided by Hoffmann-LaRoche. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was from GIBCO. All other chemicals were from Sigma.

*Preparation of human erythrocytes.* Human erythrocytes were collected from healthy volunteers, prepared as described previously [1, 15], and resuspended in 0.9% NaCl, containing 10 mM Hepes, pH 7.3, to a final hematocrit of 25%.

*Kinetics of permeant influx.* Influx assays were performed at 37° with the "inhibitor-stop" assay method as described previously [15]. Initial velocities of influx were calculated by linear regression analysis of the slopes of plots of cell-associated radioisotope versus four assay times within the linear phase of permeant influx: ddA, ≤1.2 sec (±2 μM 2′-deoxycoformycin); ddC, ≤20 sec; ddG, ≤10 sec; ddI, ≤20 sec; d4T, ≤5 sec; and ACV, ≤9 sec. Longer assay times (≤90 sec) were used when inhibitors were present.

*Kinetic analysis.* Kinetic parameters for the influx of ddNs were determined by nonlinear regression [17, 18] with the equation $v = (V_{max})(S)/(S + K_m) + (c)(S)$ using a $1/v^2$ weighting factor. "$c$" is the experimentally determined rate constant for the

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† Abbreviations: ddN, 2′,3′-dideoxynucleoside; ddA, 2′,3′-dideoxyadenosine; ddC, 2′,3′-dideoxycytidine; ddG, 2′,3′-dideoxyguanosine; ddI, 2′,3′-dideoxyinosine; d4T, 3′-deoxythymidin-2′-ene; 3′-dThd, 3′-deoxythymidine; carbovir, (−)-9-[4α-(hydroxymethyl)cyclopent-2-ene-1α-yl]guanine; ACV, acyclovir (9-[2-hydroxyethoxymethyl]guanine); and Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

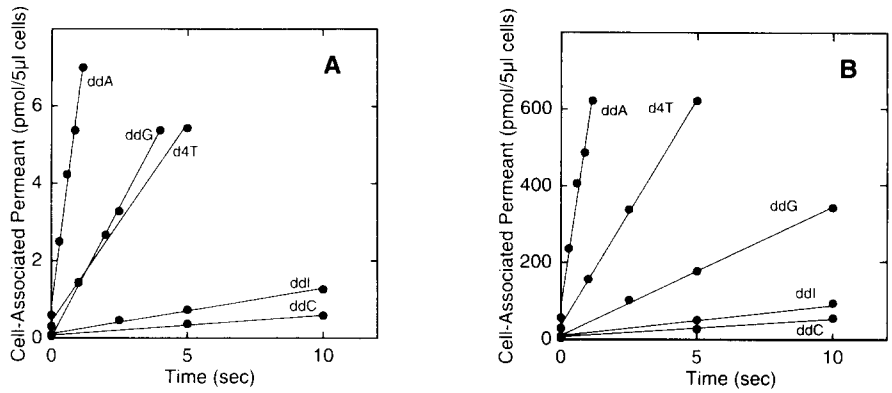


Fig. 1. Initial velocity of ddN influx into human erythrocytes. Human erythrocytes were incubated at 37°, as described in Materials and Methods, with [$^3$H]ddNs: (A) 10 μM, 500 Ci/mol; and (B) 1.0 mM, 6 Ci/mol.

residual concentration-dependent influx rates observed in the presence of either dilazep (ddC) or both adenine and dilazep (ddG, ddI). Kinetic values for the influx of ACV were determined as described previously [19]. Inhibition data were analyzed for conformity to the competitive model by the method of Spector and Hajian [20].

*Metabolism studies.* Human erythrocytes were incubated for 10 or 20 sec with permeant concentrations of 10 μM or 1.0 mM, respectively. After centrifugation of the cells through 400 μL of 1-bromododecane, cellular contents were extracted with cold acetonitrile [21], and the extracts were analyzed by reversed-phase HPLC [16]

## RESULTS

*Influx rates of ddNs.* Influx rates of 10 μM ddNs varied over a 100-fold range (Fig. 1A). At permeant concentrations of 1.0 mM, a similar profile of ddN influx was seen except that the rate of ddG influx was only 20% that of d4T (Fig. 1B). ddA influx rates measured in the presence of the adenosine deaminase inhibitor 2′-deoxycoformycin were identical to those shown in Fig. 1.

*Metabolism.* Cell-associated radioactivity after incubation of cells for 10 or 20 sec with radiolabeled ddC, ddG, ddI, and d4T was ≥89% unchanged ddN. Metabolism of ddA alone was extensive (80–90%) under these conditions, but the presence of 2.0 μM 2′-deoxycoformycin completely (99%) prevented this metabolism. ddA metabolism was not a factor in the measurements of influx rates at ≤1.2 sec, since these rates were the same in the absence or presence of 2′-deoxycoformycin.

*Inhibition of ddN influx.* Dilazep and adenine were used as specific inhibitors of the nucleoside and nucleobase transporter, respectively [22]. Adenine (3.0 mM) inhibited the influx of 1.0 mM ddG and ddI, and dilazep inhibited the influx of 1.0 mM ddC and ddI (Table 1). The influx of ddA and d4T was not inhibited significantly by either dilazep or adenine. Residual influx rates in the presence of both adenine and dilazep (presumed to represent nonfacilitated diffusion) were 3% of the total for ddG and 30–40% of the total for ddC and ddI. Inhibition results obtained with 10 μM permeants were similar (data not shown).

Table 1. Inhibition of ddN influx into human erythrocytes by dilazep and adenine

| Permeant (1.0 mM) | Inhibitors: Dilazep (1.0 μM) | Adenine (3.0 mM) | Dilazep + Adenine |
|---|---|---|---|
| | % Inhibition of influx* | | |
| ddA | 2 ± 2 | 1 ± 1 | 12 ± 3 |
| ddC | 50 ± 1 | 19 ± 6 | 58 ± 1 |
| ddG | 4 ± 4 | 95 ± 2 | 97 ± 1 |
| ddI | 43 ± 4 | 42 ± 5 | 66 ± 3 |
| d4T | 5 ± 4 | 8 ± 1 | 9 ± 7 |

Initial influx rates for each permeant were determined at 37° as described in Materials and Methods. Values are the means ± the average deviation from the mean of two determinations.

* Control influx rates, expressed as pmol/sec/5 μL packed cells, were: ddA 520 ± 50; ddA (+2.0 μM 2′-deoxycoformycin) 520 ± 40; ddC 4.1 ± 0.5; ddG 29 ± 4; ddI 9.4 ± 0.4; and d4T 150 ± 30.

*Carrier-mediated permeation.* Influx of ddG appeared saturable (Fig. 2, closed circles) and was not inhibited significantly by dilazep (Fig. 2, open circles). These data yielded a $K_m$ for ddG influx of 350 μM. Influx in the presence of adenine (Fig. 2, open triangles, inset) was markedly inhibited (>90%) and appeared saturable. The residual rate of ddG influx observed in the presence of adenine was further inhibited by ~50% by the addition of dilazep (Fig. 2, closed triangles, inset). Thus, a minor component of ddG influx appears to be nucleoside transporter-mediated. In the presence of both inhibitors, ddG influx rates were small and linearly dependent on ddG concentration ($c$ = 0.3 pmol/sec/mM ddG/5 μL cells). Further evidence for ddG

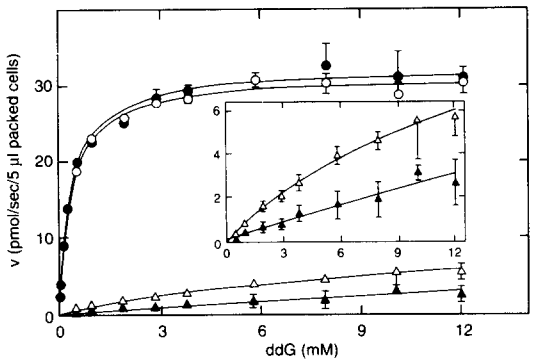


Fig. 2. Concentration dependence of ddG influx rates. Assays were performed at 37° in the absence (●) or presence of 1.0 µM dilazep (○), 3.0 mM adenine (△), or both inhibitors (▲). ddG concentrations were 24.4 µM to 12 mM (4 Ci/mol). Velocities were determined by linear regression analysis of data obtained during the linear phase of influx as described in Materials and Methods. Error bars represent the standard errors of the slopes obtained with this analysis, and these were omitted when they did not extend beyond the symbol boundaries. Inset, an expanded plot of the lower two curves.

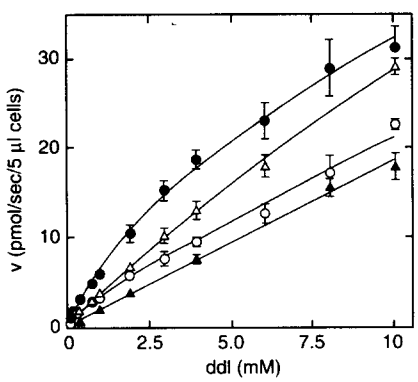


Fig. 3. Concentration dependence of ddI influx rates. Assays were performed at 37° in the absence (●) or presence of 1.0 µM dilazep (○), 3.0 mM adenine (△), or both inhibitors (▲). ddI concentrations were 0.1 to 10 mM (2.6 Ci/mol). Data analysis was as described in the legend of Fig. 2.

using the nucleobase carrier was obtained with inhibition studies using adenine and ACV, known permeants of this carrier [15, 19]. Adenine was a competitive inhibitor ($K_i = 11$ µM) of ddG influx, and ddG was a competitive inhibitor ($K_i = 360$ µM) of ACV influx (data not shown). In both cases, the $K_i$ values were similar to the respective $K_m$ values for influx.

The concentration-dependent profile of ddI influx (Fig. 3, closed circles) was biphasic, and influx was inhibited by adenine (Fig. 3, open triangles) and dilazep (Fig. 3, open circles). Influx rates in the presence of both adenine and dilazep (Fig. 3, closed triangles) were linearly dependent on ddI concentration with a low rate constant. The degree of inhibition with both inhibitors was greater than that with either adenine or dilazep alone and was consistent with additive inhibition. Kinetic parameters for the nucleobase and nucleoside transporters, obtained from data in the presence of dilazep or adenine, respectively, are presented in Table 2.

ddC influx (Fig. 4, closed circles) was not saturable to 30 mM and was inhibited by dilazep (Fig. 4, open circles). Influx rates in the presence of dilazep were linearly dependent on concentration with a low rate constant. Kinetic parameters for the nucleoside transporter are presented in Table 2.

*Nonfacilitated diffusion.* Nonfacilitated diffusion rate constants (obtained from influx rates in the presence of both adenine and dilazep) were calculated from data at two concentrations (10 µM and 1.0 mM) of ddA and d4T, and from the concentration-dependent profiles for ddG, ddI, and ddC. Expressed as pmol/sec/mM ddN/5 µL cells, these were: ddA, 440; d4T, 140; ddI, 1.9; ddC, 1.0; and ddG, <0.3*.

## DISCUSSION

The results of the present study clearly demonstrate that, in human erythrocytes, the mechanisms of ddN influx are highly individualized. ddG permeated these cells almost exclusively (>90%), and ddI partially (30–40%), via the nucleobase carrier. ddG related to the nucleobase carrier with an influx $K_m$ that was greater than that of guanine [15], carbovir [14], or ACV [19], and less than that of ganciclovir [22]. The $V_{max}$ values for all of these guanine derivatives were similar, suggesting that 9-substituents of guanine affect the binding to this transporter but not the translocation of the permeant. The kinetic parameters for ddI with respect to the nucleobase carrier were determined here for the first time, and ddI had a higher influx $K_m$ and a lower $V_{max}$ than hypoxanthine [15].

We also report for the first time the kinetic parameters for the relationship of ddG, ddI, and ddC to the human erythrocyte nucleoside transporter. Since the 3'-OH group is an important determinant of interaction with the nucleoside transporter [23], it is not surprising that ddNs are poor permeants with millimolar $K_m$ values (ddC, ddG, ddI, and carbovir [14]) or have no measurable nucleoside transport influx component (ddA and d4T).

Our results are consistent with the conclusions of others that ddA [3–5, 8] and d4T [7] enter cells by nonfacilitated diffusion and that ddC and ddI permeation occurs partially by nonfacilitated diffusion [9–13]. However, this comparative study reveals that the rates of nonfacilitated diffusion for ddC and ddI are strikingly low in comparison with ddA and d4T, and that, like carbovir [14], ddG

* The residual influx rate of ddG observed in the presence of both adenine and dilazep can be accounted for by the failure of adenine (3.0 mM) to inhibit completely the influx of ddG as calculated from the Michaelis–Menten equation for competitive inhibition using the kinetic parameters in Table 2.

Table 2. Kinetic parameters of ddNs for the nucleobase and nucleoside transporters

| Permeant | Nucleobase transporter* $K_m$ ($\mu$M) | Nucleobase transporter* $V_{max}$ (pmol/sec/5 $\mu$L cells) | Nucleoside transporter $K_m$ (mM) | Nucleoside transporter $V_{max}$ (pmol/sec/5 $\mu$L cells) |
|---|---|---|---|---|
| ddG | 390 ± 40 | 32 ± 1 | 6.6 ± 0.8† | 5.3 ± 0.7† |
| ddI | 850 ± 110 | 2.7 ± 0.2 | 7.4 ± 1.4† | 16 ± 3† |
| ddC | ND‡ | ND | 23 ± 4 | 65 ± 4 |

Initial influx rates for each permeant were determined at 37° and analyzed as described in Materials and Methods. Values are the means ± the average deviation from the mean of two determinations.

* Determined in the presence of 1.0 $\mu$M dilazep.

† Determined in the presence of 3.0 mM adenine.

‡ ND, not detected.

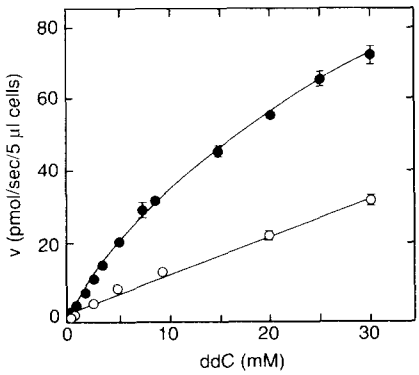


Fig. 4. Concentration dependence of ddC influx rates. Assays were performed at 37° in the absence (●) or presence of 1.0 $\mu$M dilazep (○). ddC concentrations were 0.52 to 30 mM (1.6 Ci/mol). Data analysis was as described in the legend of Fig. 2.

exhibits no detectable nonfacilitated diffusion. Thus, the nature of the nucleobase moiety appears to be a determinant in the ability of ddNs to permeate the cell membrane via nonfacilitated diffusion.

After this investigation was completed, results of a related study were published by Gati *et al.* [24]. These authors also found that ddG enters human erythrocytes (and CCRF-CEM cells) chiefly via the nucleobase carrier, although the minor influx of ddG via the nucleoside carrier was not identified in that study. Furthermore, the relative influx rates of ddA, ddG, and ddI in human erythrocytes observed by Gati *et al.* agree with those reported in this study. Quantitative differences in influx rates or kinetic constants between the two studies may be attributed to differences in methodology used in the two laboratories.

We conclude that ddNs permeate human erythrocytes in a highly individual manner via the nucleobase carrier, the nucleoside carrier, and/or by nonfacilitated diffusion. Thus, nonfacilitated diffusion is not necessarily the chief mode of cellular entry of these ddNs. With other cell types, which may contain different numbers, types, or substrate specificities of transport proteins, the relative contribution of nonfacilitated diffusion to permeation of ddNs may be more or less than that observed with human erythrocytes.

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