Enhanced Permeability of the Antimicrobial Agent 2,5-Bis(4-Amidinophenyl)Furan across Caco-2 Cell Monolayers via Its Methylamidoxime Prodrug

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Purpose. DB75 [2,5-bis(4-amidinophenyl)furan] is a promising antimicrobial agent although it has poor oral potency. In contrast, its novel prodrug, 2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime (DB289), has excellent oral potency. The mechanisms of transport of DB289 and DB75 across intestinal epithelium have been investigated in these studies to understand differences in their oral potency.

Methods. Caco-2 cell monolayers were used as an *in vitro* model to examine the mechanisms of transport of DB289 and DB75. Samples collected from the transport studies were quantified using high-performance liquid chromatography with ultraviolet and fluorescence detection.

Results. A low permeability coefficient (3.8×10^{-7} cm/s for transport in apical [AP] to basolateral [BL] direction) and high sensitivity to extracellular Ca^{2+} suggest that AP to BL transport of DB75 across Caco-2 cell monolayers occurs predominantly via a paracellular route. DB289 has an 85-fold higher transport rate (322.0×10^{-7} cm/s for transport in the AP to BL direction) across Caco-2 monolayers than that of DB75. This, with its insensitivity to extracellular Ca^{2+} indicates that AP to BL transport of DB289 across Caco-2 cell monolayers occurs predominantly via a transcellular route.

Conclusions. DB75 is transported across Caco-2 cell monolayers predominantly via paracellular pathways, whereas the prodrug DB289 is transported via transcellular pathways. This could account for the much higher oral activity of DB289 over DB75.

KEY WORDS: diamidine; O-methylamidoxime prodrug; antimicrobial; Caco-2 cells; intestinal permeability.

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INTRODUCTION

Aromatic dicationic compounds such as pentamidine (Fig. 1) are very effective antimicrobial agents with activity against a variety of pathogens (1,2). Pentamidine has been used clinically since 1939 and remains an important agent to treat primary-stage African trypanosomiasis (3), antimony-resistant leishmaniasis (4), and as a secondary drug of choice for *Pneumocystis carinii* pneumonia (5), an opportunistic infection that is prevalent among AIDS patients. Clinical use of pentamidine has been restricted by toxicity and limited oral activity (2,6). Systemic injection of pentamidine required in clinical practice causes particular difficulties in rural African settings where trypanosomiasis typically occurs. An analog of pentamidine, 2,5-bis(4-amidinophenyl)furan (DB75; Fig. 1), synthesized in the early 1970s, has markedly improved activity compared with pentamidine in an immune-suppressed rat model of pneumocystosis and in acute mouse models of trypanosomiasis (7–9). Like pentamidine, however, DB75 suffers from poor oral activity (10). At physiologic pH, the two positive charges associated with the diamidine groups (with successive pK_as = 10.4 and 9.8, Table I) make DB75 highly hydrophilic, which may lead to poor oral absorption and consequently poor oral activity.

Strategies for improving oral bioavailability for amidines have focused on attempting to mask the positive charges. Using amidoximes as prodrugs for amidines was suggested to have broad application for amidine type compounds (11,12). Several other lipophilic derivatizations of the amidino group also have been reported to produce prodrugs with improved oral bioavailability for an anti-*Pneumocystis* and an anti-platelet agent (13,14). We recently have synthesized a novel *O*-methylamidoxime prodrug of DB75, 2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime (DB289; Fig. 1; 9), which exhibits enhanced oral activity and reduced acute toxicity in animal models for pneumocystosis and trypanosomiasis. DB289 displayed potent oral anti-*Pneumocystis* activity at 33, 22, and 11 μmol/kg/day, with no overt toxicity produced in any of the animals treated for 14 days (9). In an acute mouse model of trypanosomiasis, DB289 demonstrated an excellent oral dose response with an ED_{50} of 4.8 μmol/kg (Hall *et al.*, unpublished data). Data obtained from examining its metabolic transformation using freshly isolated rat hepatocytes indicate that DB289 is efficiently converted to the DB75 via a series of stepwise *O*-demethylations and *N*-dehydroxylation (12,15). DB289, as the first bis-*O*-methylamidoxime prodrug documented, is currently undergoing phase II clinical trials as a potential new anti-*Pneumocystis* and anti-trypanosomal drug. No information is available concerning mechanisms of oral absorption for DB75 and DB289. Hence, the basis for difference in their oral activities is not clear, although one can speculate that better oral activity of DB289 is caused by better oral absorption compared with that of DB75. An understanding of how the prodrug DB289 overcomes the intestinal epithelial barrier can lead to a general approach for improving oral absorption and activity of dicationic drug candidates.

The human colon carcinoma cell line, Caco-2, shows morphologic and functional similarities to human small intestinal epithelial cells (16). Caco-2 cells have been widely used as a screening tool for predicting drug absorption across hu-

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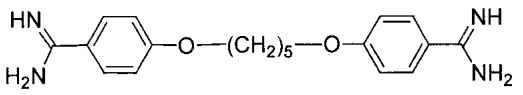
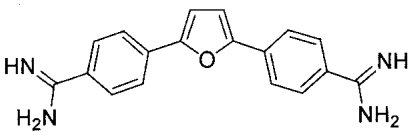
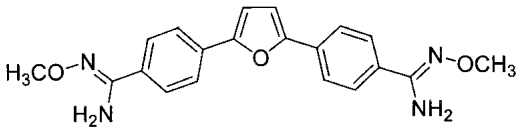


Pentamidine



2, 5-bis[4-amidinophenyl]furan (DB75)



2, 5-bis[4-amidinophenyl]furan
bis-O-methylamidoxime (DB289)

Fig. 1. Chemical structures of pentamidine, DB75, and DB289 (free base forms).

man intestines and for mechanistic studies of intestinal drug transport. In this article, both the uptake and transport of DB75 and DB289 were investigated using Caco-2 cells. As a reference compound, transport of pentamidine across Caco-2 cell monolayers was also assessed.

EXPERIMENTAL PROCEDURES

Materials

Transwells™ containing polycarbonate membrane were purchased from Corning Costar Corporation (Cambridge, MA, USA). Eagle's minimum essential medium (with Earle's salts and L-glutamine), fetal bovine serum (FBS), nonessential amino acids (NEAA, X100), 0.05% trypsin-EDTA solution, and the antibiotic/antimycotic solution (×100, 10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B) were obtained from Gibco Laboratories (Grand Island, NY, USA). Tissue culture-grade dimethyl sulfoxide (DMSO), bovine serum albumin (BSA, fraction V), Hank's balanced salt solution (HBSS), and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES, 1 M) was purchased from the Lineberger Comprehensive Cancer Center, University of North Carolina (Chapel Hill, NC, USA). Alamar Blue was supplied as a premixed solution from BioSource International (Camarillo, CA, USA). [^{14}C] Mannitol (51.1 mCi/mmol) was obtained from NEN Research Products (Boston, MA, USA). [^{14}C]Pentamidine (32 mCi/mmol) was synthesized at Research Triangle Institute (Research Triangle Park, NC). The chemical synthesis of DB75-dihydrochloride salt and DB289-monomaleate salt were performed as previously described (8,9).

Cell Culture

The Caco-2 cell line was obtained from GlaxoWellcome Research Institute (Research Triangle Park, NC, USA), and cultured as described previously (17). Briefly, Caco-2 cells were cultured at 37°C in culture medium, which contained minimum essential medium supplemented with 10% FBS, 1% NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, in an atmosphere of 5% CO_2 and 90% relative humidity. The cells were passaged at about 90% confluency using trypsin-EDTA. At passage 50–60, Caco-2 cells were seeded at a density of 60,000 cells/cm^2 on polycarbonate membranes of Transwells (12 mm i.d., 3-μm pore size) with 0.5 mL apical (AP) volume and 1.5 mL basolateral (BL) volume or seeded on 48-well plates with 1 mL of volume. The culture medium was changed the day after seeding and every other day thereafter. Caco-2 cell monolayers were used 21–25 days post-seeding.

Cell Viability

Cell viability was measured using an Alamar Blue assay to evaluate cytotoxic effects of DB75, DB289, and pentamidine to Caco-2 cells (18). Cells were cultured as described above. For experiments with 12-well transwells, the assay was started by replacing the medium in both AP and BL compartments with culture medium containing a test compound at selected concentrations. Because DB289 was only slightly soluble in culture medium, it was dissolved in culture medium containing additional 1% DMSO and 2% BSA. It was prepared by spiking prewarmed culture medium containing BSA with a concentrated DB289 stock solution made up in DMSO and then shaken at 37°C for 30 min before use. After 1-h incubation with the test compounds, 50 μL and 150 μL of the Alamar Blue solution (resulting in a 1/10 dilution in culture medium in both cases) were added to the AP and BL buffer,

Table I. Comparison of Transport of DB75 and DB289 across Caco-2 Cell Monolayers with Their Lipophilicities and *in Vivo* Oral Potency against Trypanosomiasis

Compound	ClogD (pH 7.4)[a]	pK_{a1}^a	pK_{a2}^a	Transport direction	Permeability coefficient (10^{-7} cm/s)	Recovery at 60 min (%)	Oral potency ED_{50} (μmol/kg)
DB75	−3.0	10.4	9.8	AP→BL	3.8 ± 0.2	90.5	>400[b]
				BL→AP	8.5 ± 0.2	93.1	
DB289	4.3	4.6	4.0	AP→BL	322.0 ± 10.8	95.4	4.8[c]
				BL→AP	281.7 ± 10.6	101.3	

[a] Calculated as indicated in the experimental procedures.
[b] Adapted from reference 10.
[c] Hall *et al.,* unpublished data, using the same assay system as did E. A. Steck and others in reference 10.

respectively, and the cells were further incubated for 2 h on a rocker placed in the incubator. At the end of incubation, the buffer in the AP and BL compartments was mixed and fluorescence was measured using a FLUOstar fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) at 37°C. For experiments using 48-well plates, 50 μL (resulting in 1/10 dilution as above) of Alamar Blue solution was added to the wells after 1-h incubation with test compounds. Fluorescence of the incubation buffer was measured after 2 h of further incubation. Cells incubated without the test compounds served as controls.

Transport Studies

Cell monolayers were first washed once with 0.5 mL or 1.5 mL of transport medium (HBSS supplemented with 25 mM D-glucose and 10 mM HEPES) in the AP or BL compartment, respectively. Cell monolayers were then incubated for 30 min at 37°C in the transport medium. AP to BL transport was initiated by replacing the AP medium with 0.4 mL of the transport buffer containing a test compound. The inserts were then transferred at selected times to 12-well cell culture clusters containing 1.5 mL of transport medium in each well. BL to AP transport was initiated by replacing the BL buffer with 1.5 mL of the drug solution after the AP medium had been replaced with 0.4 mL of the transport medium. The AP solution (0.2 mL) was withdrawn and the same volume of prewarmed transport medium was added to the AP sides at selected times. All transport experiments were performed at 37°C. For ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)-modulated transport experiments, the cell monolayers were incubated in the presence of 2.5 mM EGTA in both AP and BL sides for 9 min at 37°C after the initial 30 min preincubation. Cell monolayers were then washed 3 times with 0.5 mL or 1.5 mL of the transport medium. AP to BL transport was initiated in the same way as described above.

Transepithelial electrical resistance (TEER) values were measured to assess cell monolayer integrity. The EVOM (Epithelial Tissue Voltohmeter, World Precision Instruments, Sarosota, FL, USA) and Endohm12 electrode (World Precision Instruments) were used to measure TEER across Caco-2 cell monolayers. TEER values were obtained both at the beginning and end of each experiment. Only wells with TEER values between 400–500 Ω cm^2 throughout the experiment were used in the studies. Mannitol (25 μM or 100 μM) transport experiments were performed in the same manner as other transport experiments. Mannitol served as a paracellular marker and as a probe of the Caco-2 cell monolayer integrity (17). To assess the barrier properties of the polycarbonate membrane alone, transport was also determined in the absence of cells. Transport experiments with 1% DMSO and 2% BSA in the transport buffer were carried out to evaluate the effect of 1% DMSO and 2% BSA on cell monolayer integrity.

Cellular Uptake and Metabolism of DB289

Cell monolayers were prepared as described earlier for transport studies. The AP medium was replaced with 0.4 mL of the transport medium containing an additional 2% BSA and 1% DMSO to aid solubility of DB289 as described above. At selected times the wells were washed five times with ice-cold PBS (either 0.5 mL for AP or 1.5 mL or BL side). Triton X-100 (1%, 0.3 mL) was then added to the AP side, and the cell monolayers were shaken for 1 h at room temperature. Cell lysates were centrifuged (10,000 × g, 5 min) and the supernatants were analyzed using high-performance liquid chromatography (HPLC). The amount of protein in cell lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard (19).

Analytical Methods

Samples obtained from the transport studies as well as the samples from cellular uptake and metabolism studies were analyzed by reverse-phase HPLC with a photodiode array detector and a fluorescence detector (Hewlett Packard model 1100, Palo Alto, CA, USA). The column used was a 150 × 2.1 mm Zorbax Bonus-RP (5-μm particle size) column (Agilent Technologies, Wilmington, DE). Initially, ultraviolet (UV) signals in the 210–400 nm range were monitored with photodiode array detection and fluorescence signals were monitored with excitation at 359 nm and emission at multiple wavelengths (400, 430, 460, and 490 nm) to probe for possible metabolites or degradation products formed in transport or uptake experiments. After analysis of the initial data, UV detection with wavelength at 359 nm and fluorescence detection with excitation at 359 nm and emission at 461 nm were chosen for routine detection, without omitting any observable peaks. The column compartment was maintained at 40°C. The mobile phase consisted of mixtures of acetonitrile and water containing 35 mM formic acid and 15 mM ammonium formate. The concentration (v/v) of acetonitrile for analysis of DB75 transport samples was 4% at time = 0, increased to 56% after a linear gradient over 4 min. It was maintained at 56% for 3 min before recycling. The concentration (v/v) of acetonitrile for analysis of DB289 transport and uptake samples was 4% at time = 0 and increased to 56% after a linear gradient over 4 min. It was maintained at 56% for 9 min before recycling. The flow rate was 0.35 ml/min, and the injection volume was 3 μL. Five-point standard curves of DB75 or DB289 were generated with concentration ranges covering the content of DB75 or DB289 in the samples. The content of [^{14}C] mannitol and [^{14}C] pentamidine in the samples obtained from the transport samples were quantified using a liquid scintillation counter (Tri-Carb 2100TR, Packard, Downers Grove, IL, USA). Data were expressed as mean ± SD from three measurements.

Apparent Partition Coefficients and p*K*a

The calculated log octanol-water distribution coefficient at pH 7.4 (or ClogD) and p*K*a values for DB75 and DB289 were calculated using ACD/logD Suite (Advanced Chemistry Development Inc., Toronto, Canada).

Data Analysis

Results from the Alamar Blue assay were analyzed using GraghPAD Prism 3.00 (San Diego, CA, USA) to obtain a concentration that induces 50% toxic effect in cultured cells (TC_{50}). The goodness of fit (R^2) obtained from the curve-fitting procedures using the nonlinear least-squares regression analysis was in the range of 0.94–1.00. The apparent perme-

ability coefficients (P_{app}, cm/s) through Caco-2 cell monolayers were calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{A \cdot C_0}$$

where dQ/dt (nmol/s) is the appearance rate of the compound in the receiver compartment (the BL compartment for AP to BL transport; AP compartment for BL to AP transport); A is the surface area of the monolayer (1 cm^2); and C_0 is the initial concentration of the compound administered in the donor compartment (the AP compartment for AP to BL transport; BL compartment for BL to AP transport). The appearance rates (dQ/dt) were calculated by plotting the amounts of compound transported to the receiver side vs. time and determining the slopes of these plots in the linear range. The correlation coefficients (r^2) obtained from the least-squares linear regression analysis were in the range of 0.96–1.00.

Statistical Analysis

Student's *t* test was used for statistical analysis with a p value of less than 0.05 being considered statistically significant.

RESULTS

Cytotoxicity of DB75 and DB289

Diamidine compounds structurally related to pentamidine frequently elicit substantial cytotoxicity against a variety of cell lines (20). To evaluate the cytotoxicity of DB75, DB289, and pentamidine toward Caco-2 cells, cell viability was measured using the Alamar Blue assay. Alamar Blue has a wide application as a cell viability indicator. It has recently been used to assess cytotoxicity of compounds toward cell monolayers, such as Madin-Darby canine kidney cells (18). Results in Fig. 2, obtained using 12-well transwells, indicate that DB75 was relatively nontoxic to Caco-2 cells, with a TC_{50} of 302.5 μM. No toxicity was observed at concentrations of 25 μM or below. Results using 48-well plates gave similar results, with a TC_{50} of 331.0 μM. The decrease of cell viability at concentrations above 25 μM was consistent with a concomitant decrease in TEER, indicating that the TEER value is a comparable indicator for cytotoxicity of DB75 (Fig. 2). DB289 had no effect on the TEER or fluorescence intensity in the Alamar Blue assay at the test concentrations (1, 5, 10, 25 μM) shown in Fig. 2, indicating that DB289 was not cytotoxic to Caco-2 cells at 25 μM or below. Data at higher concentrations of DB289 could not be obtained because of solubility difficulties. Considering the cytotoxicity of DB75 and DB289 and the solubility limit of DB289, a single concentration of these two compounds, 25 μM, was chosen for the following studies. Pentamidine, which like DB75 was readily soluble under the assay conditions, demonstrated a TC_{50} of 93.3 μM using 12-well transwells.

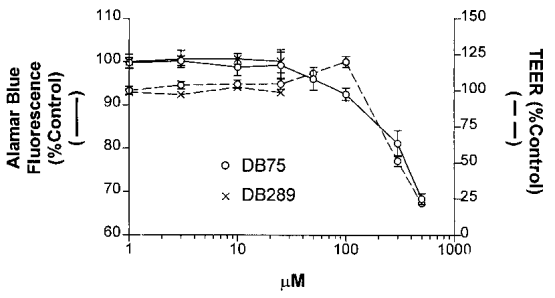


Fig. 2. Cytotoxicity of DB75 (o) and DB289 (×) to Caco-2 cell monolayers cultured on Transwells. Alamar Blue fluorescence intensity of each sample (—) was expressed as percent of control. TEER values (– –) were measured at the end of the 3-h incubation and expressed as percent of control. Cells incubated without DB75 or DB289 served as controls. The highest concentration tested for DB75 was 500 μM and for DB289 was 25 μM. All measurements were made in triplicate and expressed as mean ± S.D.

Permeability of DB75 and DB289 across Caco-2 Cell Monolayers

The time course of transport of DB75 and DB289 in both AP to BL and BL to AP directions across Caco-2 cell monolayers is shown in Fig. 3. The amount of DB75 transported in both directions increased linearly with time (Fig 3A). A permeability coefficient of 3.8×10^{-7} cm/s was obtained for transport of DB75 in AP to BL direction. The permeability in BL to AP direction was 2.3 fold greater than that in AP to BL direction (Table I). These permeability coefficients are comparable with those obtained for mannitol (paracellular marker) in these studies (2.1×10^{-7} cm/s for AP to BL direction; 2.9×10^{-7} cm/s for BL to AP direction) and reported in the literature (1.8×10^{-7} cm/s for AP to BL direction) (21).

The amount of DB289 transported in both directions also increased linearly with time after an initial lag of several minutes (Fig. 3B). As shown in Table I, DB289 had a P_{app} value of 322.0×10^{-7} cm/s for transport in the AP to BL direction, 85-fold greater than that of DB75 in the same direction. The permeability difference between DB289 and DB75 was only 33-fold for BL to AP transport (281.7×10^{-7} cm/s for DB289; 8.5×10^{-7} cm/s for DB75). This implies a greater net difference of permeability between these two compounds in the translocation across Caco-2 cell monolayers in the absorptive direction (AP to BL).

Results obtained from a study of transport of DB75 and DB289 across blank polycarbonate filters without cells showed that equilibrium between the donor and acceptor compartment was reached before 15 min for DB75 and 30 min for DB289. This indicated that translocation across the polycarbonate filter was not rate-limiting for transport of DB75 and DB289 in these studies. In an additional experiment, AP to BL transport of pentamidine was performed in the same manner. P_{app} for pentamidine was 9.3×10^{-7} cm/s when 25 μM pentamidine was initially placed in the AP compartment, which was comparable to the permeability of DB75.

The cosolvent (1% DMSO and 2% BSA) used in the transport studies with DB289 had no effect on cell monolayer integrity, based on TEER values obtained in the control experiments with 1% DMSO and 2% BSA in the transport buffer. P_{app} values for DB75 obtained from transport studies using transport medium with or without co-solvent (1% DMSO and 2% BSA) had no significant difference.

Paracellular vs. Transcellular Transport of DB75 and DB289: Effect of EGTA

To characterize the relative contributions of transcellular and paracellular transport for DB75 and DB289, the trans-

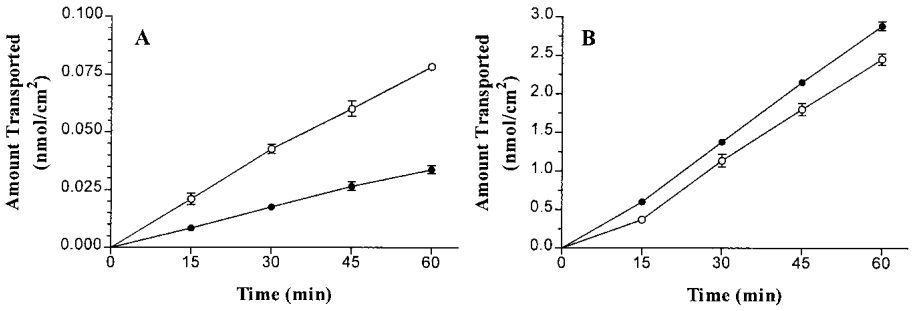


Fig. 3. Time course of transport of (A) DB75 (25 mM) and (B) DB289 (25 mM) across Caco-2 cell monolayers. AP to BL transport is indicated by filled symbols and BL to AP transport is indicated by open symbols. Values are the mean of three measurements ± S.D.

port of these two compounds was studied using Caco-2 cells monolayers pretreated with EGTA. EGTA is a selective calcium chelator, whose presence in the transport medium is known to cause opening of the intercellular tight junctions of the Caco-2 cell monolayers, thus increasing transport via the paracellular route (22). Results shown in Fig. 4 indicate that a more than 14-fold increase in P_{app} for DB75 was observed after preincubation with 2.5 mM EGTA for 9 min at 37°C. This result, together with the low P_{app} value (Table I), suggests that the paracellular transport route is the predominant route for the transport of DB75 across Caco-2 cell monolayers. No significant effect on the P_{app} value for DB289 was seen in the same studies, indicating that the paracellular route had little contribution to its transport. This result, in addition to its high P_{app} value (Table I), suggests that a transcellular route is the predominant route for DB289. Mannitol acted as a positive control in the EGTA-treatment experiments. A concomitant increase of more than 13-fold in the P_{app}, coincident with a 65% TEER decrease after EGTA-treatment, reflects the increase in junctional pore size of Caco-2 cell monolayers in this study.

Cellular Uptake and Metabolism of DB289

Results of the cellular uptake of DB289 into Caco-2 cell monolayers from the AP compartment are presented in Fig. 5, where the cumulative uptake is plotted against incubation time. Uptake of DB289 into Caco-2 cells occurred quickly, reaching a plateau after 10-min incubation with 25 μM DB289 initially administered in the AP compartment. Uptake was maintained at the same level throughout the experimental period. Maximal uptake of approximately 1.5 nmol/mg protein after 10 min incubation accounts for 4.5% of the initial amount of DB289 in the AP compartments. Gan *et al.* (21) performed cellular uptake measurements of mannitol and observed that after a 3-h incubation of 2.5 μM mannitol with a Caco-2 cell monolayer grown on a 4.71cm^2 filter, 0.83 pmol of mannitol was taken up into the Caco-2 cells. Taking into account the difference in initial incubation concentrations, incubation times and cell numbers used between these two experiments, DB289 demonstrates a substantially greater penetration across the AP membrane of Caco-2 cells than did mannitol. This supports the transcellular transport mechanism of DB289 proposed earlier in these studies.

No additional HPLC peaks were detected in either the AP or BL samples after incubation with DB289 over a time period of 2 h. Cell lysates, however, did contain three additional peaks (Fig. 6). One peak co-eluting with DB75 and two unidentified peaks eluting between DB75 and DB289 were seen at all times, and increased with time compared to the negative control of cell lysates prepared from cells incubated with DB289-free buffer for 2 h. This suggests that DB289 is metabolized in Caco-2 cells to give DB75 and two minor unidentified metabolic products. Quantitatively, only 0.2% of the cellular DB289 was measured as DB75 after 120 min of incubation. It seems that the conversion of the prodrug DB289 to DB75 is not efficient in Caco-2 cells.

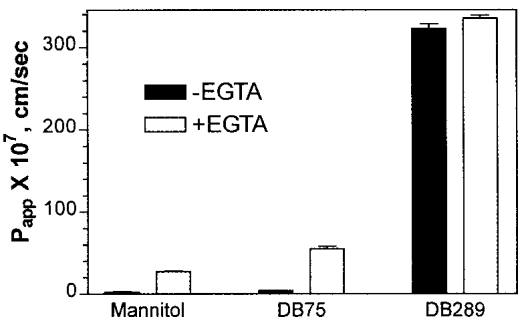


Fig. 4. Effect of EGTA, a selective Ca^+ chelator, on the apparent permeability coefficient of DB75, DB289 and mannitol (paracellular transport marker). Transport experiment conducted without EGTA pre-treatment (■) and with EGTA pretreatment (□).

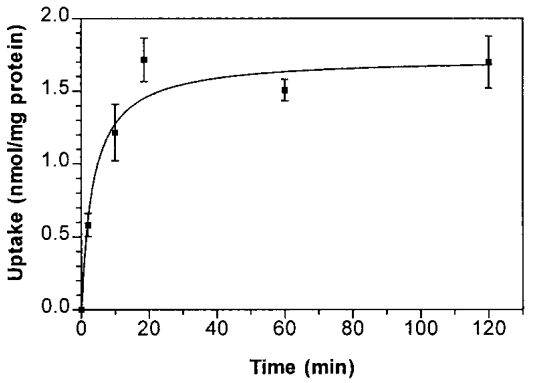


Fig. 5. Time course of cellular uptake of DB289 (25 μM) across AP membrane of Caco-2 cell monolayers. The amount of DB289 in cell lysates at selected times was measured using HPLC. The amount of protein in cell lysates was determined using the BCA protein assay kit. Values are the mean of three measurements ± S.D.

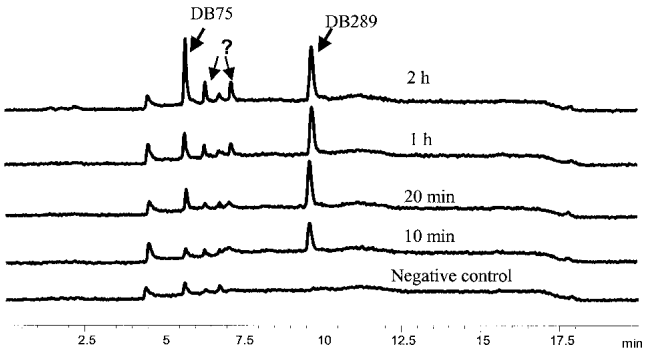


Fig. 6. HPLC chromatograms of cell lysates collected from the cellular metabolism studies with DB289 (25 mM). Fluorescence detection with excitation at 359 nm and emission at 461 nm was used (only DB289 peaks were detected by UV detection). Cell lysate obtained from incubation with transport medium without DB289 for 2 h served as the negative control.

Recovery and Stability of DB75 and DB289

To trace all of the DB75 and DB289 during transport experiments, the recovery of these two compounds after 60 min transport was examined (Table I). Percent recovery was measured from the sum of the amount of each compound in the donor and acceptor compartments after a 60-min transport experiment divided by the amount initially administered in the donor compartment. Recovery for DB75 and DB289 was between 90 and 102%. These results indicated that majority of DB75 and DB289 remained intact and stayed in the donor and acceptor compartments during transport experiments. Similarly, the recovery of pentamidine in a comparable experiment for AP to BL transport was measured as 91%. In an additional experiment, DB75 and DB289 were exposed for 12 h to cell free buffer to evaluate their stability under conditions of the transport experiments. Neither DB75 nor DB289 showed any degradation during incubation.

DISCUSSION

To traverse intestinal epithelium, the major barrier to absorption of orally administered drugs into systemic circulation, a drug molecule can use either transcellular or paracellular routes (23). Translocation using the transcellular route can occur either via a passive diffusion process or a carrier-mediated process. For transcellular passive diffusion to occur, a drug molecule must have the appropriate physicochemical properties (e.g., molecular size, charge, lipophilicity, hydrogen bounding potential) to cross both the AP and BL membranes. Lipophilic molecules cross the intestinal epithelium predominantly by this transcellular passive diffusion process. Paracellular transport, involving passive diffusion of compounds in aqueous solution, is the predominant route for hydrophilic compounds of low molecular weight to traverse the intestinal epithelium. It is less efficient than the transcellular route because of much lower surface area available to the molecules entering the intercellular space. In addition, the presence of junctional complexes (i.e., intermediate junctions, desmosomes and particularly tight junctions) in the intercellular space restricts the free passage of molecules traversing the intestinal epithelium via the paracellular route. Hence, the intestinal epithelium is a significant barrier for hydrophilic molecules because they cannot easily traverse the lipid bilayer of the cell membrane, and their passage through the intercellular space is restricted.

We have characterized the transport of DB75 and its prodrug DB289 across Caco-2 cell monolayers. A low permeability coefficient and high sensitivity to extracellular Ca^{2+} suggest that AP to BL transport of DB75 across Caco-2 cell monolayers occurs predominantly via a paracellular route. DB289 at 25 μM (same as the solubility in the transport buffer) exhibits an 85-fold greater transport rate across Caco-2 monolayers than does DB75 (solubility in transport buffer ≈ 500 μM) at the same concentration. This, along with insensitivity of its transport to extracellular Ca^{2+} indicates that AP to BL transport of DB289 across Caco-2 cell monolayers occurs predominantly via a transcellular route.

Structural characteristics of DB75 and DB289 dictate their oral absorption mechanisms. The results from this *in vitro* study using the human intestinal cell model, Caco-2 cell monolayers, indicates that DB289 is much more permeable than the diamidine analog, DB75. Masking the cationic functionalities of DB75 by *O*-methylamidoxime moieties switches the primary transport mechanism from paracellular to transcellular and, consequently, enhances the transport across Caco-2 cells significantly. This difference in the primary transport mechanism of these two compounds is consistent with the limited oral activity of DB75 and the substantially enhanced oral activity of DB289 (Table I). These results should provide an insight into prodrug design to improve oral absorption of hydrophilic dicationic drugs containing amidino functionality.

A good correlation between the transport through Caco-2 cell monolayers and the absorption in humans has been obtained for several compounds (24). According to Artursson and Karlsson, compounds like DB289 with a P_{app} value greater than 1×10^{-6} cm/s in Caco-2 cell monolayers, may be absorbed completely from the small intestine in humans. The addition of 2% albumin in the transport buffer for DB289 transport experiment, whereas increasing the solubility of DB289, may have complicated data interpretation such that the observed P_{app} value included a binding equilibrium between DB289 and albumin. However, if this albumin binding effect played a role in P_{app} determinations, it would most likely lead to an underestimation of the actual permeability of DB289 (25,26).

In these studies permeabilities of DB289 in both directions of transport are not significantly different (efflux ratio 0.88) at 25 μM, indicating that no efflux pumps were involved in the transport of DB289. The efflux ratio for DB75 at 25 μM obtained in these transport studies was 2.25, suggesting that it may be a substrate for an efflux pump (e.g., P-gp). The potential role of P-gp in transport of DB75 and related dications is currently being investigated. However, DB75's predominantly paracellular transport would suggest that the efflux pump is not attenuating its transport in AP to BL direction. Ranitidine, a cationic hydrophilic compound, exhibits a similar behavior (Thakker *et al.*, unpublished data).

It has been reported that phase I drug metabolic enzymes (CYP1A1 and CYP3A) and some phase II enzymes that exist in the small intestine are also expressed in Caco-2 cells (23,27). However, the activity/expression levels of some of these enzymes, e.g., CYP 3A, are known to be considerably lower than those found in intestinal epithelium. Therefore,

the limited ability of Caco-2 cells to metabolize DB289 to DB75 observed in these studies does not provide sufficient information to draw conclusions about how the small intestine would metabolize DB289.

In conclusion, the results presented here mechanistically indicate that employing a bis-*O*-methylamidoxime moiety as a prodrug for these diamidine compounds is a useful strategy for overcoming the intestinal membrane barrier.

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