Stereoselective and Concentration-Dependent Polarized Epithelial Permeability of a Series of Phosphoramidate Triester Prodrugs of d4T: An in Vitro Study in Caco-2 and Madin-Darby Canine Kidney Cell Monolayers

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ABSTRACT

Nucleoside analogs are successful, widely used antiviral and anticancer therapeutics. Nucleotide prodrugs (i.e., pronucleotides) have increasingly been used to improve in vivo efficacy of nucleoside analogs. In this study, we evaluated the permeability of a series of phosphoramidate triester prodrugs of the anti-HIV drug 2',3'-didehydro-2',3'-dideoxythymidine across monolayers of Caco-2, Madin-Darby canine kidney (MDCKII) epithelial cell line, and its recombinant clone containing the human MDR1/P-gp gene (MDR1-MDCKII). Transport was studied in the apical-to-basolateral (A-B) and the basolateral-to-apical directions (B-A). The impact upon transport of differences in stereochecmy at the chiral phosphate center was evaluated. In the Caco-2 and MDCK models the A-B permeability was lower than expected based on the lipophilicity of the compounds, suggesting the involvement of a polarized efflux system and/or metabolic degradation in limiting the absorption of these ester-based prodrugs. Average permeability values through cell monolayers obtained in the A-B direction were lower than in the B-A direction. The inclusion of the P-glycoprotein (P-gp) inhibitor verapamil in the transport medium markedly increased the permeability in the A-B direction, whereas decreasing it in the opposite direction, suggesting an efflux mechanism mainly mediated by P-gp. Stereoselective permeability was significant for the most lipophilic compounds, where the diastereoisomer possessing the slower eluting time on a reverse-phase high-performance liquid chromatography column was transported through Caco-2 and MDCK monolayers at higher rate.

2',3'-Dideoxynucleoside analogs (ddN) that are active against HIV reverse transcriptase are important components in the highly active antiretroviral therapy regimens used for the treatment of acquired immunodeficiency syndrome. Stavudine (d4T) is among the ddN analogs with significant clinical utility (Cheer and Goa, 2002). The ddN analogs have to be converted to their corresponding 5'-triphosphate derivatives to act as inhibitors of the retroviral reverse transcriptase. Aminoacyl arylxy phosphoramidate derivatives of nucleoside analogs (pronucleotides) have been shown to lead to an elevated potency for a range of antiviral and antitumor nucleoside analogs, in part through the successful by-passing of the dependence upon initial nucleoside kinase-mediated phosphorylation (i.e., thymidine kinase in the case of d4T) in the target cell (Wagner et al., 2000). The success of such an approach is based upon the ability to mask the phosphate atom with neutral hydrophobic groups to generate a membrane permeable mononucleotide prodrug able to access intracellular target sites where the free mononucleotide is released (McGuigan et al., 1993; Balzarini et al., 1996).

The first step in the release of the mononucleotide is thought to be a carboxylesterase-mediated hydrolysis of the carboxylic ester function in the amino acid moiety (Fig. 1). Spontaneous elimination of the phenol would then produce an amino acyl metabolite (AAM). An enzymatic cleavage of the P-N bond will then release the mononucleoside monophosphate (Saboulard et al., 1999). For pharmacological action, this activation, leading to a charged hydrophilic active species, must occur within the target cell itself, although poten-
tial clearly exists for significant metabolism of the pronucleotide during mucosal transport and distribution to the target cell.

In the current work, we have investigated the potential epithelial permeability of a series of pharmacologically active aryloxy phosphoramidate derivatives of the anti-HIV agent d4T (Fig. 1) using the Caco-2 and MDCK cell lines as in vitro model systems (Artursson and Karlsson, 1991; Irvine et al., 1999). Because these molecules exist as a mixture of two diastereomers produced by chirality at the phosphate, the possibility for stereoselective transport was also examined.

Materials and Methods

Materials. Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). The parental MDCKII cell line and its human MDR-1 recombinantly transformed derivative, MDR1-MDCKII, were obtained as a kind gift from P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Phosphate-buffered saline, Dulbecco’s modified Eagle’s medium (DMEM), and penicillin/streptomycin were obtained from Invitrogen (Paisley, UK). Tissue culture plastics and polycarbonate Transwell filters were from Corning-Costar (Bucks, UK). d4T phosphoramidate prodrugs were synthesized according to previously published procedures (McGuigan et al., 1996). Labeled [14C]propranolol, and verapamil and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Labeled [3H]mannitol was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). All reagents used were of analytical grade.

Measurement of Log Octanol-Water Partition Coefficients (Log P). The partition coefficients of pronucleotides were determined in 1-octanol/aqueous buffer (pH 7.0) as described previously (Siddiqui et al., 1999b) using UV-spectroscopy for quantitation.

Cell Culture. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and grown at 37°C in an atmosphere of 90% relative humidity and 5% CO2. Culture medium was changed every other day during T-flask culture and cells passaged every 3 to 5 days by trypsin-EDTA exposure. For permeability experiments Caco-2 cells and MDCKII or MDR1-MDCKII cells were seeded at 500,000 and 250,000/cm2, respectively, onto polycarbonate Transwell inserts (6.5 mm in diameter) with culture medium (0.25 ml in the apical donor chamber of the insert and 1 ml in the basal well chamber) replaced every 24 h. The Caco-2 transport studies were conducted upon confluent monolayers between 17 to 21 days postseeding when transepithelial electrical resistance (TEER) was typically 300 to 500 Ωcm2. Caco-2 experiments were conducted on cells between passage 30 and 40. MDCKII and MDR1-MDCKII cells were used in experiments at passage 5 to 8 after receipt from the Netherlands Cancer Institute. Cells were used in transport experiments at days 4 to 6 postseeding when MDCKII TEER was typically 40 to 50 Ωcm2 and MDR1-MDCKII TEER was typically 120 to 140 Ωcm2.

Permeability Experiments. Permeability studies were conducted with Caco-2, or the MDCK cells incubated in DMEM (without serum) on an orbital shaker (200 rpm). At 30 min before experiments, the culture medium was replaced with warm DMEM, and at the end of this preincubation period the TEER was measured and inserts distributed evenly between treatments on the basis of the TEER measurements. Labeled [3H]mannitol (0.088 μM, 0.147 MBq) and labeled [14C]propranol (35 μM, 0.185 MBq) were used as probes for low-permeability paracellular transport and high-permeability transcellular transport, respectively. Radioactivity was determined by liquid scintillation counting. Transport experiments in the apical-to-basal (A-B) direction were initiated by adding 250 μl of drug solution to the apical chamber of the insert. Transport experiments in the basal-to-apical (B-A) direction were initiated by adding 1 ml of drug solution to the basal well chamber.

Pronucleotide drug solutions were prepared by spiking DMEM with a concentrated stock of prodrug dissolved in DMSO. The final concentration of DMSO within the solutions used for the experiments was always adjusted to 1% (v/v) maximum. At predetermined times over the course of the experiment, samples (100 μl) were taken from the respective receiver chamber and replenished with fresh DMEM. Transport studies conducted in the presence of the P-gp inhibitor verapamil (50 μM) and the carboxylesterase inhibitor PMSF (1.5 mM) were conducted as described above, with the inhibitors present throughout the experiment in both apical and basal compartments and also preincubated with the cells for 30 min before addition of the pronucleotides.

The cumulative mass (M) of drug transported from donor to receiver chambers was assessed over the time course of the experiment (dM/dt). Using linear regression analysis, the following equation was fit to the data: dM/dt = p · A · C0 , where p is the permeability coefficient (centimeters per second), A is the surface area of the Transwell membrane, and C0 the initial drug concentration in the donor chamber (nanomoles per cubic centimeter). In all experiments, the amount of drug transported from donor to receiver was always
≤6% of the donor drug mass, thus ensuring the validity of C₀ as a constant in the equation.

**HPLC and LC-MS Analysis of d4T and Phosphoramidate Prodrugs.** The concentration of phosphoramidate pronucleotide in the incubation media was determined by HPLC-UV using a Thermoquest HPLC system consisting of a P4000 quaternary pump, autosampler, and a UV6000LP photodiode array detector. To address qualitative issues of pronucleotide degradation, an HPLC-electrospray mass spectrometry (LC-MS) system, using a Finnigan LCQDeca mass spectrometer operated in the positive electrospray ionization mode, in the case of the parent pronucleotides, or in the negative electrospray ionization mode for the AAM, was used. The HPLC column was used with Phenomenex Luna (3 μm) C18 (50 × 3.00 mm). Elution was performed isocratically using a mobile phase consisting of water/methanol/acetonitrile 60:25:15 (v/v/v) with a flow rate of 0.2 mL/min; the injection volume was 30 μL. The compounds were identified according to peak retention times, UV spectra, and m/z signals. The concentration of compounds were determined by UV detection (wavelength 266 nm) against linear (r² > 0.99) calibration curves. The limit of detection for each diastereoisomer of the pronucleotide series and for AAM was approximately 2.5 pmol/ml (1.0–1.5 ng/ml). Calibration standards in the range 0.025–25 nmol/ml per each compound were prepared in the incubation media (DMEM) and run on each day of analysis, together with a set of quality control samples at high and low concentration. The relative standard deviation for within-day and between-day precision was assessed for high and low concentrations and was less than 10% for each compound. The accuracy was determined by subtracting the measured concentration from its theoretical value; the mean relative error of the difference from theoretical was less than 10%.

**Statistical Analysis.** Comparisons between two groups were made by nonpaired Student’s t test with a level of significance at p < 0.05. Comparisons across more than two groups were made by analysis of variance and Duncan’s multiple range test with significance level of p < 0.05.

**Results**

For each pronucleotide, the phosphate diastereoisomers were separated and quantified by reverse-phase HPLC-UV detection. According to the retention times the diastereomers were assigned as FE (fast eluting; less lipophilic) and SE (slow eluting; more lipophilic). Figure 5 shows a representative UV chromatogram with retention times, and mass spectra for the p-Me derivative (C₀ of 0.5 mM) and the amino acyl metabolite (AAM) recovered in the apical chamber of the Transwell system after a 120 min transport experiment. The parent pronucleotides display a retention time of 22.65 and 50.2 min for FE and SE diastereoisomers, respectively, with the SE diastereoisomer (the more lipophilic isomer) showing a permeability approximately 1.6-fold greater for the more hydrophobic derivatives (p-Me derivative, a group containing the p-Me, p-Cl, and p-Br derivatives, and then as a distinct member of the series the p-I derivative showing a markedly higher permeability. Intriguingly, for the more hydrophobic derivatives (p-Cl, p-Br, and p-I), clear differences (p < 0.05) were also seen in Caco-2 permeability between the corresponding FE and SE diastereoisomers, with the SE diastereoisomer (the more lipophilic isomer) showing a permeability approximately 1.6-fold greater for the p-Cl derivative, 3-fold greater for the p-Br derivative, and 1.9-fold greater for the p-I derivative. Such diastereoisomer selective permeability was not observed for the less hydrophobic members of the series.

At a 10-fold higher C₀ concentration of 5 mM the perme-
The measured log P values for the compounds analyzed and permeability coefficients through Caco-2 cell monolayers obtained in a 120-min transport experiment in the A-B direction are shown in Table 1. Values represent the mean ± S.D. of n = 6.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log P</th>
<th>Permeability Coefficients</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>FE 5 mM</td>
</tr>
<tr>
<td>Sn324 (H)</td>
<td>1.04</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>CF 1526 (p-OMe)</td>
<td>1.09</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>CF 1525 (p-Me)</td>
<td>1.19</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>CF 1523 (p-Cl)</td>
<td>1.43</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>CF 1517 (p-Br)</td>
<td>1.60</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td>CF 1572 (p-I)</td>
<td>1.93</td>
<td>12.0 ± 0.7</td>
</tr>
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</table>

* Significantly increased compared with FE diastereoisomer.

The permeability coefficients for the series show a clear correlation (r² = 0.9672) to the experimentally determined log P values of note once again for the more hydrophobic derivatives (p-Cl, p-Br, and p-I) the SE diastereoisomer shows a greater log P values (p < 0.05) (approximately 1.6-fold) Caco-2 permeability than the respective FE diastereoisomer. Increasing the C₀ concentration from 0.5 mM to 5 mM seemed generally not to result in any preferential diastereoisomer-selective increases in permeability, e.g., for the p-I derivative a 1.8-fold increase for the SE isomer versus a 2.1-fold increase for the FE isomer. Although an exception to this is apparent for the p-Br derivative where the FE isomer permeability increases some 7-fold versus a 3.8-fold increase for the SE isomer. The data collection was not compromised by differential sequestration, with the terminal mass balance recovery of the pronucleotides from both the apical and basal chambers of the Transwell and from the cellular tissue itself ranging from 80–100% of the initial dose, with importantly, no differences observed between the SE and FE diastereoisomers. In summary therefore the above Caco-2 data shows a concentration dependence in A-B permeability for all members of the pronucleotide series, and stereoselectivity in transport for the more hydrophobic derivatives.

From a more biopharmaceutical perspective comparisons of the permeability to pronucleotide can be made to data in Table 2, which shows the permeability of our Caco-2 monolayers to the parent nucleoside analog d4T and to the transeellular and paracellular probes propranolol and mannitol. The lower molecular weight d4T analog applied at a 5 mM concentration displayed a permeability coefficient corresponding to that of the p-Br pronucleotide (5 mM; Table 1) and appreciably less than the p-I derivative. All the pronucleotides showed permeability greater than the paracellular probe mannitol. This highlights that these molecules could be considered for oral delivery and that they could clearly provide a biopharmaceutical advantage through the absorption process and not just through increased target cell permeation.

To probe the potential bidirectional permeability of the Caco-2 cells toward the pronucleotide diastereoisomers, we used the p-Me derivative. Figure 4 shows that at a C₀ concentration for the pronucleotide of 0.5 mM, the permeability coefficients in the A-B direction were about 15- to 20-fold lower (approximately 0.7 × 10⁻⁶ cm/s for both the FE and SE diastereoisomers) than the respective permeability coefficients for the series.

![Fig. 3.](image1.png) **Fig. 3.** Polarized transport of the p-Me derivative across Caco-2 monolayers at donor C₀ of 0.5 and 5 mM. Transport studies (120 min) were undertaken in the A-B and B-A directions in the absence or presence of verapamil (50 μM) added to both the apical and basal compartments. Data are mean ± S.D. of three monolayers. No difference (p > 0.05) between the FE and SE diastereoisomers was observed within any treatment. The asterisk indicates statistical difference (p < 0.05) compared with the respective diastereoisomer in the corresponding A-B treatment control.

![Fig. 4.](image2.png) **Fig. 4.** Comparison of the permeability coefficients of MDCK II monolayers (120 min) to the p-Me derivative (CF1525) in the A-B and B-A directions at C₀ of 0.5 and 5 mM. The asterisk indicates statistical difference (P < 0.05) between diastereoisomers. Data are mean ± S.D. of five monolayers.
cients in the B-A direction (10.2 and 13.8 \times 10^{-6} \text{ cm/s} for the FE and SE diastereoisomers, respectively), demonstrating a polarization in the Caco-2 permeability toward these molecules and indicating a predominantly secretory rather than absorptive transport. Using these data, a [B-A/A-B] permeability ratio can be determined that is relatively high, averaging 14.6 and 19.7 for the FE and SE diastereoisomers, respectively. Using the classic P-gp substrate vinblastine, we have previously reported [B-A/A-B] permeability ratios of approximately 4 in these Caco-2 cell monolayers (Campbell et al., 2003). Inclusion of the P-gp inhibitor verapamil (50 \mu M) in both apical and basal chambers abolished the profound polarized \( p \)-Me pronucleotide transport, with the A-B permeability coefficients for the FE and SE diastereoisomers increasing to 4.7 and 6.7 \times 10^{-6} \text{ cm/s}, respectively, and the B-A permeability coefficients decreasing to 2.1 and 3.1 \times 10^{-6} \text{ cm/s}, respectively (Fig. 3). Of note is that when increasing the \( C_0 \) concentration of the \( p \)-Me pronucleotide to 5 mM, then the directional-dependent permeability was eradicated, with the permeability coefficients in the A-B direction determined at 3.3 and 3.7 \times 10^{-6} \text{ cm/s} for the FE and SE diastereoisomers, respectively, and in the B-A direction, 4.4 and 4.2 \times 10^{-6} \text{ cm/s}, respectively (Fig. 3), leading to a [B-A/A-B] permeability ratio of 1.33 for the FE diastereoisomer and 1.13 for the SE diastereoisomer. This finding is consistent with the presence of a saturable efflux transport mechanism, potentially P-gp, capable of directing the pronucleotide (at least for the \( p \)-Me derivative) out of the Caco-2 cell into apical (luminal) medium.

To further examine the nature of the polarized transport of the pronucleotides, permeability studies were conducted in MDR1-MDCKII cells which stably and functionally overexpress the human MDR1 gene product, and their wild-type parental cell line MDCKII, which expresses constitutive canine P-gp, but at a much lower level to that in the recombinant MDR1-MDCKII cells (Polli et al., 2001; Guo et al., 2002). Table 3 shows the transport data across these two MDCK cell lines for selected derivatives from the pronucleotide series \((C_0 \text{ concentration of } 0.5 \text{ mM})\), including that for the parent compound So324 (log P 1.04), the \( p \)-Me derivative (Cf1525; log P 1.19) and the most hydrophobic \( p \)-I derivative (Cf1572; log P 1.93). Consistent with the data for the \( p \)-Me
derivative in the Caco-2 studies (Fig. 3), each of the three pronucleotides studied in the MDCKII cells at 0.5 mM C0 concentration showed a significantly (p < 0.05) greater permeability in B-A direction compared to that in the A-B direction (Table 3). For So324 the permeability ratio [B-A/A-B] averaged 3.5 for both diastereoisomers. For the p-I derivative (CI572) the [B-A/A-B] ratio for the FE diastereoisomer averaged 3.3 and for the SE diastereoisomer 2.3. For the p-Me derivative (CI525) the extent of the polarized transport would seem to be more profound with a permeability in the A-B direction determined to be no greater than 0.1 (×10−6 cm s−1), whereas that in the B-A direction was some 30-fold greater. Table 3 also shows the transport of the three pronucleotides (C0 concentration of 0.5 mM) across the recombinant MDR1-MDCKII cells, which translationally and functionally overexpress P-gp. Most apparent is that the extent of polarized transport is now more profound, with a permeability ratio [B-A/A-B] for So324 averaging 12 for both diastereoisomers. For the p-Me and p-I derivatives, the permeability in the A-B direction was determined to be no greater than 0.1 (×10−6 cm s−1), whereas that in the B-A direction was up to 200-fold greater. In all cases, the permeability of the pronucleotides in the A-B direction in the MDR1-MDCKII cells were significantly (p < 0.05) greater than the respective B-A transport in the MDCKII cells.

Similar to the observations in the Caco-2 model (Table 1), differences in the permeability between SE and FE diastereoisomers were observed in the MDCK cells (Table 3). In the MDCKII cells, a significant (p < 0.05) difference in the A-B permeability between the SE and FE diastereoisomers of the most hydrophobic derivative (p-I) was noted, whereas no such stereoselective difference was seen for the less hydrophobic derivative So324. The A-B transport of the diastereoisomers of the p-Me derivative (CI525) could not be compared because the mass transfer to the receptor compartment was too low to allow quantitation. The B-A transport of the SE and FE diastereoisomers of the p-Me derivative, like those of So324, did not display stereoselectivity (p > 0.05). Notably, stereoselective differences were also observed for the hydrophobic p-I derivative with regard to the B-A cell permeation, with again greater transport for the SE diastereoisomer over the FE diastereoisomer, although the stereoselective differences seemed to be less pronounced (a SE/FE ratio of 1.51) than that seen in the opposing A-B direction (an SE/FE ratio of 2.19). Whereas these data indicate that the pronucleotides are substrates for a secretory transport mechanism acting in the basal-to-apical direction, it does not support such a mechanism serving as the sole or even the main basis for the greater permeability of the SE diastereoisomer over the FE diastereoisomer. For example, whereas the A-B transport across MDCKII cells of the SE diastereoisomer of the p-I derivative is greater than the FE diastereoisomer, it is also the SE diastereoisomer that shows the greater transport in the B-A direction. With a more profound polarized transport in the MDR1-MDCKII cells, it is of note that this stereoselectivity in B-A transport for the p-I derivative evident in the MDCKII cells is no longer manifest in the MDR1-MDCKII cells. Furthermore, when the A-B transport of the p-I derivative across MDCKII monolayers was conducted in the presence of verapamil (50 μM), the permeability toward both SE and FE diastereoisomers increased, approximately 1.6- and 2.6-fold, respectively, and although the differences between the diastereoisomers was reduced there was still evidence of a greater permeability to the SE isomer (SE/FE permeability ratio of 1.5 in the presence of verapamil versus 2.4 in the absence).

To explore the finding that the p-Me derivative in the MDCKII cells showed a uniquely low A-B permeation, transport studies were undertaken at the higher C0 concentration of 5 mM. Figure 4 shows the transport results for the p-Me derivative across the MDCKII cells at C0 concentrations of 0.5 and 5 mM. As for the Caco-2 data, increasing the C0 concentration resulted in an obviously higher A-B permeability with coefficients calculable at between 1.5 and 2.0 (×10−6 cm s−1) and with the SE diastereoisomer showing a slightly greater (p < 0.05) permeability than the FE diastereoisomer. As previously mentioned, this indicates the overcoming of a saturable barrier to the A-B transport of the pronucleotide within the MDCKII monolayer.

Using LC-MS, we observed, for every pronucleotide, significant amounts of the charged precursor to d4T monophosphate, the AAM, in the donor chamber of the Transwell inserts, when both Caco-2 and MDCK cells were used in the transport experiments (Fig. 5). For example, for 125 nmol of the p-I derivative spiked in the donor compartment of a Transwell system (MDCKII monolayers), the amount of unchanged pronucleotide found in the same compartment after 120 min was (mean ± S.D. of three wells) 44.2 ± 5.6 and

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDCKII</th>
<th>MDR1-MDCKII</th>
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<tr>
<td></td>
<td>FE</td>
<td>SE</td>
</tr>
<tr>
<td>So324 (H)</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>A-B</td>
<td>3.5 ± 0.5</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>CI525 (p-Me)</td>
<td>&lt;0.1*</td>
<td>&lt;0.1*</td>
</tr>
<tr>
<td>A-B</td>
<td>3.5 ± 1.0</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>B-A</td>
<td>1.6 ± 0.3</td>
<td>3.5 ± 1*</td>
</tr>
<tr>
<td>CI572 (p-I)</td>
<td>5.3 ± 0.8</td>
<td>8.0 ± 1.2*</td>
</tr>
</tbody>
</table>

> Statistical difference of the SE to the respective FE diastereoisomer (p < 0.05).

* Concentrations in the receiver chambers were below the limit of detection of the HPLC assay and only upper limits of permeability could be calculated.
47.5 ± 7.6 nmol of FE and SE, respectively. About 68% of the 33 nmol of pronucleotide that had disappeared was found as the AAM (i.e., 22.7 ± 1.3 nmol). The amount of AAM in the basolateral chamber was estimated to be <1 nmol, which corresponds to less than 5% of the entire AAM produced. In both Caco-2 and MDCKII monolayers, no difference compared with untreated control was seen in the appearance of the AAM metabolite or in the mass balance of unchanged prodrug recovered at the end of the transport studies when verapamil was present in the transport studies (data not shown).

The susceptibility to hydrolysis by carboxylesterases of the pronucleotides was investigated in Caco-2 and MDCKII studies performed in the A-B direction with the p-Me and p-I derivatives in the presence of the carboxylesterase inhibitor PMSF (1.5 mM). PMSF did not significantly increase (p > 0.05) the transport rate of any compound in both the Caco-2 and MDCKII models.

**Discussion**

In this study, we evaluated for the first time the permeability of an epithelial barrier to a series of pharmacologically active anti-HIV pronucleotide molecules. Specifically, the polarized stereoselective permeability of Caco-2 and MDCK monolayers to a series of aryloxy phosphoramidate prodrugs of d4T.

The results of this study highlight the need to consider the role of transport proteins and metabolizing enzymes in attenuating the absorption of this class of prodrugs. We found that the permeability of the Caco-2 monolayers to the mixed phosphate diastereoisomers is nonlinearly related to concentration in the 0.5 to 5.0 mM range. The deviation from linearity suggests the presence of a polarized efflux pump and/or a saturable metabolic barrier to absorption. For the pronucleotide series the permeability of the Caco-2 monolayers at C₀ 5 mM correlated well with the log octanol-water partition coefficient (log P) as one would expect in a strictly diffusion controlled process (Artursson et al., 2001). At this concentration, however, the Caco-2 permeability was comparatively low. For example, the p-Cl derivative displayed a similar measured log P (1.43; Siddiqui et al., 1999a) to propranolol (1.54; Artursson and Karlsson, 1991), but displayed a permeability for the diastereoisomer mixture some 27% of that for propranolol (5.1 versus 19.2, respectively). At C₀ 5 mM, all of the pronucleotide derivatives resulted in a permeability greater than that for the hydrophilic paracellular probe mannitol, ranging from a 40% greater permeability for the p-OH derivative to a 5-fold greater permeability for the p-I derivative. One possible explanation for the comparatively low permeability for these lipophilic molecules may be that they are susceptible to first pass metabolism within the enterocyte. Intestinal epithelial cells are recognized to be rich in esterase activity and previously shown to limit the oral absorption of a number of ester-based prodrugs (Annaert et al., 1997, 1998). Mass balance analysis for the parent prodrug species conducted in this work accounted for 85 to 95% of the original dose applied to the cell, but given the low percentage of transport, the apparent imbalance may represent significant degradation.

When we tested for the A-B transport of the p-Me derivative at C₀ 0.5 mM, we found that the permeability coefficients were about 15-fold lower than the permeability coefficients in the B-A direction. Moreover, the addition of the P-gp inhibitor verapamil reduced the transport of each diastereoisomer of p-Me in the B-A direction, resulting in a polarized permeability ratio (B/A-A-B) of approximately 0.5 for both diastereoisomers. At C₀ 5.0 mM, we found no evidence of directionally dependent permeability. Although verapamil is considered to not be a potent P-gp inhibitor this is in the context of the limiting in vivo concentrations above which cardio toxicity is observed, i.e., at concentrations >5 μM, and not at the concentrations commonly used in vitro, i.e., 40 to 50 μM. Furthermore, although other ATP-binding cassette transporters reported to be modulated by verapamil include MRP1 and MRP4, it seems not to effect MRP2 or breast cancer resistance protein function (Walgren et al., 2000; Berger et al., 2003; O’Leary et al., 2003), the latter two being the key ATP-binding cassette efflux transporters present with P-gp on the apical membrane of Caco-2 (Faber et al., 2003). Recently, using recombinant cell expression models, Reid et al. (2003) have shown that these molecules are not substrates for MRP4 and MRP5. Therefore, although we do not absolutely exclude these molecules as being substrates for other efflux transporters the Caco-2 data are consistent with the presence of a saturable P-gp efflux pump on the apical membrane of Caco-2 capable of directing the pronucleotide out of the cell and back in the apical (luminal) medium.

Transport data in the MDR1-MDCKII model further supported the hypothesis that these compounds are substrates for P-gp, So324 (H), Cf1525 (p-Me), and Cf1572 (p-I) permeabilities were measured in both wild-type MDCKII and MDR1-transfected cells (MDR1-MDCKII), the latter overexpressing the MDR-1 gene product (P-gp). All compounds tested were more permeable in the B-A than in the A-B direction in both cell lines. The permeability coefficients in the B-A direction were significantly higher in the cell line overexpressing P-gp.

Of particular note in this study is the finding of stereoselectivity in the permeability of the more lipophilic derivatives, p-I (Cf1572) and p-Br (Cf1517) at the lower concentration (i.e., 0.5 mM) and p-I (Cf1572), p-Br (Cf1517), and p-Cl (Cf1523) at the higher concentration (i.e., 5.0 mM). For these molecules, the SE diastereoisomer showed significantly greater permeation across the Caco-2 monolayers, with for example the SE isomer for the p-I derivative being 1.6-fold and 1.9-fold higher than the FE isomer at C₀ 5.0 and 0.5 mM, respectively. In the MDCKII model, the A-B ratio (SE/FE) for the p-I derivative was approximately 2; addition of verapamil reduced the ratio to approximately 1.5, and the differences were not statistically significant (p > 0.05). In the B-A direction the ratio was also 1.5 (p < 0.05). In the MDR1-MDCKII cell line, no passage could be detected in the A-B direction, to demonstrate that both diastereoisomers are effectively good substrates for the efflux pump. In the same cell line, however, the ratio in the B-A direction was reduced to approximately 1.2 and differences between diastereoisomers were not significant (p > 0.05). It is therefore possible that P-gp is involved in at least part of the stereoselective effect seen for the transport of these molecules.

In displaying a slower elution from the reverse-phase HPLC column, the SE diastereoisomer clearly possesses different chemical properties (higher lipophilicity) from its FE diastereoisomer. Although the slightly more hydrophobic na-
The degradation pattern of the pronucleotides in the Caco-2 and MDCK transport models was consistent with a significant hydrolytic cleavage on the methyl ester bond resulting in the formation of the AAM, as previously shown in different cell lines (Saboulard et al., 1999). AAM was found in both the apical and the basolateral side of the Transwell. It is possible that AAM was produced in the donor apical chamber by hydrolisis of the parent phosphoramidate by hydrolytic enzymes bound to the cell membranes. From here, AAM could have reached the basolateral compartment by passive diffusion, or by means of some active transport. Alternatively, AAM could have formed intracellularly and then exuded by efflux transporters located in the basolateral side. Recently, AAM has been shown to be substrate for MRP5, which has been shown to mediate the efflux of d4TMP and AAM from MRP5-overexpressing human embryonic kidney 293 cells (Reid et al., 2003).

The carboxylester inhibitor PMSF failed to increase the overall permeability of the p-I derivative or reduce its stereoselective transport. Concentration of PMSF of 0.5 mM has been reported to effectively increase the transport of menamecine-guaiacol ester across Caco-2 cell monolayers (Tantishayakul et al., 2002). However, it is possible that the degradation of the d4T pronucleotides in the apical compartment of the Transwell system is carried out by membrane-bound hydrolytic enzymes that are insensitive to PMSF.

In summary, certain derivatives from a series of potent anti-HIV phosphoramidate triester prodrugs of d4T permeated the Caco-2 cell model at a rate usually characteristic of drugs showing a moderate-to-high in vivo intestinal permeation (Artursson and Karlsson, 1991; Artursson et al., 2001). The more lipophilic derivatives in the series demonstrated significant stereoselectivity in Caco-2 and MDCK transport, most likely due to a stereoselective active efflux mechanism, or to metabolism by carboxylesterase enzymes. In these model systems, specifically MDRI-MDCKII, we can conclude that the phosphoramidate triester prodrugs are good substrates for P-gp, although they do not exclude these molecules as being substrates for other efflux transporters to varying extents in other cell lines or tissue systems. Further work addressing the in vivo absorption, stereoselective metabolism, and pharmacology of these clinically interesting molecules is warranted.

References


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