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Stereoselective and concentration-dependent polarised epithelial permeability of a series of phosphoramidate triester prodrugs of d4T: an *in vitro* study in Caco-2 and MDCK cell monolayers.

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Running Title: Epithelial permeability of d4T pronucleotides.

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List of abbreviations: HIV, human immunodeficiency virus; d4T, 2',3'-didehydro-2',3'-dideoxythymidine, P-gp, P-glycoprotein; FE, fast eluting; SE, slow eluting; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulphonyl fluoride.

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Abstract

Nucleoside analogues are successful, widely used antiviral and anti-cancer therapeutics. Nucleotide prodrugs (i.e. pronucleotide) have increasingly been used to improve in vivo efficacy of nucleoside analogues. 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 (d4T) 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 stereochemistry at the chiral phosphate centre 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, while 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 HPLC column was transported through Caco-2 and MDCK monolayers at higher rate.

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2',3'-Dideoxynucleoside analogues (ddN) that are active against HIV reverse transcriptase are important components in the highly active antiretroviral therapy (HAART) regimens used for the treatment of AIDS. Stavudine (d4T) is among the ddN with significant clinical utility (Cheer and Goa, 2002). The ddN analogues have to be converted to their corresponding 5'-triphosphate derivatives to act as inhibitors of the retroviral reverse transcriptase. Aminoacyl aryloxy phosphoramidate derivatives of nucleoside analogues (pronucleotides) have been shown to lead to an elevated potency for a range of antiviral and antitumor nucleoside analogues, in part through the successful by-passing of the dependency 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 (Figure 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 nucleoside 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 potential clearly exists for significant metabolism of the pronucleotide during mucosal transport and distribution to the target cell.

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

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MATERIALS AND METHODS

Materials

Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC). 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). Phosphate buffered saline (PBS), 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 synthesised according to previously published procedures (McGuigan et al., 1996). Labelled ^{14}[C]-propranolol, and verapamil and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co (Poole, UK). Labelled ^{3}[H]-Mannitol was obtained from Ampharm (Little Chalfont, UK). All reagents used were of analytical grade.

Measurement of LogP

The partition coefficients of pronucleotides were determined in 1-octanol/aqueous buffer (pH 7.0) as previously described (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

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90% relative humidity and 5% CO_2. 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/cm^2, respectively, onto polycarbonate membrane Transwell® inserts (6.5 mm 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 hr. The Caco-2 transport studies were conducted upon confluent monolayers between 17 to 21 days post seeding when transepithelial electrical resistance (TEER) was typically 300-500 Ω. cm^2. Caco-2 experiments were conducted on cells between passage number 30-40. MDCKII and MDR1-MDCKII cells were used in experiments at passage number 5 to 8 after receipt from the Netherlands Cancer Institute. Cells were used in transport experiments at days 4-6 postseeding where MDCKII TEER was typically 40-50 Ω. cm^2 and MDR1-MDCKII TEER was typically 120-140 Ω. cm^2.

Permeability Experiments

Permeability studies were conducted with Caco-2, or the MDCK cells incubated in DMEM (without serum) on an orbital shaker (200 r.p.m.). At 30 min prior to experiments, the culture medium was replaced with warm DMEM, and at the end of this pre-incubation period the TEER was measured and inserts distributed evenly between treatments on the basis of the TEER measurements. Labelled ^{3}[H]-mannitol (0.088 µM, 0.147 MBq) and labelled ^{14}[C]-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

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experiments in the apical to basal (A - B) direction were initiated by adding 250 µl 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 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 prior to 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 Equation 1 was fit to the data.

$$dM/dt = p \cdot A \cdot C_0 \qquad (1)$$

where p is the permeability coefficient (cm/s), A is the surface area of the Transwell membrane, C_0 the initial drug concentration in the donor chamber (nmol/cm^3). 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 C_0 as a constant in equation 1.

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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 ESI mode, in the case of the parent pronucleotides, or in the negative ESI mode for the amino acyl metabolite (AAM), was used. The HPLC column used was a Phenomenex Luna (3µm) C18 (50 x 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 ($r^2 > 0.99$) calibration curves. The LOD for each diastereoisomer of the pronucleotide series and for AAM was approximately 2.5 pmol/ml (1.0 to 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 (MRE) of the difference from theoretical was less than 10%.

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Statistical Analysis

Comparisons between two groups were made by non-paired Student T-test with a level of significance at $p < 0.05$. Comparisons across more than 2 groups were made by ANOVA and Duncan's multiple range test with significance level of $p < 0.05$.

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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 (C_0 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 24.08 min for FE and SE diastereoisomers, respectively, with a MS peak at m/z 502, corresponding to the sodium ion-added molecule. The peak at 11.30 min corresponds to the amino acyl metabolite (AAM), with a deprotonated molecular ion of m/z 374 .

For the 6 analogues tested the retention times showed a strong correlation with the respective measured log octanol-water partition coefficients (logP) with a $r^2 > 0.99$ for each of the two diastereoisomers.

Based upon assessment of transepithelial electrical resistance (TEER) and solute permeabilities, e.g. mannitol, we did not observe 1% DMSO used as a co-solvent to compromise epithelial cell monolayer integrity (data not shown). For all pronucleotides the permeability profiles (dM/dt) across Caco-2 monolayers measured at both 0.5 and 5.0 mM C_0 donor chamber concentrations displayed a linear phase and an apparent lack of a lag phase. A concentration-dependence in transepithelal Caco-2 transport was however observed for all the pronucleotides (Table 1) and the more comprehensive dose-

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dependency study for the p-Me pronucleotide derivative is shown in Figure 2 depicting the % of dose transported of the p-Me derivative from the apical to basal compartment over 30 min expressed as function of five different C_0 concentrations between 0.5 and 5 mM. Above a C_0 of 2.5 mM there is a clear increase ($P<0.05$) for both the FE and SE derivatives in the normalised mass of pronucleotide transported indicating at the higher C_0 concentration the surmounting of a a saturable permeability barrier within the Caco-2 monolayer.

Table 1 itself shows the permeability coefficients (at C_0 concentrations of 0.5 mM and 5 mM) for the series of pronucleotides determined in the A - B direction across the Caco-2 monolayers. At a C_0 concentration of 0.5 mM the permeability coefficients for the series showed a poor correlation to the experimentally determined logP values, although at the extremes of hydrophobicity for this series the permeability of the p-I derivative (logP 1.93) is still some 5 to 10 times greater than the less hydrophobic parent compound (So324; logP 1.04). Statistical differences ($P < 0.05$) in the permeability coefficients were noted between the FE diastereoisomers within the series, as was the case for the SE diastereoisomers. Essentially the compounds could be ranked into 3 groups based upon statistical comparisons of the permeability coefficients, i.e. a group comprising the parent compound (So324) and the p-OMe derivative, a group comprising 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

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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 C_0 concentration of 5 mM the permeability coefficients for the series show a clear correlation ($r^2 = 0.9672$) to the experimentally determined logP values. Of note once again is that for the more hydrophobic derivatives (p-Cl, p-Br and p-I) the SE diastereoisomer shows a greater ($P<0.05$) (approximately 1.6-fold) Caco-2 permeability than the respective FE diastereoisomer. Increasing the C_0 concentration from 0.5 mM to 5 mM appeared 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 dependency in A – B permeability for all members of the pronucleotide series, and stereoselectivity in transport for the more hydrophobic derivatives.

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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 analogue d4T and to the transcellular and paracellular probes propranolol, and mannitol. The lower molecular weight d4T analogue applied at a 5 mM concentration displayed a permeability coefficient corresponding to that of the p-Me 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 employed the p-Me derivative. Figure 4 shows that at a C_0 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 x 10^{-6} cm/s for both the FE and SE diastereoisomers) than the respective permeability coefficients in the B - A direction (10.2 and 13.8 x 10^{-6} cm/s for the FE and SE diastereoisomers, respectively), demonstrating a polarisation in the Caco-2 permeability toward these molecules and indicating a predominantly secretory rather than absorptive transport. Using this 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

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the P-gp inhibitor, verapamil (50 μM) in both apical and basal chambers abolished the profound polarised p-Me pronucleotide transport, with the A-B permeability coefficients for the FE and SE diastereoisomers increasing to 4.7 and 6.7 ($x10^{-6}$ cm/s), respectively, and the B-A permeability coefficients decreasing to 2.1 and 3.1 ($x10^{-6}$ cm/s), respectively (Figure 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 ($x10^{-6}$ cm/s) for the FE and SE diastereoisomers, respectively, and in the B - A direction, 4.4 and 4.2 ($x10^{-6}$ cm/s), respectively (Figure 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 polarised 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 concentration of 0.5 mM) including that for the parent compound So324 (logP 1.04), the p-Me derivative (Cf1525; logP 1.19) and the most hydrophobic p-I derivative (Cf1572; log P 1.93). Consistant with the data for the p-Me

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derivative in the Caco-2 studies (Figure 3), each of the 3 pronucleotides studied in the MDCKII cells at 0.5 mM C_0 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 (Cf1572) 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 (Cf1525) the extent of the polarised transport would appear to be more profound with a permeability in the A - B direction determined to be no greater than 0.1 ($x10^{-6}$ cm . sec-1) while that in the B - A direction was some 30-fold greater. Table 3 also shows the transport of the three pronucleotides (C_0 concentration of 0.5 mM) across the recombinant MDR1-MDCKII cells which translationally and functionally overexpresses P-gp. Most apparent is that the extent of polarised 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 ($x10^{-6}$ cm . sec^{-1}) while that in the B - A direction was up to 200-fold greater. In all cases the permeability of the pronucleotides in the B - A 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, while no

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such stereoselective difference was seen for the less hydrophobic derivative So324. The A - B transport of the diastereoisomers of the p-Me derivative (Cf1525) could not be compared as 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 appeared to be less pronounced (a SE/FE ratio of 1.51) than that seen in the opposing A - B direction (a SE/FE ratio of 2.19). While this data indicates 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, while 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 which shows the greater transport in the B - A direction. With a more profound polarised 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. Further, 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-fold and 2.6-fold, respectively, and although the differences between the diastereoisomers was

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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 C_0 concentration of 5 mM. Figure 4 shows the transport results for the p-Me derivative across the MDCKII cells at C_0 concentrations of 0.5 mM and 5 mM. As for the Caco-2 data, increasing the C_0 concentration resulted in an obviously higher A - B permeability with coefficients calculable at between 1.5 and 2.0 ($x10^{-6}$ cm . sec^{-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 amino acyl metabolite (AAM), in the donor chamber of the Transwell inserts, when both Caco-2 and MDCK cells were used in the transport experiments (Figure 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 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

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MDCKII monolayers no difference compared to 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.

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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 polarised steroselective 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 metabolising 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 non-linearly 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 C_0 5 mM correlated well with the log octanol-water partition coefficient (logP) 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 logP (1.43; (Siddiqui et al., 1999a) to propranolol (1.54; (Artursson and Karlsson, 1991), but displayed a permeabililty for the diastereoisomer mixture some 27% of that for propranolol (5.1 versus 19.2, respectively). At C_0 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-OMe derivative to a 8-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 1st pass

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metabolism within the enterocyte. Intestinal epithelial cells are recognised 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; Annaert et al., 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 % transport, the apparent imbalance may represent significant degradation.

When we tested for the A - B transport of the p-Me derivative at C_0 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 C_0 5.0 mM we found no evidence of directional-dependent permeability. Although verapamil is considered not to be a potent P-gp inhibitor this is in the context of the limiting *in vivo* concentrations above which cardiotoxicity is observed, i.e. at concentrations > 5 µM, and not at the concentrations commonly used in in-vitro, i.e. 40-50 µM. Further, while other ATP-Binding Cassette (ABC) transporters reported to be modulated by verapamil include MRP1 and MRP4, it appears not to effect MRP2 or BCRP function (Walgren et al., 2000; Berger et al., 2003; O'Leary et al., 2003), the latter two being the key ABC 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. (Reid et al., 2003) have shown that these molecules are not substrates for MRP4 and MRP5. Therefore while we do not absolutely exclude these molecules

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as being susbtrates for other efflux transporters the Caco-2 data is 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 AP (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 MDR-1 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), p-Br (Cf1517) at the lower concentration (i.e. 0.5 mM) and p-I (Cf1572), p-Br (Cf1517), 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 C_0 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

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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 nature of the SE diastereoisomer is unlikely to account for the substantial permeation differences seen, for example, with the p-I derivative, it is possible that at least to a limited extent this factor could be involved in the relative difference in permeation. The stereoselectivity in cellular permeability of the pronucleotides found in this study suggests that the in vivo pharmacokinetics of the diastereoisomers may be also different. Clearly potential pharmacological differences between the diastereoisomers need to be investigated, and if found would provide greater endorsement for these compounds to be treated as different entities in the drug discovery setting.

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 amino acyl metabolite (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

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by hydrolysis 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 extruded 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 HEK293 cells (Reid et al., 2003).

The carboxylesterase 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 mefenamic-guaicol ester across Caco-2 cell monolayers (Tantishaiyakul 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 which 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 metabolism by carboxylesterase enzymes. In these model systems, specifically MDR1-MDCKII, we can conclude that the phosphoramidate triester prodrugs are good susbtrates for P-gp although do not exclude

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these molecules as being susbtrates 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.

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Footnotes

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LEGENDS FOR FIGURES

Figure 1. The aryloxy phosphoramidate prodrugs (pronucleotides) of d4T examined in this study. The pronucleotides are designed to deliver d4T 5'-monophosphate (d4TMP) inside cells. Chirality at the P atom generates two diastereoisomers. The enzymatic cleavage of the methoxy group is considered the first step in the hydrolytic activation of the compounds, with formation of an intermediate metabolite (IM), which is spontaneously converted into the amino acyl metabolite (AAM) by liberating the phenyl ring through intramolecular nucleophilic attack of the phosphorus by the charged carboxyl moiety. d4TMP is in part converted to d4T, or further phosphorylated to d4T 5'-diphosphate (d4TDP) and finally to the active d4T 5'-triphosphate (d4TTP) by intracellular kinases.

Figure 2. Concentration-dependence in A - B transepithelial transport across Caco-2 monolayers for the p-Me derivative (Cf1525). Data represent % of dose transported in 30 minutes for the FE (A) and SE (B) diastereoisomers (mean ± S.D. of three monolayers). The * symbol indicates significant difference ($p < 0.05$) compared to the 0.5 mM C_0 concentration.

Figure 3. Polarised transport of the p-Me derivative across Caco-2 monolayers at donor C_0 of 0.5 and 5 mM. Transport studies (120 min) were undertaken in the apical to basal direction (A - B) and basal to apical direction (B - A) 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 were observed within any treatment. The * symbol indicates statistical difference ($P < 0.05$) compared to the respective diastereoisomer in the corresponding A - B treatment control.

Figure 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 C_0 of 0.5 and 5 mM. The symbol * indicates statistical difference ($P < 0.05$) between diastereoisomers. Data are mean ± S.D. of five monolayers.

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Figure 5. Reverse-phase HPLC-UV chromatogram (266 nm) and mass spectra of the p-Me derivative (C_0 of 0.5 mM) recovered in the apical chamber of the Transwell system after a 120 min transport experiment (A - B) using MDCKII cells. The parent pronucleotides were analysed by LC-MS using positive ion electrospray (ESI) ionization, which produced the sodium ion-added molecule as the major fragment (m/z 502 for FE and SE diastereoisomers of the p-Me derivative). The peak at 11.33 min corresponds to the amino acyl metabolite (AAM), with a deprotonated molecular ion of m/z 374 .

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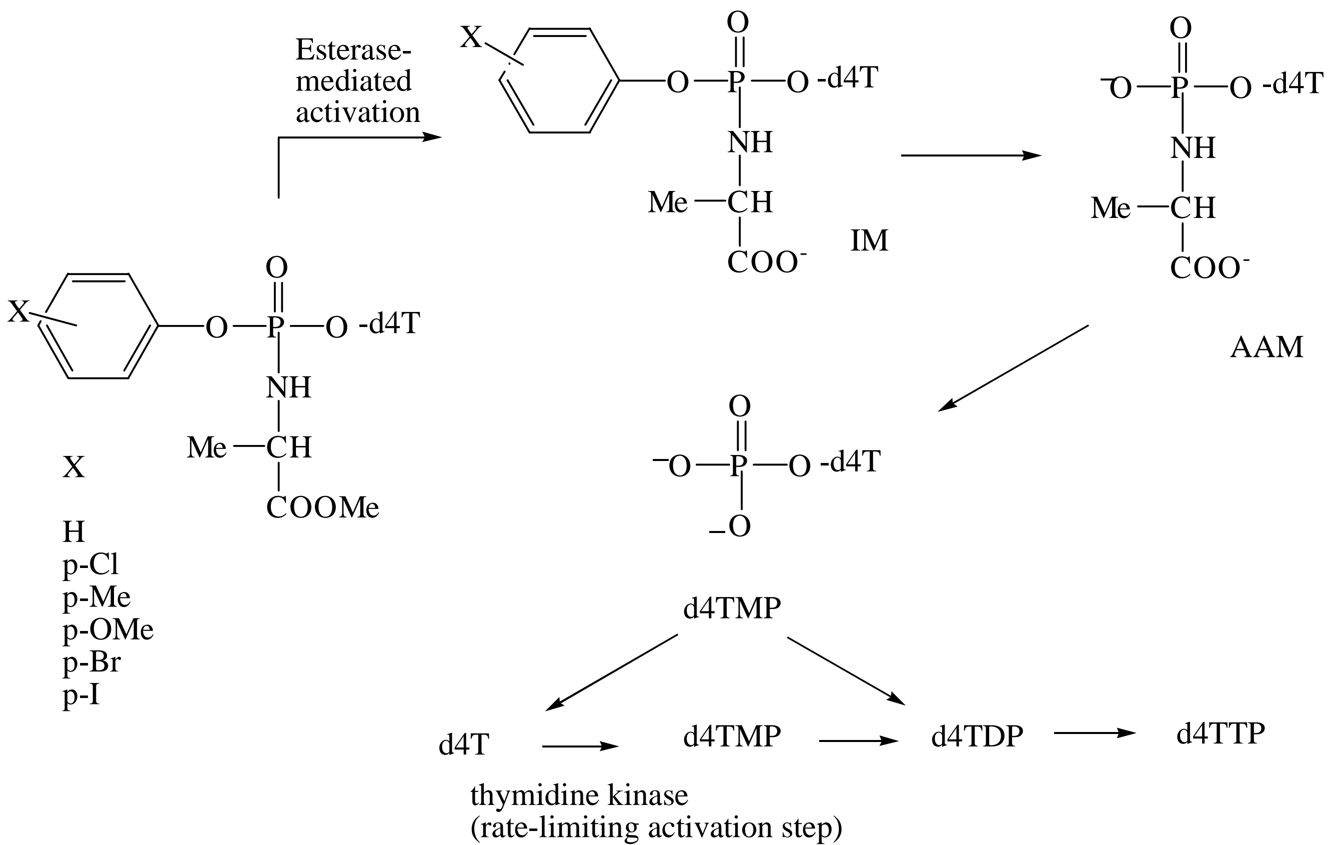

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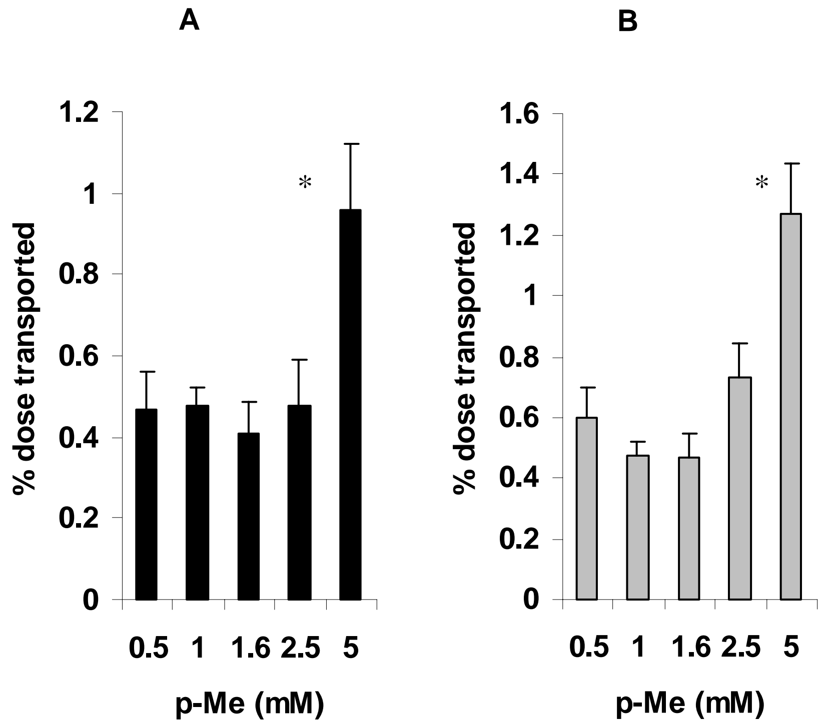

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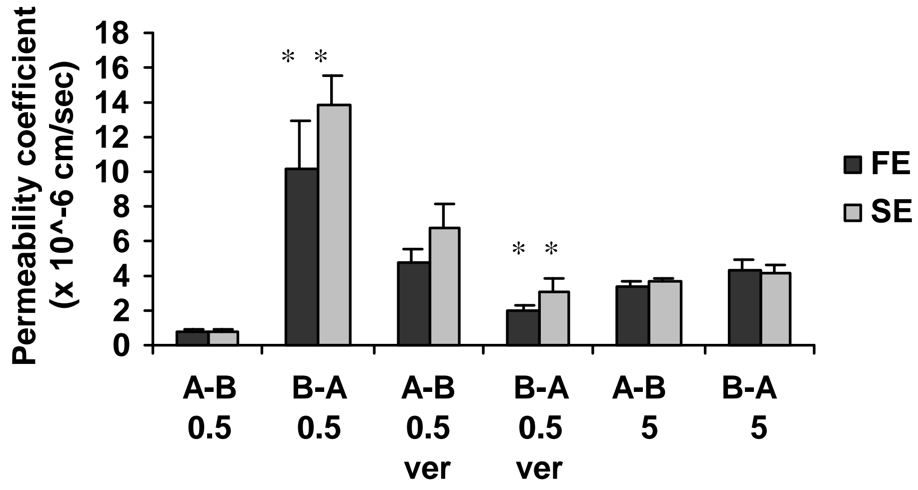

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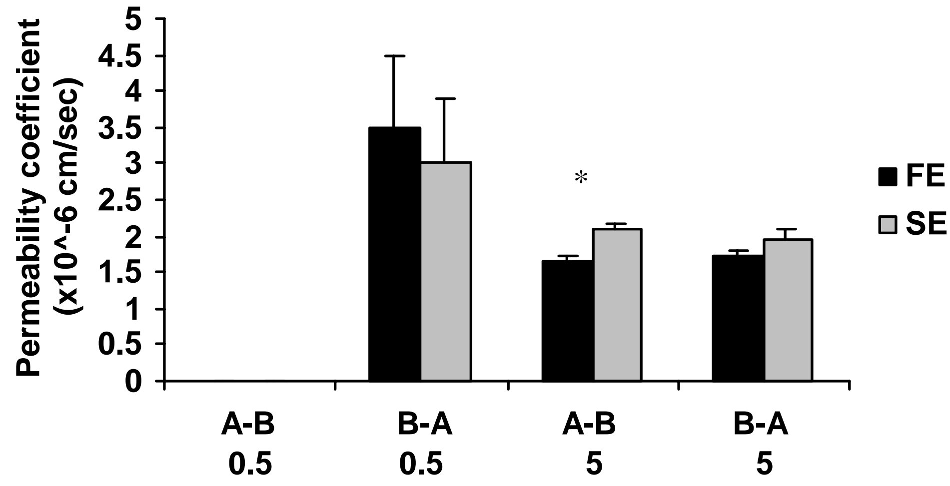

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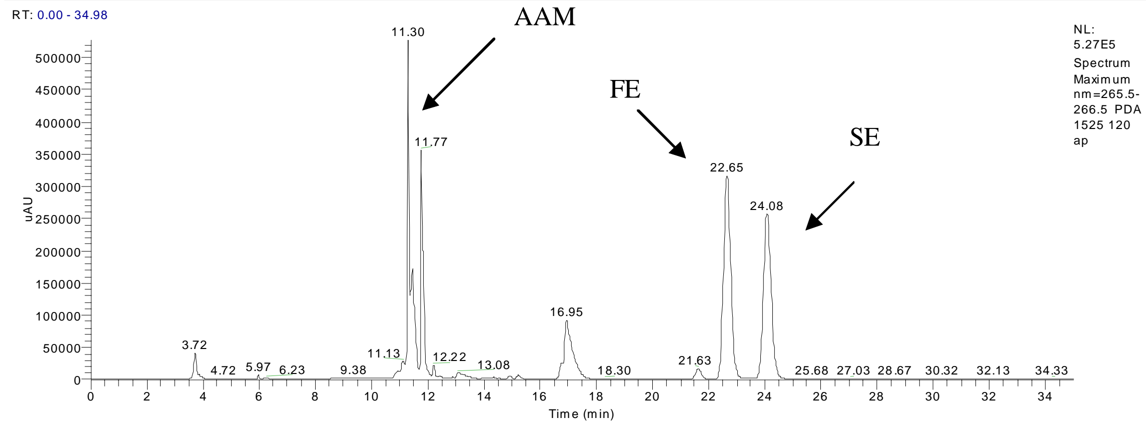

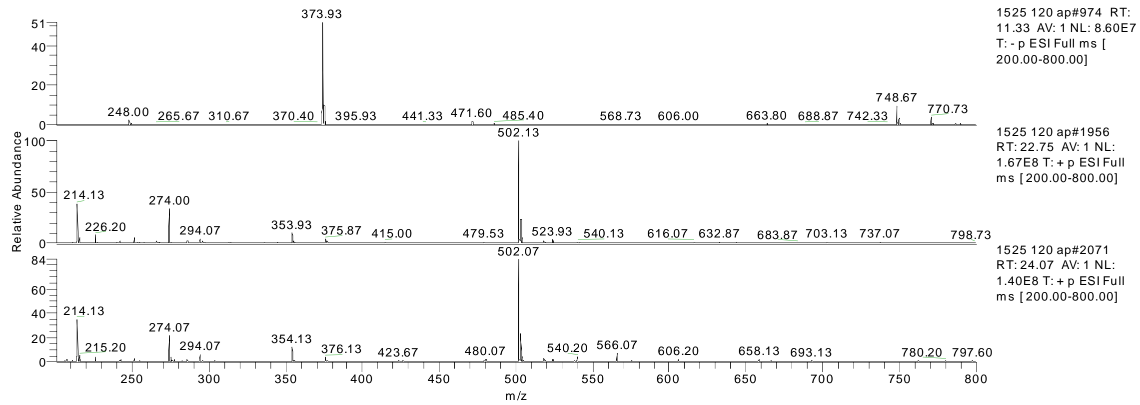

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Table 1. Measured logP values for the compounds analysed and permeability coefficients through Caco-2 cell monolayers obtained in a 120 min transport experiment in the A – B direction.

		Permeability Coefficients (cm/sec x 10^{-6})[a]			
		5 mM		0.5 mM	
Compound	logP	FE	SE	FE	SE
So324 (H)	1.04	1.7(±0.5)	1.8(±0.5)	0.7±0.2	0.6±0.06
CF 1526 (p-OMe)	1.09	1.6(±0.2)	1.7(±0.2)	0.2±0.02	0.2±0.06
CF 1525 (p-Me)	1.19	3.7(±0.3)	4.2(±0.3)	0.7±0.2	0.7±0.2
CF 1523 (p-Cl)	1.43	4.1(±0.2)	*6.1(±0.3)	0.7±0.2	1.1±0.1
CF 1517 (p-Br)	1.60	4.3(±0.2)	*6.8(±0.2)	0.6±0.01	*1.8±0.05
CF 1572 (p-I)	1.93	7.5(±0.1)	*12.0(±0.7)	3.6±1.1	*6.7±1.1

[a]Values represent the mean ± S.D. of n = 6.

*, significantly increased compared to FE diastereoisomer.

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Table 2. LogP values and permeability data for d4T and the probes mannitol and propranolol in Caco-2 cell monolayers obtained in 120 min transport experiments.

	logP	Permeability (cm/sec x 10^{-6})[a]
d4T	0.5 [b]	4.5(±0.5)
Mannitol	-2.0 [c]	1.2(±0.1)
Propranolol	1.5 [d]	19.2(±0.4)

[a]Values represent the mean ± S.D. of n = 6.

[b,c]Values calculated using CS ChemDraw Ultra 7.0 (Cambridge Soft, USA).

[d]Measured octanol/buffer partition coefficient at pH 7.4 (Artursson and Karlsson 1991)

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Table 3. Permeability data for So324 (H), Cf1525 (p-Me) and Cf1572 (p-I) derivatives at C_0 0.5 mM through MDCKII and MDR1-MDCKII monolayers measured in a 120 min transport experiment. Results are mean ± S.D. of four monolayers.

		Permeability Coefficients ($x\ 10^{-6}$ cm/sec)			
Compound		MDCKII		MDR1-MDCKII	
		FE	SE	FE	SE
So324 (H)	A - B	1.1±0.1	1.0±0.1	0.6±0.1	0.6±0.1
	B - A	3.5±0.5	3.5±0.1	7.4±1.9	7.1±2.1
Cf1525 (p-Me)	A - B	< 0.1†	< 0.1†	< 0.1†	< 0.1†
	B - A	3.5±1.0	3.0±0.9	13.4±2.3	14.5±2.0
Cf1572 (p-I)	A - B	1.6±0.3	3.5±1*	< 0.1†	< 0.1†
	B - A	5.3±0.8	8.0±1.2*	16.8±2.0	19.9±2.4

†Concentrations in the receiver chambers were below the limit of detection of the HPLC assay and only upper limits of permeability could be calculated.

* Indicates statistical difference of the SE to the respective FE diastereoisomer ($p < 0.05$).

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