The Influence of Donor and Reservoir Additives on Caco-2 Permeability and Secretory Transport of HIV Protease Inhibitors and other Lipophilic Compounds

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Purpose. To optimize the conditions for determining Caco-2 permeation of HIV protease inhibitors and other lipophilic compounds, and to compare cyclic urea HIV protease inhibitors with marketed compounds.

Methods. Absorptive and secretory Caco-2 membrane permeation studies were performed with HIV protease inhibitors and various reference compounds, examining the effects of adding the solubilizing agents dimethylacetamide (DMAC) and albumin in donor and reservoir compartments, respectively.

Results. DMAC was useful as an additive in the donor vehicles, increasing the dissolved concentrations of poorly water-soluble HIV protease inhibitors, and enabling more reliable determination of P_{app} values. Donor vehicles containing up to 5% DMAC could be used without altering Caco-2 barrier function, as indicated by the lack of effect on permeabilities of reference compounds with diverse absorption characteristics. The utilization of a reservoir containing albumin resulted in marked increases in absorptive P_{app} values for some HIV protease inhibitors as well as other lipophilic, highly protein bound compounds, consistent with albumin increasing the release of these compounds from the cell monolayer.

Conclusions. Poorly soluble, lipophilic, highly bound compounds may require using solubilizing agents in the donor and reservoir compartments of Caco-2 permeation experiments for estimating in vivo absorption potential. If the reservoir does not provide adequate sink conditions, cellular retention could over-emphasize the contributions of secretory transport. The cyclic ureas, DMP 450, DMP 850, and DMP 851, have Caco-2 permeabilities suggestive of moderate-to-high oral absorption potential in humans.

KEY WORDS: HIV protease inhibitor; cyclic urea; Caco-2; absorption; transport.

INTRODUCTION

One of the most commonly used regimens for drug therapy of HIV disease is the use of two reverse transcriptase inhibitors combined with an HIV protease inhibitor (1). The HIV protease inhibitors currently available in the U.S. are indinavir, ritonavir, saquinavir, nelfinavir, and amprenavir. Structurally, each of these could be considered linear peptidomimetics. A different structural class of HIV protease inhibitors is based on a rigid cyclic urea scaffold (2). We were interested in comparing Caco-2 permeabilities of cyclic urea HIV protease inhibitors and the currently available HIV protease inhibitors to assess their relative absorption potentials, particularly in view of the known in vivo bioavailabilities of the marketed products.

These HIV protease inhibitors have several properties that could complicate the evaluation of Caco-2 permeability. First, Caco-2 permeation studies require adequate solubility to ensure a driving force for diffusion, but these compounds have low solubilities at physiologic pH. Aqueous solubility of indinavir was reported as 0.07 mg/ml at pH 7.4 (3), and nelfinavir was similarly reported to have very low solubility above pH 4 (4). Ritonavir had solubility in the 5-7 μg/ml range at pH 7.4 (5). The cyclic ureas also have low solubilities at physiologic pH. We therefore examined the influence of solubilizing agents on Caco-2 permeation of HIV protease inhibitors.

Secondly, these HIV protease inhibitors are very lipophilic at physiologic pH. Previously, the in vitro MDCK epithelial membrane permeation of chlorpromazine, also a highly lipophilic compound, was shown to be highly dependent upon the composition of the reservoir (6). Addition of albumin to the reservoir reduced the membrane storage of chlorpromazine and increased its apparent permeability coefficient (6). We therefore investigated the effect of albumin on Caco-2 permeation of HIV protease inhibitors. Previous literature reports of Caco-2 permeation of HIV protease inhibitors have not utilized albumin or other solubilizing agents in the reservoir.

Finally, several of the marketed HIV protease inhibitors have been shown to be substrates for intestinal secretory transport, which appears to be mediated by P-glycoprotein. Indinavir, nelfinavir, and saquinavir each had greater Caco-2 permeation in the secretory direction than in the absorptive direction, and quinidine and PSC 833, known inhibitors of P-glycoprotein, reduced secretory permeation (7). The secretory permeation of saquinavir through Caco-2 membranes was 25-fold greater than that in the absorptive direction, and ritonavir had 15-fold greater secretory permeation than absorptive permeation (8). Ritonavir, saquinavir, and indinavir also inhibited photoaffinity labeling of P-glycoprotein in MDR1-transfected insect cells (9). Furthermore, in mice not expressing P-glycoprotein, plasma concentrations after oral dosing of these compounds were 2- to 5-fold greater than in wild-type mice, a difference not seen after i.v. dosing (7). Therefore, another aspect of the present investigation was to examine secretory transport.

The compounds included in this investigation were indinavir, ritonavir, nelfinavir, amprenavir, and three compounds from the cyclic urea structural category, DMP 450, DMP 850, and DMP 851. Structures of the cyclic ureas are given in Figure 1. In addition to the HIV protease inhibitors, other structurally unrelated compounds were studied as reference compounds for various experiments to further evaluate the influences of donor and reservoir additives.

METHODS

Chemicals

DMP 450, DMP 850, DMP 851, indinavir, ritonavir, nelfinavir, and amprenavir were synthesized at DuPont Pharma-

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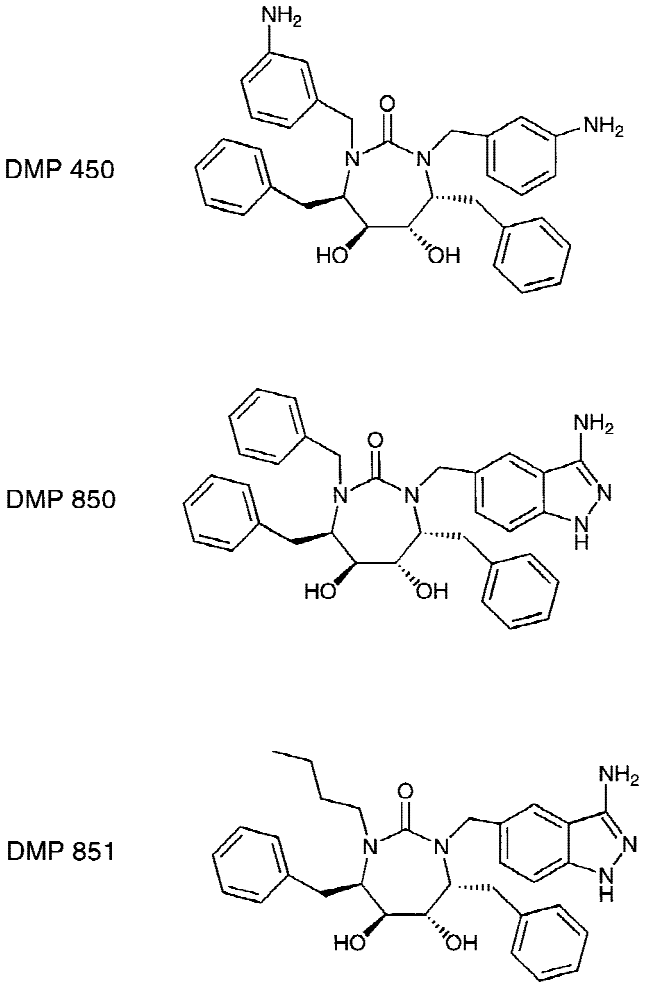

Fig. 1. Structures of the cyclic urea HIV protease inhibitors DMP 450, DMP 850, and DMP 851.

ceuticals Research Laboratories. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Gibco BRL (Grand Island, NY) supplied tissue culture reagents and buffers.

Tissue Culture

Caco-2 cell stock was provided by ATCC (Rockville, MD). Cells were grown in culture flasks and maintained at 37°C in an atmosphere of 5% CO_2 at 95% relative humidity. The culture medium was Dulbecco's Modified Eagle's Medium, supplemented with non-essential amino acids, L-glutamine, penicillin-streptomycin, and fetal bovine serum. Cells were used at passage number 35–50. Cells were seeded onto 12 mm diameter, 0.4 μm pore size, Transwell polycarbonate membranes (Corning Costar, Acton, MA). Cell membranes were used 18–22 days post-seeding.

For permeation studies, cell culture media was removed and replaced with Hank's balanced salt solution buffered at pH 7.4 with 10 mM morpholinopropanesulfonic acid (HBSS/MOPS). Cell membrane confluence was confirmed by measuring transepithelial electrical resistance (TEER) using a resistance meter (Endohm, World Precision Instruments, Sarasota, FL). Normal TEER values were in the range of 250-400 Ωcm^2, not subtracting the filter TEER. Donor vehicles were prepared by adding the compound of interest at a concentration of 200 μM to HBSS/MOPS at pH 7.4. Donors were mixed overnight and filtered before the permeation study, if not completely dissolved. When filtered, the filtrate drug concentration was assayed. In some studies the compound was first dissolved in dimethylacetamide (DMAC) and then diluted with the HBSS/MOPS to give DMAC concentrations of 2% or 5%, with the compound at 200 μM. The reservoir was pH 7.4 HBSS/MOPS with or without 4% bovine serum albumin added. The apical volume was 0.5 ml and the basolateral volume was 1.0 ml. Generally, studies were of 60 minutes duration, and samples were taken from the reservoir at 15 minute intervals. Permeation was in the apical-to-basolateral (A-to-B) or B-to-A direction. Caco-2 membrane integrity was monitored after each experiment by determining the permeation of lucifer yellow in a 30 minute period.

Analyses

Drug concentrations in permeation samples and in donor filtrates were determined by HPLC or LC/MS. Lucifer yellow and rhodamine 123 concentrations were determined using a fluorescence plate reader. Samples containing albumin were treated with 1 volume of acetonitrile and centrifuged. Permeability was expressed as the apparent permeability coefficient (P_{app}), which was obtained by dividing the amount permeating/cm^2/sec by the membrane surface area and the drug concentration in solution in the donor. P_{app} values are reported as mean ± S.E.M., and generally represent four or more replicates. Statistical comparisons were made using analysis of variance and student's t-tests.

RESULTS

Dimethylacetamide (DMAC) as a Solubilizing Agent

Dimethylsulfoxide (DMSO) is often used to prepare stock solutions or to solubilize compounds for in vitro tests. We examined DMAC as an alternative cosolvent. Some preliminary studies were performed comparing the effects of DMAC and DMSO, using TEER and lucifer yellow permeability to monitor Caco-2 membrane integrity. With equal solvent concentrations in donor and reservoir compartments, DMSO altered TEER values at concentrations of approximately 2% and greater (results not shown). However, DMAC had no effect on TEER values or lucifer yellow permeability at donor and reservoir concentrations up to 4%. Using 5% DMAC in the donor and 2% DMAC in the reservoir caused no change in TEER or lucifer yellow permeation. We also performed Caco-2 permeation studies with numerous reference compounds using 0%, 2%, and 5% DMAC in the donor vehicle. Permeability coefficients of these compounds are presented in Table I. These reference compounds range from having poor permeability and poor oral absorption to being highly permeable and well absorbed. In addition, the permeabilities of rhodamine 123, a P-glycoprotein substrate, in the absorptive (A-to-B) and secretory (B-to-A) directions were determined. Although there were a few instances in which P_{app} with DMAC was significantly different from control, these effects of DMAC are considered minor. We therefore chose to utilize DMAC as a cosolvent to prepare donor vehicles of the HIV protease inhibitors. The benefit of utilizing

Table I. Caco-2 Monolayer P_{app} Values for Various Reference Compounds and the Effects of DMAC in the Donor Vehicle

Donor	Caco-2 P_{app} (10^{-6} cm/sec)[a]		
	Buffer only	+ 2% DMAC	+ 5% DMAC[b]
Amiloride	0.49 ± 0.03	0.68 ± 0.03	0.74 ± 0.08
Atenolol	0.65 ± 0.05	0.67 ± 0.02	0.84 ± 0.12
Losartan	0.88 ± 0.03	0.62 ± 0.04[c]	1.0 ± 0.10
Lucifer yellow	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Phenol red	0.41 ± 0.12	0.24 ± 0.01	0.59 ± 0.18
Rhodamine 123 (A-to-B)	0.55 ± 0.07	0.33 ± 0.05[c]	0.82 ± 0.04[c]
Rhodamine 123 (B-to-A)	3.50 ± 0.53	4.12 ± 0.40	2.86 ± 0.12
Theophylline	27.8 ± 1.2	23.0 ± 1.2	21.6 ± 0.9[c]
Warfarin	30.0 ± 3.1	26.9 ± 1.8	28.2 ± 2.9

[a] Mean ± S.E.M. of at least 4 replicates. Donors contained compound at 200 μM and were at pH 7.4, except for losartan and phenol red which were at pH 6.5.
[b] Donor contained 5% DMAC and reservoir contained 2% DMAC.
[c] Significantly different ($p < 0.05$) from buffer only group.

a cosolvent to increase the dissolved concentration of test compound in the donor is that it also increases the concentrations subsequently found in the reservoir. With very poorly soluble compounds, the reservoir concentrations resulting during a permeation study could be at or below the limits of quantitation, even though that compound may be highly permeable. DMAC resulted in higher solution concentrations in the donor phase.

Absorptive and Secretory Permeation of HIV Protease Inhibitors

Caco-2 permeabilities of the HIV protease inhibitors were determined in the absorptive and secretory directions and are reported in Table II. DMAC was used in some, but not all of these studies, as the previous results indicated that DMAC does not have a strong influence on membrane permeability. Each of these HIV protease inhibitors had absorptive P_{app} values greater than 2×10^{-6} cm/sec, which indicates the potential for at least moderate oral absorption. Nelfinavir and amprenavir showed little difference between absorptive and secretory permeabilities. Indinavir, ritonavir, DMP 450, DMP 850, and DMP 851 had secretory permeabilities significantly greater than their respective absorptive permeabilities. The ratio of secretory P_{app} to absorptive P_{app} was 4 for indinavir, 20 for ritonavir, 1.3 for DMP 450, 2.5 for DMP 850, and 20 for DMP 851 in buffer alone and 4 in the presence of 5% DMAC. Permeabilities of indinavir and ritonavir were comparable to values previously reported in the literature. Indinavir was reported to have an absorptive P_{app} of 2.96×10^{-6} cm/sec and a secretory P_{app} of 21.47×10^{-6} cm/sec (10). Alsenz et al. (8) reported ritonavir absorptive P_{app} as 3.5×10^{-6} cm/sec and a secretory P_{app} of 59×10^{-6} cm/sec.

Table II also indicates that the concentrations of ritonavir and DMP 851 dissolved in the donor vehicle increased approximately 4- to 5-fold with the addition of 5% DMAC. DMAC did not significantly affect ritonavir absorptive and secretory permeabilities. DMP 851 had significantly greater absorptive permeability and significantly lower secretory permeability when using 5% DMAC. This could have been due to the increased donor solution concentration and concentration-dependent efflux.

Effect of Albumin in the Reservoir

Absorptive Caco-2 permeabilities of these HIV protease inhibitors were also determined using a reservoir containing 4% albumin. Figure 2 compares P_{app} values measured without and with albumin in the reservoir. Albumin significantly increased the absorptive P_{app} values of nelfinavir, ritonavir,

Table II. Drug Concentrations in Solution in the Donor Vehicles Used in Caco-2 Permeation Studies of HIV Protease Inhibitors, and Their P_{app} Values in the Absorptive (A-to-B) and Secretory (B-to-A) Directions

	Drug conc. in solution (μM)	P_{app} A-to-B (10^{-6} cm/sec)	P_{app} B-to-A (10^{-6} cm/sec)
Amprenavir	200	21.6 ± 0.6	22.1 ± 0.7
Indinavir (2% DMAC)	200	6.0 ± 1.3	30.0 ± 3.0[a]
Nelfinavir (2% DMAC)	4	3.4 ± 0.1	4.4 ± 0.4
Ritonavir	11	3.9 ± 1.2	78.4 ± 16.1[a]
Ritonavir (5% DMAC)	42	2.1 ± 0.1	41.4 ± 1.8[a]
DMP 450 (2% DMAC)	200	36.8 ± 1.8	48.3 ± 1.2[a]
DMP 850 (2% DMAC)	45	12.4 ± 0.8	30.7 ± 1.2[a]
DMP 851	37	2.1 ± 0.4	46.8 ± 1.7[a]
DMP 851 (5% DMAC)	200	5.2 ± 0.4	21.9 ± 0.7[a]

[a] P_{app} B-to-A is significantly different ($p < 0.05$) from P_{app} A-to-B.

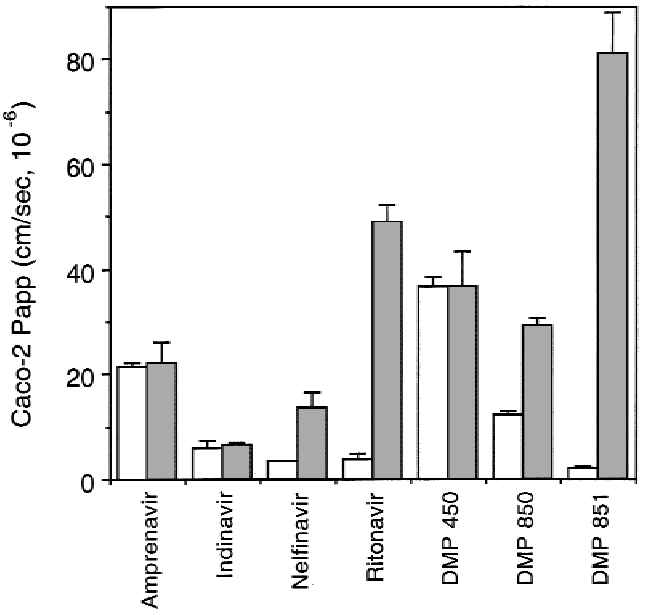

Fig. 1. Caco-2 monolayer permeation of HIV protease inhibitors in the absorptive direction using reservoirs of buffer alone (open symbol) or buffer with 4% albumin added (filled symbol).

DMP 850, and DMP 851. P_{app} values for ritonavir and DMP 851 increased 10-fold and 40-fold, respectively. However, there was no significant effect of albumin on P_{app} values of indinavir, amprenavir, or DMP 450.

We next investigated the influence of reservoir albumin on Caco-2 permeation of reference compounds with diverse properties. For atenolol, cimetidine, chlorothiazide, and warfarin, Caco-2 P_{app} values were not different when using reservoirs of buffer alone or with 4% albumin added (Table III). However, the permeabilities of chlorpromazine and tamoxifen were increased 5-fold or more when albumin was added to the reservoir. Similar albumin effects on chlorpromazine permeation of MDCK cells were previously described and shown to be mediated by a reduction of the membrane storage of chlorpromazine (6,11). Taken together, these results suggest that some compounds can be absorbed into the Caco-2 cell monolayer and retained there. The presence of albumin in the reservoir buffer increases drug diffusion out of the cells through the basolateral membrane. In vivo, plasma proteins could have a similar effect of removing compounds absorbed into the intestinal epithelium.

Table III. Effects of Using Albumin in the Reservoir on Caco-2 Permeation of Various Reference Compounds[a]

	P_{app} (10^{-6} cm/sec)	
Albumin concentration in reservoir	0%	4%
Atenolol	0.68 ± 0.04	0.53 ± 0.04
Cimetidine	1.87 ± 0.17	1.84 ± 0.15
Chlorothiazide	0.21 ± 0.01	0.17 ± 0.03
Chlorpromazine	9.1 ± 1.7	46.5 ± 2.0[b]
Losartan	0.42 ± 0.03	0.57 ± 0.04[b]
Phenytoin	15.4 ± 0.3	21.5 ± 1.2[b]
Tamoxifen	bql[c]	47.4 ± 21.0
Warfarin	28.2 ± 1.5	33.1 ± 0.5

[a] Donors were prepared to contain 200 μM drug concentrations at pH 7.4, or pH 6.5 for chlorothiazide, and donors were filtered if incompletely dissolved.
[b] Significantly different ($p < 0.05$) from result at 0% albumin.
[c] Permeation was below quantifiable limit.

To see whether albumin was indeed affecting the cell monolayer-to-reservoir release rates, a study was performed in which Caco-2 cell monolayers were loaded with DMP 851 for 60 minutes and the effects of albumin on subsequent release were examined. A DMP 851 suspension was put onto the apical side of the Caco-2 cells, with buffer alone on the basolateral side, and removed after 60 minutes. Cell monolayers were rinsed, and buffer containing 0%, 0.1%, or 4% albumin was put into the reservoir. Drug concentrations in the reservoir were determined at various times. Albumin increased the release of DMP 851 from Caco-2 monolayers, as illustrated in Figure 3.

DISCUSSION

There is very little published information on the effects of solvents on Caco-2 integrity or transport. We showed DMAC to be useful to increase the concentrations of our test compounds dissolved in the donor vehicle, increasing the driving force for diffusion, and increasing the amounts permeating the membrane. At donor and reservoir DMAC concentrations up to 4%, or with a donor of 5% DMAC and a reservoir of 2% DMAC, Caco-2 permeabilities of reference compounds that were selected to represent a range of permeabilities were insignificantly or only slightly altered. Therefore, low concentrations of DMAC did not affect Caco-2 integrity. DMSO could not be used at concentrations greater than 2% without impairing Caco-2 integrity, using TEER and lucifer yellow as integrity markers. To the best of our knowledge, DMAC has not previously been utilized as a solubilizing agent for Caco-2 studies.

Our samples were often analyzed by mass spectrometry. One potential drawback of using DMAC is that when HPLC with uv absorbance was utilized for sample analysis,

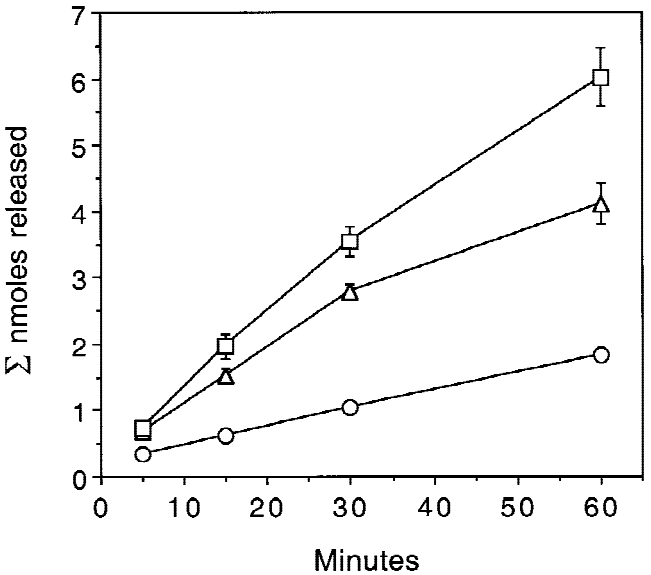

Fig. 1. Profiles of DMP 851 release from Caco-2 monolayers using reservoirs of buffer alone (○) or buffer containing 0.1% (△) or 4% (□) albumin.

Table IV. The Magnitude of Albumin Effect on Caco-2 Permeabilities of HIV Protease Inhibitors and Reference Compounds, and Their Octanol/Water Partition Coefficients, Plasma Protein Binding, and Human Oral Bioavailability[a]

	Albumin effect on Caco-2 Papp	Log P (o/w)	Plasma protein binding (%)	Oral bioavailability in human (%)
Amprenavir	Not significant	NA[b]	93 (12)	NA
Indinavir	Not significant	2.92 (6)	61 (6)	65 (1)
Nelfinavir	4.1-fold	4.1 (7)	>99 (12)	20–80 (1)
Ritonavir	13-fold	3.1	98–99 (12)	>60–80 (13)
DMP 450	Not significant	3.0	90–93 (14)	NA
DMP 850	2.4-fold	3.60	99	NA
DMP 851	38-fold	3.81	99.2	NA
Chlorpromazine	5.1-fold	3.46 (15)	95–98 (9)	10–25 (16)
Losartan	1.4-fold	1.3	>98 (17)	33 (17)
Phenytoin	1.4-fold	2.5 (18)	89 (19)	90 (19)
Tamoxifen	NA	NA	>98 (19)	NA
Warfarin	Not significant	0.12 (20)	99 (19)	98 (20)

[a] Data were obtained from the references given in parentheses, or if no reference is cited the result was obtained within The DuPont Pharmaceuticals Co.
[b] Not available.

DMAC sometimes created interference due to its uv absorbance, particularly if the compound was to be assayed at low wavelengths. DMSO also has similar potential for uv interference.

We also systematically investigated the effects of adding albumin to the reservoir in Caco-2 permeation studies. Caco-2 P_{app} values increased more than 2-fold for nelfinavir and DMP 850, and more than 10-fold for ritonavir and DMP 851, compared to results obtained with no added albumin. P_{app} values of chlorpromazine and tamoxifen were also greatly increased when using a reservoir containing albumin. A compilation of the lipophilicity and protein binding properties of various compounds tested is given in Table IV. The addition of albumin to the reservoir affected Caco-2 permeation of only the most lipophilic and highly protein bound compounds, those with log P(o/w) greater than 3 and plasma protein binding greater than 95%.

Published results evaluating the mechanism of albumin effects on chlorpromazine permeation of MDCK monolayers, as well as our results examining the effect of albumin on DMP 851 release from Caco-2 monolayers, indicate that albumin increases P_{app} values by reducing cellular accumulation. The presence of albumin in the reservoir would seem to best mimic the sink conditions that occur in vivo where plasma acts as the reservoir. Therefore, it is suggested that when evaluating Caco-2 permeability of lipophilic, highly protein bound compounds, the use of albumin in the reservoir produces P_{app} values more consistent with in vivo absorption properties. Similarly, it was recently reported that MDCK cell monolayer permeation of highly lipophilic antioxidants did not adequately mimic in vivo properties unless serum proteins were added to the reservoir (21). Membrane desorption was rate-limiting for in vitro permeation of those compounds. In addition, albumin could reduce or prevent compound adsorption to the plastic wells.

Some of the HIV protease inhibitors we examined are substrates for secretory transport by P-glycoprotein. It would seem that the rates of secretory drug transport would depend upon intracellular drug concentrations. Conditions that increase cellular accumulation of drug could result in greater rates of secretory transport. The effects of secretory transport as an impediment to drug absorption could be exaggerated when appropriate sink conditions are not maintained. Caco-2 permeation of indinavir and ritonavir are highly secretory oriented, based on P_{app} values with buffer alone on both sides. However, both compounds are greater than 60% absorbed orally. In vitro cellular metabolism could also be influenced by albumin in the reservoir. For example, the addition of 4% albumin to the reservoir reduced the intracellular accumulation of midazolam after apical application to cyp3A4-expressing Caco-2 monolayers, and reduced the formation of its hydroxylated metabolite (22).

In conclusion, the evaluation of Caco-2 permeation of poorly water soluble, lipophilic compounds can be improved using DMAC in the donor compartment and albumin in the reservoir. DMAC does not influence Caco-2 permeability at donor concentrations up to 5% and reservoir concentrations up to 2%. Caco-2 permeation of highly lipophilic compounds was increased using albumin in the reservoir, and this may better reflect in vivo conditions. In comparing the cyclic urea HIV protease inhibitors with marketed reference compounds, we expect that they should be well absorbed in vivo.

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