Transepithelial Transport of Prodrugs of the HIV Protease Inhibitors Saquinavir, Indinavir, and Nelfinavir across Caco-2 Cell Monolayers

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Purpose. This study is dedicated to the permeation of various amino acid-, D-glucose-, and PEG-conjugates of indinavir, saquinavir, and nelfinavir across monolayers of Caco-2 cells as models of the intestinal barrier. This screening is aimed at detecting the most promising prodrugs for improving the intestinal absorption of these protease inhibitors.

Methods. The bidirectional transport of the prodrugs was investigated using P-gp-expressing Caco-2 monolayers grown on membrane inserts using high-performance liquid chromatography for quantitation.

Results. The L-valyl, L-leucyl, and L-phenylalanyl ester conjugates led to an enhancement of the absorptive flux of indinavir or saquinavir. These results are likely attributable to an active transport mechanism and/or to a decrease of their efflux by carriers such as P-gp. Connection of tyrosine through its hydroxyl, of D-glucose, or of polyethylene glycol decreased their absorptive and secretory diffusion.

Conclusions. Conjugation of the protease inhibitors to amino acids constitutes a most appealing alternative that could improve their intestinal absorption and oral bioavailability. Whether it could improve their delivery into the central nervous system remains to be explored. D-Glucose conjugation will most probably not improve their intestinal absorption or their crossing of the blood–brain barrier. If some pharmacologic benefits are to be expected from PEG-protease inhibitor conjugates, they must then be administered intravenously.

KEY WORDS: antiproteases; intestinal transport; P-glycoprotein; amino acid; glucose; polyethylene glycol.

INTRODUCTION

Polytherapies that rely on the use of HIV reverse transcriptase and protease inhibitors combinations are the most efficient chemotherapies against AIDS known at present. Despite such polytherapies, HIV replication continues indicating, among others, resistance issues and the existence of reservoirs or sanctuaries for the virus (such as the lymphatic and central nervous system [CNS]) in which the antivirals do not penetrate at an efficient inhibitory level or do not penetrate at all (1,2). This is particularly the case of the current protease inhibitors used in clinical trials, i.e., indinavir (Ind), saquinavir (Saq), nelfinavir (Nelf), ritonavir, amprenavir, and lopinavir. Thus, to reduce total viral replication, an attractive alternative is to improve and optimize their pharmacologic properties. This implies, among others, increasing their oral bioavailability and delivery into the CNS sanctuary by facilitating, for example, their active permeation through the intestinal and blood–brain barriers (3,4).

Aiming at these goals, we have designed and performed the synthesis of various series of prodrugs deriving from Saq, Ind, and Nelf (Scheme 1). The prodrug approach is indeed widely used to obtain enhanced oral delivery of poor membrane-permeable compounds (5). The diffusion through the cell membrane, the intestinal and blood–brain barriers of pharmacologically significant amounts of drugs can be facilitated passively by increasing drug lipophilicity or actively by using the nutrient (D-glucose, amino acids) carrier-mediated transport systems located at these barriers (6). This could be achieved by conjugation of the protease inhibitors to fatty acid chains or to amino acids or D-glucose, respectively. To circumvent their *in vivo* binding to plasma proteins and their inactivation and rapid elimination from the blood circulation, their conjugation to hydrophilic polymers, such as polyethylene glycols (PEG), may represent attractive alternatives for increasing their circulation time in the body and bioavailability (7). The connection of various substituents (i.e., fatty acid chains, amino acids, D-glucose, or PEG) onto the hydroxyl functions of these protease inhibitors has been performed through a labile ester or a chemically more stable carbamate bond. Most of these prodrugs have been evaluate for their *in vitro* anti-HIV activity (see Scheme 1) and their chemical stability with respect to hydrolysis, hence liberation of the active free drug (8,9). These studies indicated that these prodrugs display a considerable therapeutic potential, providing their use improves the pharmacological properties of the parent protease inhibitors (e.g., permeation across intestinal and/or blood–brain barrier, hence oral bioavailability and/or the penetration into the HIV sanctuary sites), their safety, and pharmacokinetic profiles.

The present study is dedicated to the permeation of various amino acid-, D-glucose-, and PEG-conjugated Ind, Saq, and Nelf prodrugs across a monolayer of human intestinal epithelial Caco-2 cells in comparison with the transport of their parent drug. This screening is aimed at selecting and detecting the most promising prodrugs that could improve the intestinal absorption of the parent protease inhibitors and for further *in vivo* studies.

Caco-2 monolayers are widely accepted as an *in vitro* model for intestinal drug absorption. This human cell line forms polarized monolayers, which have indeed been shown to express most of the enzymatic, functional, and morphologic characteristics of the intestinal mucosa (10,11). The Caco-2 system has already been used in numerous studies to characterize the permeation of various drugs and prodrugs (12), including Ind, Saq, and Nelf (13,14). Moreover, these cells express, on the apical side of the monolayer, efflux carrier systems, such as the multidrug-resistant P-glycoprotein (P-gp), which is also responsible for the limited oral bioavailability and brain penetration of the protease inhibitors (13,14). The influence of efflux carriers on the permeability of the different prodrugs was also examined by comparing the absorptive apical (AP) to basolateral (BL) transport to the secretory BL to AP one. As Caco-2 cells are also rich in esterases, the hydrolysis of the ester prodrugs during their perme-

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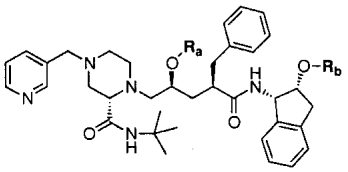
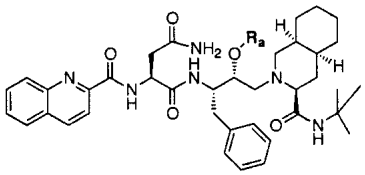
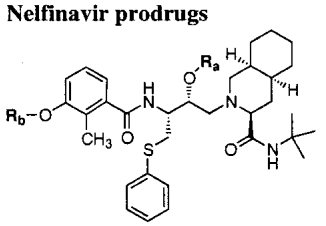
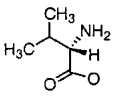
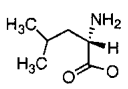
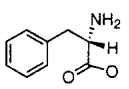
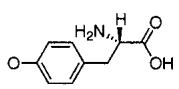
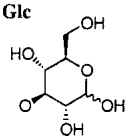
	R_a and R_b	Code name	IC_{50}(nM) (CEM-SS)	IC_{50}(nM) MT4
Indinavir prodrugs				
 Indinavir (R_a = R_b = H)	R_a = H; R_b = H	**Ind**	≤ 10	22
	R_a = H; R_b = Val	**Ind(8)-Val**	17	150
	R_a = Val; R_b = H	**Ind(14)-Val**	90	27
	R_a = C(O)CH_2Tyr; R_b = H	**Ind(14)-C(O)C1Tyr**	10	80
	R_a = H; R_b = C(O)$(CH_2)_4$-Tyr	**Ind(8)-C(O)C4Tyr**	9	47
	R_a = R_b = C(O)$(CH_2)_4$-Tyr	**Ind-[C(O)C4Tyr]2**	810	1,650
	R_a = C(O)NH$(CH_2)_4$-Tyr; R_b = H	**Ind(14)-C(O)NC4Tyr**	6,400	12,000
	R_a = H; R_b = C(O)$(CH_2)_4$-Glc	**Ind(8)-C(O)C4Glc**	48	190
	R_a = C(O)NH$(CH_2)_4$-Glc; R_b = H	**Ind(14)-C(O)NC4Glc**	52,000	42,000
	R_a = C(O)(C_2H_4)C(O)$(OC_2H_4)_{44}OCH_3$; R_b = H	**Ind(8)-PEG2000**	85	730
Saquinavir prodrugs				
	R_a = H	**Saq**	9	18
	R_a = Val	**Saq-Val**		
	R_a = Phe	**Saq-Phe**		
	R_a = Leu	**Saq-Leu**		
	R_a = C(O)NH$(CH_2)_4$-Tyr	**Saq-C(O)NC4Tyr**	2,300	6,000
	R_a = C(O)$(CH_2)_4$-Glc	**Saq-C(O)C4Glc**	< 11	62
	R_a = C(O)NH$(CH_2)_4$-Glc	**Saq-C(O)NC4Glc**	3,600	7,100
	R_a = C(O)(C_2H_4)C(O)$(OC_2H_4)_{44}OCH_3$	**Saq-PEG2000**	40	32
Nelfinavir prodrugs				
	R_a = H; R_b = H	**Nelf**	2*	
	R_a = H; R_b = C(O)$(CH_2)_4$Tyr	**Nelf(1)-C(O)C4Tyr**	325	465
	R_a = R_b = C(O)$(CH_2)_4$Tyr	**Nelf-[C(O)C4Tyr]2**		
	R_a = C(O)NH$(CH_2)_4$-Tyr; R_b = H	**Nelf(18)-C(O)NC4Tyr**	$>10^4$	$>10^4$
	R_a = C(O)NH$(CH_2)_4$-Glc; R_b = H	**Nelf(18)-C(O)NC4Glc**	8,200	$>10^4$

Val 

Leu 

Phe 

Tyr 

Glc 

Scheme 1. Chemical structures, code names, and anti-HIV activity of the prodrugs deriving from the Ind, Saq, and Nelf antiproteases. The anti-HIV activity data were taken from references 8 and 9. The data for Saq-Val, Saq-Phe, Saq-Leu, and Nelf-[C(O)C4Tyr]2 are not yet available. *Data from reference 15.

ation across Caco-2 monolayers was taken into account and carefully checked.

MATERIALS AND METHODS

Materials

Saquinavir, indinavir, and nelfinavir (as their methanesulfonate salt or sulfate salt) were kindly supplied by Hoffmann-La Roche, E. Merck, and Agouron, respectively. All the prodrugs used in this study came from our laboratory. Their synthesis, characterization, and anti-HIV activity are described in references 8 and 9. The anti-HIV activity data of Nelf was taken from reference 15. 1-Pentanesulfonic acid sodium salt, sodium acetate trihydrate, and acetonitrile were high-performance liquid chromatography (HPLC) grade. Fetal bovine serum, Dulbecco's phosphate-buffered saline (DPBS), penicillin:streptomycin solution (5000 U/mL:5000 μg/mL), trypsin-EDTA 0.25% solution (2.5 g trypsin and 0.2 g EDTA in 1 L DPBS), D(+)-glucose, and *N*-[2-hydroxyethyl]piperazine-*N*-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical (St Quentin Fallavier, France). Dulbecco's modified eagle's medium (DMEM) and nonessential amino acids DMEM 100 × (NEAA) were purchased from Gibco-Life Technologies (Cergy-Pontoise, France). Transport Medium (TM) consisted of DPBS containing 25 mM glucose and 10 mM HEPES (pH 7.4). Cell culture medium consisted of DMEM supplemented with 20% fetal bovine serum, 1% NEAA, and 2% penicillin-streptomycin.

Caco-2 Cell Culture

Caco-2 cells, clone TC7, were kindly provided by Dr. A. Zweibaum (INSERM U178, Villejuif, France). The cells were

routinely maintained in 75 cm^2 culture flasks at 37°C in an atmosphere containing 5% CO_2 and 95% relative humidity. Cells were split every 7 days at a density of 1.5×10^6 cells/flask.

For the transepithelial transport experiments, Caco-2 cells (passage 36–50) were harvested with trypsin-EDTA and seeded on Anopore membrane inserts (0.2-μm pore diameter, 25-mm diameter; Nunc, Roskilde, Denmark) at a density of 5×10^5 cells/insert (cm^2). AP and BL chamber volumes were maintained at 2 mL. Culture medium was changed every 3 to 4 days and cells were used for the experiments between days 14 and 27 post-seeding. Monolayer formation was monitored by measurement of Transepithelial electrical resistance (TEER) using a Millicel ERS apparatus (Millipore).

Transport Experiments

Before the transepithelial transport experiments, the Caco-2 monolayers were rinsed twice with the TM (both chambers) and pre-incubated for 30 min in the TM. After this equilibration period, the monolayer integrity was checked by measuring its TEER. For the transport experiments, only monolayers displaying TEER values above 174 $\Omega.cm^2$ and for which TEER values fell by less than 15% from the value measured at the end of the equilibration period were used. Under these conditions, the age of the cell monolayer within the 14 to 27 day's postseeding range did not affect the transport results. Transport was initiated by replacing the TM in the AP or in the BL " donor " compartment with 2 mL of the drug or prodrug solution. AP and BL chamber volumes were maintained at 2 mL. The test solutions were prepared by mixing a known amount of TM with a concentrated MeOH stock solution of the drug or prodrug under investigation to reach a final concentration of the drug or prodrug in the 75–330 μM, 12.5–113 μM, and 15–56 μM range for the Ind, Saq, and Nelf derivatives, respectively (the exact concentrations used for each derivative are given in the figures showing their transepithelial transport). The final MeOH concentration in the drug or prodrug solutions in contact with the monolayers never exceeded 3% MeOH (TEER monitoring and transport experiments have shown that MeOH at concentrations up to 5% did not affect cell monolayer integrity during the 3-h period of transport experiment nor the amount of drug and/or prodrug transported, respectively). Two hundred microliter samples were withdrawn from the "acceptor" compartment (opposite to the addition "donor" chamber) every 1 h over a period of 3 h and replaced by the same amount of fresh transport medium in order to maintain the same volume. The dilution was taken into account for the calculations. To prevent hydrolysis of the prodrugs, all these samples were stored at 4°C awaiting for prodrug and/or parent drug HPLC analysis (see next section). At the end of the experiments (3 h), samples were also taken from the "donor" compartment for HPLC analysis and the monolayers were checked for integrity by measuring TEER values. The concentrations of the prodrug and parent drug that were measured in the donor and acceptor chamber at the end of the transport experiment indicated that no non-specific adsorption on glass or on plastic had occurred. The experiments for which TEER has decreased by more than 8% from the value measured at the beginning of the experiment were discarded. Transport was expressed as a percentage of the initial amount added to the donor compartment. For the Saq or Ind prodrugs for which the parent drug was detected in the acceptor compartment, the transport data are expressed as Saq- or Ind-equivalent percentages of the amount of prodrug initially added to the donor chamber. All flux experiments were conducted at least in triplicate in the AP to BL and BL to AP directions. If possible, a concentration of the prodrug in the donor chamber the closest to that of the parent drug was privileged but solubility issues of the prodrug (which needed in some cases the use of MeOH) and detection issues of the prodrug and/or parent drug in the acceptor chamber was also considered for the selection of the prodrug concentration.

HPLC Analysis

Ind, Saq, and Nelf and their prodrugs were analyzed using a HPLC HP1100 apparatus (flow rate of 1 ml/min) equipped with a Kromasil C18 (100-5 μm; 150 × 3 mm) column for Ind, Ind(8)-Val, Ind(14)-Val, Ind(14)-C(O)C1Tyr, Ind(8)-C(O)C4Glc, Ind(8)-PEG2000, Saq, Saq-C(O)C4Glc, Saq-PEG2000, and with a Licrospher RP18 (100-5 μm; 250 × 3.2 mm) column for Ind(8)-C(O)C4Tyr, Ind-[C(O)C4Tyr]2, Ind(14)-C(O)NC4Tyr, Ind(14)-C(O)NC4Glc, Saq-Val, Saq-Phe, Saq-Leu, Saq-C(O)NC4Tyr, Saq-C(O)NC4Glc, Nelf, Nelf(1)-C(O)C4Tyr, Nelf-[C(O)C4Tyr]2, Nelf(18)-C(O)NC4Tyr, and Nelf(18)-C(O)NC4Glc. The mobile phase consisted of H_2O (15 mM $CH_3COONa.3H_2O$ and 15 mM $CH_3(CH_2)_4SO_3Na$)-CH_3CN (v:v) pH 6.0 buffer [59:41 for Ind, Ind(14)-Val, Ind(14)-C(O)C1Tyr, Ind(8)-C(O)C4Tyr, Ind-[C(O)C4Tyr]2, Ind(14)-C(O)NC4Tyr, Ind(8)-C(O)C4Glc, Ind(14)-C(O)NC4Glc; 41:59 for Ind(8)-Val, Saq, Saq-Val, Saq-Phe, Saq-Leu, Saq-C(O)NC4Tyr, Saq-C(O)C4Glc, Saq-C(O)NC4Glc, Saq-PEG2000; 35:65 for Ind(8)-PEG2000; 45:55 for Nelf, Nelf(1)-C(O)C4Tyr, Nelf-[C(O)C4Tyr]2, Nelf(18)-C(O)NC4Tyr, Nelf(18)-C(O)NC4Glc]. The prodrugs and/or parent drugs were detected by measuring their UV absorption at 210 (for Ind, Nelf, and their derivatives) or 240 nm (for Saq and its derivatives), and the obtained signals were integrated by the software provided. No other metabolites of the protease inhibitors were detected by HPLC. Under their respective HPLC conditions, the retention times measured for the different compounds were 11.0 min for Ind, 3.8 min for Ind(8)-Val, 5.4 min for Ind(14)-Val, 6.6 min for Ind(14)-C(O)C1Tyr, 8.5 min for Ind(8)-C(O)C4Tyr, 8.5 min for Ind-[C(O)C4Tyr]2, 8.0 min for Ind(14)-C(O)NC4Tyr, 5.0 min for Ind(8)-C(O)C4Glc, 5.1 min for Ind(14)-C(O)NC4Glc, 5.1 min for Ind(8)-PEG2000, 7.3 min for Saq, 11.8 min for Saq-Val, 14.2 min for Saq-Phe, 14.0 min for Saq-Leu, 4.3 min for Saq-C(O)NC4Tyr, 4.4 min for Saq-C(O)C4Glc, 3.8 min for Saq-C(O)NC4Glc, 7.0 min for Saq-PEG2000, 12.5 min for Nelf, 9.6 min for Nelf(1)-C(O)C4Tyr, 13.6 min for Nelf-[C(O)C4Tyr]2, 7.6 min for Nelf(18)-C(O)NC4Tyr, and 6.5 min for Nelf(18)-C(O)NC4Glc. The concentrations of the prodrug and of the parent compound in each sample were determined using calibration curves made up by standards of each molecule. The calibration curves were linear (correlation coefficients from 0.9957 to 0.9999) in a concentration range of 0.4 to 350 μM for Ind and its prodrugs, 0.1 to 300 μM for Saq and its prodrugs, and 0.1 to 300 μM for Nelf and its prodrugs, the lower limit corresponding to the limit of detection that can be quantified with accuracy. Above the lower concentration limit, the analytical method was reproducible.

Data Analysis

The apparent permeability coefficients P_{app} (cm/s) were calculated by linear regression analysis on the time course plot of amount of (pro)drug transported from equation $P_{app} = k^*(V_D/A)$, where k is the slope of the linear curve $\ln(C_t/C_o) = -kt$, C_t being the (pro)drug concentration in the receiver chamber at time t, C_o the initial concentration in the donor chamber, A the membrane surface area (4.52 cm^2), and V_D the volume of the donor chamber (2 cm^3). These calculations were performed for each transport experiment and the values reported in Table I represents the means (± SD) of at least three independent experiments.

RESULTS AND DISCUSSION

There is currently a considerable interest in increasing the intestinal and/or CNS absorption of the HIV protease inhibitors and in reducing their recognition by the efflux P-gp carrier (13,14), which is a possible reason for their poor or variable absorption into the blood circulation and/or into the CNS. In this regard, esterase-rich and efflux carrier-expressing Caco-2 cell line monolayers are being used as models of the intestinal epithelium for screening drug and prodrug candidates and thus for evaluating prodrug approaches for enhanced drug absorption (10,16). Evidence has also been provided that Caco-2 cells express amino acid and glucose carriers (10). Potential approaches for enhancing oral drug delivery involve modulation of the lipophilic/hydrophilic balance of the drug or the utilization of the body's own carrier systems for facilitating its passive or active diffusion across cell membranes, respectively.

Translocation of the various protease inhibitor-derived prodrugs across Caco-2 cell monolayers was evaluated at a concentration where they are soluble. It was initiated by adding the test solution to the AP or BL side of the monolayer (donor chamber). Investigation of the transport across the polarized Caco-2 cell monolayer in the BL to AP direction constitutes a mean of evaluating the influence of P-gp and related efflux carriers which are located on the AP side of the monolayer. As the Caco-2 cells express esterases and other hydrolases (16), special attention has been paid to the detection and quantification of the parent drug metabolite in both the acceptor and donor chambers during the transport studies of the ester and carbamate prodrugs.

Table I. Percentages of (Pro)Drug Transported from the Donor Chamber after the 3 h of Experiment and Apparent Permeability Coefficients P_{app}

			BL to AP		AP to BL	
(Pro)drug	Concentration in donor (μM)	Percent hydrolysis	Percent (±SD) in receiver	P_{app} (±SD) (cm/s, ×1E-8)	Percent (±SD) in receiver	P_{app} (±SD) (cm/s, ×1E-8)
Indinavir	175		38 (2)	1550 (234)	2.6 (0.3)	103 (10)
	5[a]		27[a]		2.7[a]	
Ind(14)-Val	177	0	14.6 (0.5)	708 (147)	14.0 (0.4)	513 (50)
Ind(8)-Val	330	0	18.1 (0.3)	737 (26)	16.6 (0.4)	664 (44)
Ind(14)-C(O)C1Tyr	225	85	4.1 (1.4)	162 (26)	1.4 (0.2)	44 (6)
Ind(8)-C(O)C4Tyr	125	≤5	nd	—	nd	—
Ind(14)-C(O)NC4Tyr	60	0	nd	—	nd	—
Ind-[C(O)C4Tyr]2	75	≤5	nd	—	nd	—
Ind(8)-PEG2000	213	0	4.1 (1.0)	177 (63)	1.1 (0.1)	40 (5)
Ind(8)-C(O)C4Glc	265	≤5	6.2 (1.5)	251 (110)	2.7 (1.3)	66 (31)
Ind(14)-C(O)NC4Glc	180	0	nd	—	nd	—
Saquinavir	9.4		10.3 (1.9)	398 (117)	2.6 (0.4)	110 (31)
	5[a]		33[a]		2.6[a]	
Saq-Val	21	100	9.6 (0.1)	343 (22)	6.1 (0.3)	232 (22)
Saq-Phe	21	100	17.6 (5.1)	840 (313)	7.5 (0.4)	332 (31)
Saq-Leu	25	100	11.6 (0.7)	428 (25)	7.2 (0.4)	265 (0)
Saq-C(O)NC4Tyr	22	0	nd	—	nd	—
Saq-PEG2000	106	60	1.2 (0.2)	40 (5)	nd	—
Saq-C(O)C4Glc	12.5	55	22.1 (7.4)	950 (720)	nd	—
Saq-C(O)NC4Glc	113	0	1.6 (0.1)	442 (10)	nd	—
Nelfinavir	8		6.3 (1.4)	251 (68)	2.7 (1.1)	103 (66)
	5[a]		20[a]		4[a]	
Nelf(1)-C(O)C4Tyr	15	0	nd	—	nd	—
Nelf(18)-C(O)NC4Tyr	56	0	nd	—	nd	—
Nelf-[C(O)C4Tyr]2	21	0	nd	—	nd	—
Nelf(18)-C(O)NC4Glc	29	0	8.5 (0.8)	339 (51)	1.6 (0.2)	44 (6)

Note. The percentage value is the ratio of (pro)drug concentration in receiver vs. donor chamber × 100. The P_{app} coefficients were calculated from the slope of cumulative receiver concentration with time for each transport experiment (see Materials and Methods section). The percentage and P_{app} values given represent the means (±SD) from at least three independent transport experiments.

nd: not detected, i.e., below the detection limit which is 0.4, 0.1, and 0.08 μM for the indinavir, saquinavir, and nelfinavir derivatives, respectively.

[a] Data taken from reference 13.

Hydrolysis

The ester Saq-Val, Saq-Leu, Saq-Phe, Saq-C(O)C4Glc, Saq-PEG2000, and Ind(14)-C(O)C1Tyr prodrugs were found to be extensively hydrolyzed in the donor compartment during the transport experiments, resulting in the exclusive formation of their corresponding parent drug (100, 100, 100, 55, 60, and 85%, respectively, after the 3-h incubation period), as shown by HPLC analysis. For the most sensitive Saq-Val, Saq-Leu, Saq-Phe, and Ind(14)-C(O)C1Tyr prodrugs, only the parent drug was detected in the acceptor compartment, even after one hour of incubation. For Saq-C(O)C4Glc, both the prodrug (40%) and Saq (60%) were found in the acceptor chamber. By contrast, for Saq-PEG2000, which is as sensitive to hydrolysis as Saq-C(O)C4Glc, only the parent Saq was detected in the acceptor compartment.

The Ind(8)-C(O)C4Tyr, Ind-[C(O)C4Tyr]2, and Ind(8)-C(O)C4Glc esters were also found to be hydrolyzed in the donor chamber but to a much lesser extent ($\leq$ 2%). For Ind(8)-C(O)C4Tyr, Ind (nearly 5% of the amount transported) was detected in the acceptor compartment only when its transport was investigated in the BL to AP direction. For Ind-[C(O)C4Tyr]2 and Ind(8)-C(O)C4Glc, no Ind could be detected in the acceptor chamber.

By contrast, no hydrolysis was detected for the ester Ind(8)-Val, Ind(14)-Val, Ind(8)-PEG2000, Nelf(1)-C(O)C4Tyr, and Nelf-[C(O)C4Tyr]2, nor for any of the carbamate prodrugs, even in the acceptor compartment, hence after cellular absorption and secretion. This indicates a relative high resistance against Caco-2 esterases and hydrolases for these latter derivatives.

Transport

The results of the bidirectional transport studies of amino acid-, D-glucose-, and PEG-conjugated prodrugs and of their parent protease inhibitor are presented in Table I. This table collects the percentages of (pro)drug that have been transported after the 3 h of experiment and the apparent permeability coefficients P_{app} that were calculated from the slope of a plot of the cumulative receiver concentration with time. Selected transport profiles, including the most promising prodrugs, are illustrated in Figs. 1 and 2. Figures 3 and 4 compare the permeability coefficients P_{app} of the various prodrugs with that of their respective parent protease inhibitor.

In line with the literature data (13,14), the transepithelial transports of Ind, Saq and Nelf across the Caco-2 cell monolayer in the absorptive (AP to BL) direction, and their respective P_{app} coefficients were low and significantly much lower than in the secretory (BL to AP) direction (Table I and Figs. 1A, 1E, 2G). These asymmetric permeation profiles indicate that Ind, Saq, and Nelf are substrates of the apically localized efflux P-gp carrier (13,14), which limits their oral bioavailability by transporting the absorbed drug back into the intestinal lumen and most probably limits their delivery into the brain. However, some discrepancies between the BL to AP secretory data and those published in the literature were found (see Table I). These differences are likely related to different experimental conditions and to the characteristics of the Caco-2 cell monolayers used for the transport studies. In our case, the transport studies were initiated using monolayers 14–27 days post-seeding (vs. 4 days in the literature, 13,14), the incubation period affecting the P-gp expression, its functional behavior (17) and the integrity of the monolayer (10).

Transport of the L-Amino Acid-Conjugates

Among all the prodrugs investigated here, the L-valyl, L-phenylalanyl, and L-leucyl esters of Ind and Saq were found to display the most promising transport profiles that could improve the intestinal absorption of these two protease inhibitors. It is indeed particularly noteworthy that a 3- to 6-fold enhancement of the AP to BL flux of Ind- or Saq-equivalents was observed with their L-valyl (Fig. 1, B, C, and F), L-phenylalanyl and L-leucyl esters (Fig. 1, G and H). This is even the case of the Saq conjugates despite their extensive hydrolysis during the transport experiments. This is further supported by the substantial increase of the absorptive P_{app} coefficients of Ind and Saq upon conjugation to these amino acids (Table I and Fig. 3).

These absorptive transport enhancements are likely attributable 1) to an active transport mechanism, indicating that these amino acid derivatives are substrates of the amino acid carriers located at the brush border side of the Caco-2 cell monolayer, and/or 2) to a substantial decrease of their efflux owing to a lower affinity of the efflux carriers for these conjugates. The former point is strongly supported by the lower transport of the chemically stable Ind(8)-Val and Ind(14)-Val prodrugs in the BL to AP secretory direction (Fig. 1B and C) and, concomitantly, by their lower P_{app} coefficients (Table I and Fig. 3) as compared with those of "free" Ind (Fig. 1A). The second point is confirmed by the superimposable absorptive and secretory permeation profiles found for these two Val-Ind prodrugs (Fig. 1B and C), and by the slightly asymmetric translocation profiles measured for the Val-, Leu-, and Phe-Saq conjugates (Fig. 1, F–H) by contrast to the highly asymmetric translocation profiles of Ind (Fig. 1A) and Saq (Fig. 1E), respectively. That the translocation profiles of the Saq conjugates were not superimposable can be attributed to some extent to their poor stability during the transport experiments. Indeed, their progressive hydrolysis into Saq (which is substrate of P-gp) in the apical donor chamber contributes significantly in lowering their absorptive transport. Further experiments are nevertheless required for evaluating the impact of these amino acids on the affinity of their Saq conjugates for P-gp as compared with that of "free" Saq.

An increase of passive diffusion upon conjugation of the protease inhibitors to the L-valyl, L-phenylalanyl, and L-leucyl amino acids could also constitute an alternative hypothesis for the absorptive transport enhancement. If this transport mechanism is operative, one expects more particularly an enhancement of the secretory BL to AP diffusion of the Ind and Saq conjugates, as compared with that of the "free" drugs. Of the three types of conjugates tested, Saq-Phe is the sole prodrug for which significantly higher levels of parent drug equivalents in the AP acceptor chamber were measured (Figs. 1 and 3). This secretory transport enhancement indicates likely an increase of passive diffusion upon conjugation to phenylalanine, which thus may also account for the transport enhancement observed for Saq-Phe in the absorptive direction, as compared with that of "free" Saq.

Where the translocation of L-Tyr-conjugates across the Caco-2 cell monolayer is concerned, the connection of tyro-

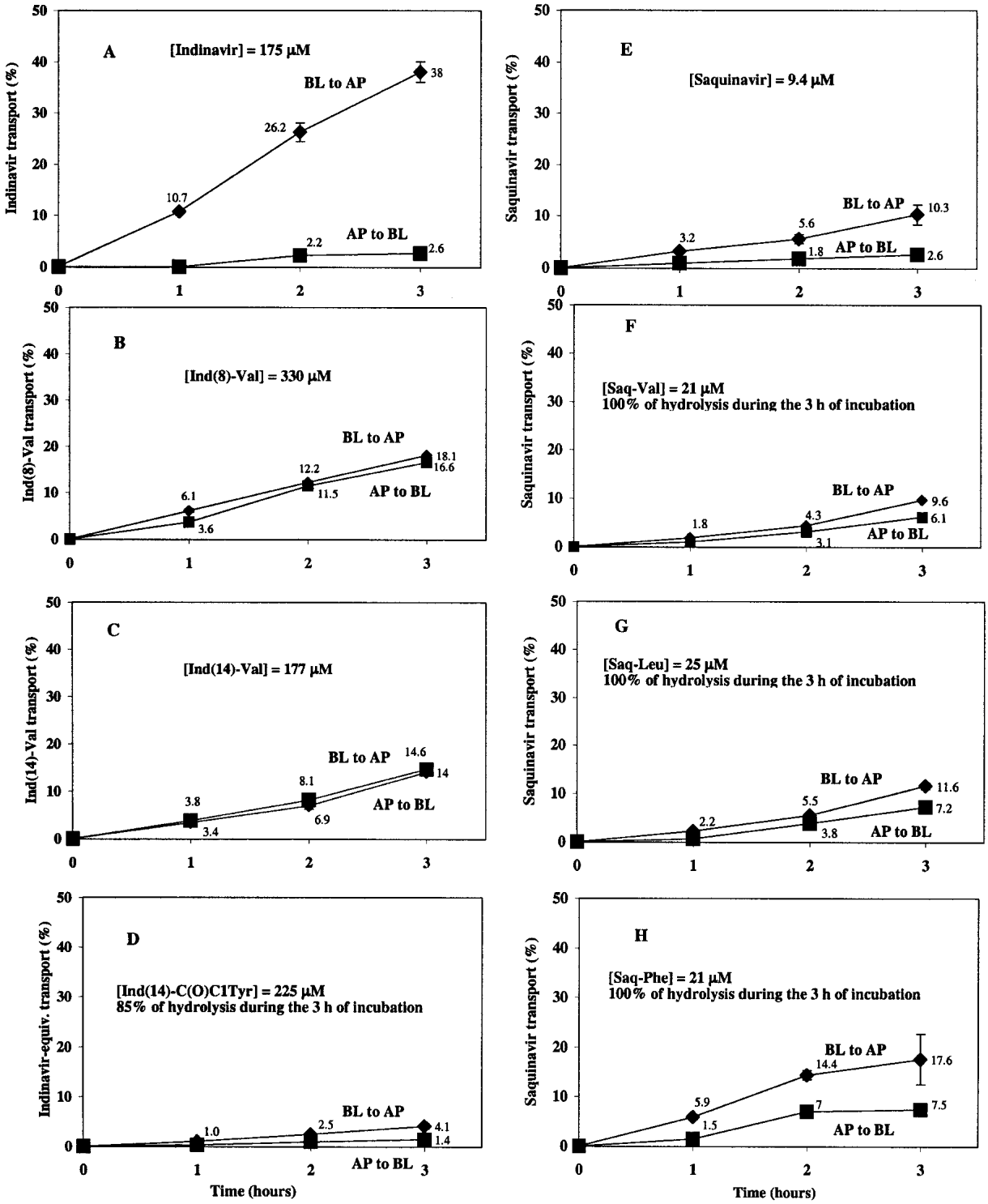


Fig. 1. Bidirectional transepithelial transport across a monolayer of Caco-2 cells of Ind (A) and Saq (E) and of some of their amino acid-conjugates shown in Scheme 1 (B–D and F–H). Filled squares, absorptive translocation [apical (AP) to basolateral (BL) compartment]; filled diamonds, secretory translocation (BL to AP). The results are expressed as prodrug or drug-equivalent transport percentages vs. time. The percentage values represent the ratios of (pro)drug concentration in receiver vs donor chamber × 100. The initial concentration of the prodrug in the donor compartment is indicated on each panel. Results are means ± SD from three experiments. All incubations were performed at 37°C and pH 7.4.

sine to Ind, Saq, or Nelf through the tyrosine aromatic hydroxyl (using different linkers) was found to decrease drastically their transport in both directions, and consequently their P_{app} coefficient, and whatever the linker used (see Table I, Figs. 1D and 3). This is even the case for the Ind(14)-C(O)C1Tyr ester, although it was extensively hydrolyzed during the transport experiment (Fig. 1D). The different transepithelial transport behaviors observed for the L-Val, L-Phe,

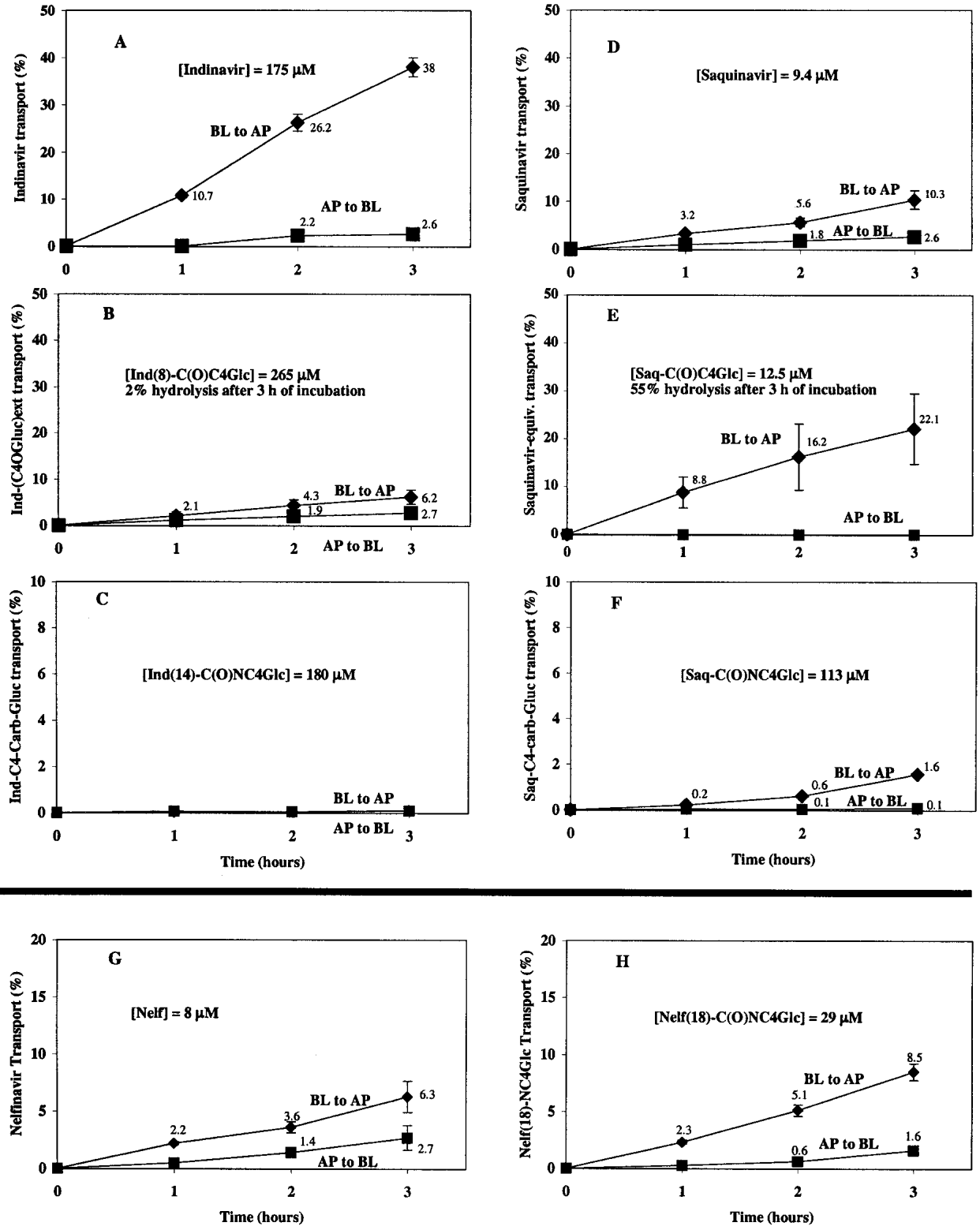


Fig. 2. Bidirectional transepithelial transport across a monolayer of Caco-2 cells of indinavir- (B, C), saquinavir- (E, F), and nelfinavir-glucose (H) conjugates shown in Scheme 1, as compared with that of their respective parent protease inhibitor (A, D, and G, respectively). For more details, see caption of Fig. 1.

or L-Leu conjugates and the L-Tyr ones are most probably related to the way these amino acids are connected to the protease inhibitors. Whereas L-valine, L-phenylalanine and L-leucine were linked through their acid function, L-tyrosine was coupled through its hydroxyl leaving its amino and acid functions free. This was preferred to fully preserve the recognition of tyrosine and its transport capability by the large amino acid carrier system located at the blood–brain barrier

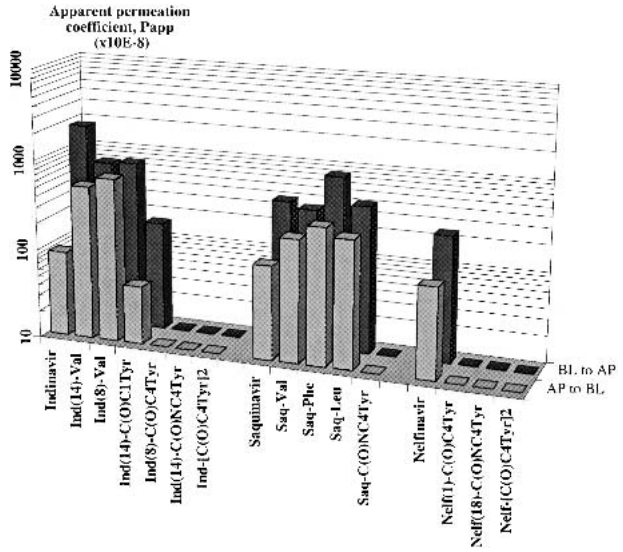


Fig. 3. Caco-2 cell monolayer apparent permeation coefficients (P_{app}) in the absorptive (AP to BL; grey bars) and secretion (BL to AP; dark grey bars) direction of Ind, Saq, Nelf, and of their amino-acid conjugates displayed in Scheme 1. The P_{app} coefficients were calculated from the slope of cumulative receiver concentration with time for each transport experiment (see Materials and Methods section). The P_{app} values represent the means from three independent transport experiments.

(18,19). Unfortunately, this way of conjugation of tyrosine to Ind, Saq or Nelf was found to inhibit the passive and/or active transport of these protease inhibitors across the Caco-2 cell monolayer model of the intestinal barrier.

Transport of the Glucose-Conjugates

The glucose prodrug strategy is mainly aimed at improving the penetration of the protease inhibitors into the up to now preserved CNS sanctuary for HIV. D-Glucose, which is actively transported across the intestinal and blood–brain barriers, was connected through its 3-hydroxyl to the protease inhibitors. This connection should preserve its recognition and transport capability by the GLUT-1 carrier system located at these barriers (5,6,12). The transport experiments, whether performed with or without D-glucose in the incubation medium, gave comparable results in both the absorptive and secretory directions.

Most importantly, the conjugation of Ind, Saq or Nelf to glucose led to a decrease of their absorptive permeation across Caco-2 cell monolayers (Table I and Fig. 2) and, consequently, of their P_{app} coefficient (Fig. 4). Indeed, and although tested at higher concentration than their respective parent drug, the translocation of the Ind-, Saq-, and Nelf-glucose conjugates from the AP to BL compartment was at the most comparable to (e.g., for Ind(8)-C(O)C4Glc, Fig. 2B or for Nelf(18)-C(O)NC4Glc, Fig 2H) or substantially much lower than that of Ind, Saq, and Nelf, respectively. These results indicate that the glucose conjugates, if recognized by the GLUT-1 carrier, are not transported efficiently by this system and/or are substrates of the efflux carrier systems.

The conjugation of glucose to Ind, as in Ind(8)-C(O)C4Glc or Ind(14)-C(O)NC4Glc, and Saq, as in the carbamate Saq–C(O)NC4Glc, was further found to reduce drastically their passive permeation, as expected from an increase of hydrophilicity. This is supported by their much lower secretory BL to AP efflux and P_{app} coefficient as compared with that of their corresponding "free" protease inhibitor (see Fig. 4).

However, contrasting results were obtained for the ester Saq-C(O)C4Glc and carbamate Nelf(18)-C(O)NC4Glc conjugates. These two glucose conjugates led to higher level of Saq and Nelf equivalents in the AP acceptor compartment when the translocation was investigated in the BL to AP secretory direction (Fig. 2, E and H). Furthermore, they displayed also a much more pronounced asymmetric secretory and absorptive profile (Fig. 2D and G, respectively), hence a larger difference of their BL to AP and AP to BL P_{app} coefficients than their respective parent protease inhibitor (Fig. 4). Concerning more particularly the ester Saq-C(O)C4Glc, these results cannot only be attributed to its sensitivity to hydrolysis. They indicate more likely that the connection of glucose to Saq and also to Nelf increases their recognition by the efflux carriers.

All these results demonstrate clearly that the conjugation of glucose to the protease inhibitors will not improve their intestinal absorption. Conjugation of glucose to Ind is also detrimental for its delivery into cells that do not express efflux carriers. However, conjugation of glucose to Nelf or to Saq (depending the chemical nature of the moiety linking glucose to Saq) could improve the penetration of these two protease inhibitors into cells that do not express efflux carriers.

Transport of the PEG–Conjugates

The PEG-protease inhibitor conjugates were mostly designed for reducing the interactions of the protease inhibitors with plasma proteins, and improving their *in vivo* blood circulation time. The connection of highly hydrophilic polyethylene glycol moieties to a drug is further expected to decrease

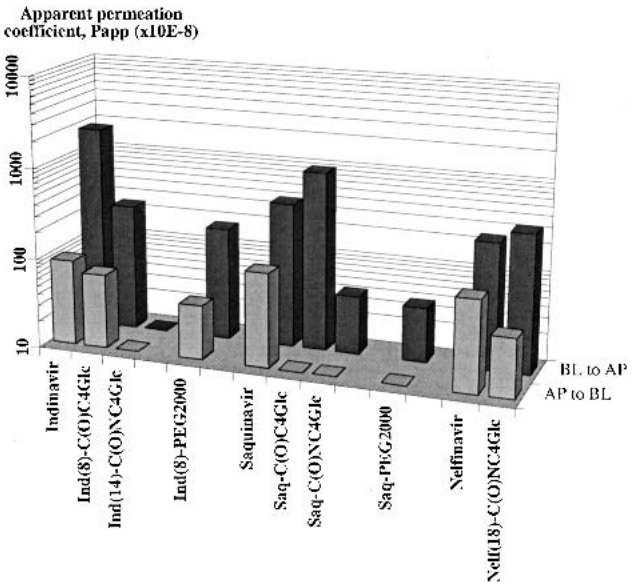


Fig. 4. Caco-2 cell monolayer apparent permeation coefficients (P_{app}) in the absorptive (AP to BL; grey bars) and secretion (BL to AP; dark grey bars) direction of indinavir, saquinavir, nelfinavir, and of their glucose conjugates displayed in Scheme 1. For more details, see legend to Fig. 3.

its solubility into the cell membrane and consequently its passive diffusion across a cell monolayer and across the intestinal barrier. Such PEG-modified prodrugs are therefore rather destined for an intravenous administration. Their translocation across a Caco-2 cell monolayer was nevertheless investigated. As expected from a hydrophilicity increase, the conjugation of PEG2000 to Saq and Ind reduced drastically both their absorptive and secretory diffusion and P_{app} coefficients across the Caco-2 monolayer (Table I and Fig. 4). This is even the case of Saq-PEG2000 despite its extensive hydrolysis (60%) in the donor compartment during the transport experiments.

CONCLUSION

The transepithelial transport of various protease inhibitor (Ind, Saq, and Nelf) conjugates was examined using Caco-2 cell monolayers as a model of the intestinal barrier. We found that conjugation of L-valine, L-leucine or L-phenylalanine (through their carboxyl) to the protease inhibitors constitutes a most appealing alternative, which improves their absorptive diffusion and reduces their recognition by efflux carriers. These results are further of interest considering that drug translocation across Caco-2 monolayers is commonly assumed to be lower as compared to their transport across the human intestine (20). The 3- to 6-fold absorption enhancement resulting from the conjugation of the protease inhibitors to these amino acids indicates that potentially lower doses of protease inhibitors (as their conjugates) can be administered orally to HIV(+) patients.

By contrast, conjugation of L-tyrosine through its aromatic hydroxyl to the protease inhibitors was found to inhibit their translocation across the Caco-2 cell monolayer. Whether it could improve their delivery into the CNS can however not be excluded and remains worth to be explored.

We established also that D-glucose conjugation to the protease inhibitors will most probably not improve their intestinal absorption nor it will be beneficial for their crossing of the BBB, even after direct administration into the blood circulation thus bypassing intestinal absorption. Interestingly, its conjugation to Nelf or Saq could constitute a mean of improving the penetration of these protease inhibitors into cells that do not express efflux carrier systems. But this was detrimental in the case of Ind.

As expected from a hydrophilicity increase, the conjugation of PEG2000 to the protease inhibitors reduced drastically their translocation across the Caco-2 monolayer. If some pharmacological benefits are to be expected from such prodrugs, they must then be administered intravenously.

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