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Research paper

Absorption of poorly water soluble drugs subject to apical efflux using phospholipids as solubilizers in the Caco-2 cell model

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Abstract

The purpose of this work was to determine the influence of liposomal solubilization of poorly water soluble drugs exhibiting apical efflux on permeation kinetics and cell toxicity in Caco-2 cells. The HIV-protease inhibitors indinavir and saquinavir were incorporated in phosphatidylcholine liposomes at maximal drug-to-lipid mass ratios and their absorption was determined in Caco-2 cell cultures grown on Transwell inserts using purely aqueous drug solutions as reference. A novel mathematical model was developed to quantitatively delineate the contribution of passive membrane permeation and carrier mediated efflux to transport across the cell monolayer and passive permeability coefficient and maximal efflux rate and affinity constant of the transporter system were determined. Cell toxicity of phospholipids was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and the lactate dehydrogenase (LDH) assay. Cell integrity was not significantly affected by phospholipid concentrations of up to 150 mg/ml with respect to the used standard tests. Maximum drug concentration was increased 10- and 750-fold for indinavir and saquinavir, respectively, by the use of liposomes. The passive membrane permeability coefficient differed between the two drugs in accordance with their lipophilicity and the affinity for apical efflux transporters was on average 4-fold greater for saquinavir than for indinavir. Liposomal solubilization diminished the passive permeability coefficient of both drugs but the passive apical-to-basal delivery rate was increased by the liposomes compared to the purely aqueous solutions at maximal donor concentrations for at least one of the two drugs. Efflux rate reached a maximum for the liposomal formulations reflecting transporter saturation. Hence, liposomal solubilization considerably increased drug concentration in the media and altered absorption behavior by affecting both the passive diffusion and the carrier mediated efflux components of cell monolayer permeation.

Keywords: Caco-2; Drug absorption; HIV-protease inhibitors; Efflux; Liposomes; Phospholipids; Kinetic modeling

1. Introduction

As guide for formulation development work aimed at optimizing bioavailability, drugs are classified by the biopharmaceutic classification system (BCS), which considers four classes of drugs having either a low or high solubility in water and either a high or low permeability for the biological membrane [1,2]. The intestinal permeation kinetics of candidate drugs is routinely measured using the Caco-2 cell model and the PAMPA method [3]. The characteristics of the Caco-2 cell monolayer model have been well described [4–6]. The Caco-2 cell monolayer can be easily used for testing water soluble drugs. The necessary concentration gradient can be generated by simply using high concentrations of the drug in the donor compartment. However, poorly water soluble compounds are much harder to test in the Caco-2 cell model because of the insufficiently steep concentration gradient over the cell monolayer requiring very sensitive analytical tools such as radio-labelling for monitoring drug permeation. For this reason, addition of solubilizing agents is necessary to allow

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measurement of the permeation at readily detectable drug levels in the receiver compartment. Unfortunately, the concentration of solubilizing excipient cannot be infinitely increased because of the detrimental effects of solubilizers such as surfactants, chelating agents and organic solvents to the cell membrane [7–10]. In general, most solubilizers could only be used at very low levels. Consequently, it is to be expected that for some poorly soluble drugs it may be almost impossible to assess the membrane permeation characteristics using the Caco-2 cell model. This deficit is reflected in a recent article [11] showing that a major part of the drugs listed by the WHO as essential drugs cannot be classified in the BCS because of lack of reliable membrane permeation data of these drugs.

Liposomes are phospholipid vesicles, comprising a phospholipid bilayer surrounding an aqueous compartment. In the lipid domain of the bilayer membrane, lipophilic drugs can be dissolved. This principle has been used to solubilize lipophilic drugs like amphotericin B [12] and cyclosporin A [13]. Compared to detergents or solvents, it might be expected that liposomes do not perturbate the plasma membrane of the Caco-2 cells.

A major objective of this work was to explore the possibility of using liposomal solubilization of lipophilic, poorly water soluble drugs for studying their permeation in the Caco-2 model and to investigate the influence of liposomal solubilization on the Caco-2 permeation. This included determining the maximally applicable phospholipid concentration with respect to Caco-2 cell toxicity for increasing drug concentration in solution. Two HIV-protease inhibitors, indinavir and saquinavir with an aqueous solubility at pH 7.4 of 70 and 36 μg/ml, respectively, were used as model poorly water soluble compounds [14]. Both drugs are known to be subject to apical cellular efflux mediated by P-glycoprotein and MRP2 [15–18]. These transporters have been identified in Caco-2 cells [19–21]. The measured cell monolayer permeability in this case is the result of two transport mechanisms taking place in parallel, i.e., passive cell membrane permeation and active apical efflux. In order to gain a basic understanding of the effect of liposomes on absorption, the contribution of each of these two mechanisms to cell layer permeation of the two drugs must be determined, which should allow the effect of liposomes on them to be separately assessed. Therefore, the second major objective of this work was to develop a method for quantitatively delineating passive membrane permeation and carrier mediated apical efflux from experimental transport data in the Caco-2 cell model. This was accomplished by introducing a mathematical model for the kinetics of cellular transport.

2. Materials and methods

2.1. Materials

The human colon adenocarcinoma cell line Caco-2 was a gift of Prof. H. P. Hauri, Biocenter, University of Basel, and originated from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose without foetal calf serum (FCS) and without sodium pyruvate, L-glutamine, MEM non-essential amino acids solution (100×), FCS and phosphate-buffered saline (PBS) were all purchased from Gibco (Gaithersburg, MD, USA). For cell culture, DMEM was supplemented with 2 mM L-glutamine, 1% MEM solution and 10% FCS.

Transport media used for the permeation studies were made with Dulbecco's modified Eagle's medium (DMEM) base (Sigma, St. Louis, MO, USA) which did not contain phenol red, sodium pyruvate, FCS and $NaHCO_3$. This was dissolved in bi-distilled water and supplemented with glucose (4.5 g/l), Hepes (4.76 g/l), NaCl (1.987 g/l) and L-glutamine (0.876 g/l), the pH was adjusted to 7.4 and the final medium was subjected to sterile filtration.

Plastic dishes (56.7 cm^2) and 24-well plates were from Nunc (Roskilde, Denmark) and the 12 and 6-well Transwell plates were from Costar, Corning (NY, USA).

Indinavir was kind gift of the University of Applied Sciences, (FHNW, Muttenz, Switzerland), and was synthesized by Sequoia Research Products (Oxford, UK). The base of saquinavir was a kind gift of F. Hoffmann-La Roche, Ltd. (Basel, Switzerland). Vinblastine sulfate was obtained from Sigma Chemicals (St. Louis, MO, USA). Thiazolyl blue for the MTT assay was purchased from Fluka (Buchs, Switzerland) and the LDH Cytotoxicity Detection Kit from Roche Diagnostics (Mannheim, Germany).

Phosphatidylcholine (PC) (>94%) and phosphatidylglycerol (PG) (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol ammonium salt) were purchased from Lipoid AG (Ludwigshafen, Germany).

2.2. Cell culture

Caco-2 cells were grown in petri dishes and maintained at 37 °C in an atmosphere of 8% CO_2 and in equilibrium with distilled water. Their doubling time under these conditions was about 20 h. They were passaged by treatment with 0.25% trypsin and 2.65 mM EDTA with a splitting ratio of 1:14. The medium was changed every alternate day until the petri dishes reached 90% confluence. Cells were used in the experiments at passage number 60–65.

2.3. Preparation of liposomes

Liposomes for the experiments with indinavir were prepared by adding PC to transport media and homogenizing the dispersion with a Polytron mixer (Kinematica AG, Littau, Switzerland) for 5 min at 15,000 rpm. Subsequently the dispersions were extruded at room temperature through polycarbonate filters (Nucleopore® Track-Etch Membrane, Sterico, Wangen, Switzerland) with pore size of 0.4 μm (3 extrusions), 0.2 μm (5 extrusions) and 0.1 μm (20 extrusions) by applying a pressure of 10 bar. The

liposome dispersions finally underwent sterile filtration (0.2 μm sterile filter, Minisart® Sartorius AG, Goettingen, Germany). The phospholipid concentration of the produced vesicle dispersions was 150 mg/ml.

Liposome dispersions with a PC of 50 mg/ml were loaded with indinavir at a pre-defined drug-to-lipid weight ratio (see Section 4 below). A 200 mg/ml solution of the drug was first prepared in DMSO which also contained 20 mg/ml PG. This solution was slowly added to the liposomes under vigorous agitation with a vortex mixer whereas the indinavir partitions spontaneously into the liposomal membrane. The final liposome formulation contained no more than 0.25% DMSO.

To prepare saquinavir-loaded liposomes, the film method was used. PC, saquinavir and PG were dissolved in ethanol 96%. The PC concentration was 100 mg/ml and those of saquinavir and PG varied depending on the desired final drug-to-lipid weight ratio (see below). Ethanol was removed in a rotary evaporator at 40 °C and an initial pressure of 800 mbar that was gradually reduced to 20 mbar. The film was dispersed in transport medium by agitation with a vortex mixer and mild heating to a final PC concentration of 50 mg/ml. This dispersion was extruded and subjected to sterile filtration as described above.

The size of the liposomes was determined with a Zetasizer 1000 HSA photon correlation spectrometer (Malvern Instruments Ltd, Malvern, UK). Prior to the measurements, vesicle dispersions were diluted in transport media until counting rates between 100 and 200 KCts/s were reached. Measurements were performed at room temperature. Vesicle size was typically between 130 and 150 nm with a polydispersity index of around 0.1. The addition of the drugs had no measurable effect on the size of the liposomes.

2.4. MTT-assay

Mitochondrial activity of the cell was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. This assay is based on the reduction of MTT by hydrogenase activity in functionally intact mitochondria. The hydrogenase catalyses the conversion of the yellow MTT reagent to blue formazan crystals. Caco-2 cells were seeded on 24-well plates or 12-well Transwell plates at a density of 114,000 cells/cm^2 and allowed to attach for 7–9 days. Medium was changed every other day. Liposome formulations were added to the cells using 4 wells and 3 wells per group for the 24-well plate and the Transwell plate, respectively, and were incubated for 2.5 and 5 h. The liposomes were removed, the cells washed twice with PBS and MTT solution at a concentration of 0.45 mg/ml in culture medium that was prepared from a stock solution of MTT in PBS was added to the cells and incubated for 4 h. Subsequently, lysis buffer containing 10% SDS in 0.01 M HCl was added to solubilize the cells and the formed formazan crystals. The mixture was kept overnight in humidified atmosphere at 37 °C and the amount of formazan was quantified in quadruplicate by transferring to a 96-well plate and measuring the absorbance at 580 nm (reference wavelength 650 nm) using an ELISA plate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Mitochondrial activity was expressed relative to a control group treated with transport media.

2.5. LDH-assay

Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the medium supernatant of the cells was used as a marker for cell membrane integrity. LDH activity was assayed in the supernatants by a reaction in which the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride is reduced to a red formazan salt. Cells were grown as for the MTT-assay. Liposome formulations were added to the cells using 3 wells per group and were incubated for 5 h at 37 °C. After incubation the supernatant of each well was diluted 1:40 with transport media, transferred into a 96-well plate and LDH activity of the diluted medium was determined using the Cytotoxicity Detection Kit according to the manufacturer's instructions. The absorbance of the red formazan was measured at 492 nm (reference wavelength 650 nm) with a plate reader. Absorption values were in the linear range.

Cultures exposed to 1% Triton X-100 were used as high control (100% lysis) and cultures exposed to transport media as low control. Transport medium was also used as background. Cytotoxicity was expressed as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{sample value} - \text{low control}}{\text{high control} - \text{low control}} \times 100 \qquad (1)$$

A potential interaction of the liposomes with the assay was tested using pure LDH (hog muscle, Roche Diagnostics). A LDH solution diluted to 0.05 U/ml with transport media was mixed with liposome formulations that were diluted to concentrations corresponding to the assay with the cells and LDH activity was determined as above. Transport media instead of liposomes were used as a control.

2.6. TEER

The transepithelial electrical resistance (TEER) of cultured cells on Transwell inserts was monitored before and after each permeation experiment with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Before the experiment, Caco-2 monolayers were washed with Dulbecco's PBS (containing Ca^{2+} and Mg^{2+}) and incubated with transport media for 1 h at 37 °C before the TEER measurement. Physiologically and morphologically well-developed confluent Caco-2 monolayers (at least 19 days old) with TEER values typically between 500 and 650 Ω cm^2 were used in the experiments.

2.7. Drug permeation across Caco-2 monolayers

Liposomal formulations with an indinavir concentration of 500 μg/ml and a saquinavir concentration of 7.5 and 2.2 mg/ml were used in the permeation studies. The concentration of PC was always 50 mg/ml and the concentration of PG was in each case one-tenth of the corresponding drug concentration. These formulations were stable under the conditions of the experiment while at higher drug loadings precipitation of the drug was observed. In addition, purely aqueous solutions of the drugs in transport media were used for permeation studies. Concentrations of these solutions were 50 and 10 μg/ml for indinavir and 10 and 3 μg/ml for saquinavir. These solutions were prepared by adding stock solutions of the drugs in DMSO to the transport media under stirring. The final DMSO concentration in these solutions was 0.25% for indinavir and 1% for saquinavir. The respective high concentration of each drug approached its solubility in the media.

Cells were seeded at a density of 114,000 cells/cm^2 onto 6-well Transwell® plates with an insert area of 4.7 cm^2 and a pore size of the polycarbonate membrane of 0.4 μm. Culture medium was changed every other day and cell monolayers were used between 19 and 21 days post seeding.

The drug solution/formulation was added either in the apical or in the basal compartment with respect to the cell monolayer. Three Transwells were used in each group. When liposomal drug formulations were used, the respective receiver compartment contained a blank liposomal dispersion in transport media with the same phospholipid and DMSO concentration. For the non-liposomal drug solutions the receiver compartment contained transport medium with the same DMSO concentration as the drug solution. In one experiment, the efflux inhibitor vinblastine was added at a concentration of 70 μM to the solution in both compartments.

Permeation of drug across the cell monolayer was monitored by sampling the solutions in both compartments at predefined time points for a duration of 5 h. Sampling volume was 100 μl for the non-liposomol drug solutions and 25 or 50 μl for the liposomal formulations. The withdrawn volume was not replaced. The starting volume of the apical solution was 1.6 ml and of the basal solution 2.8 ml. During the permeation experiment the Transwell plates were kept at 37 °C in a vapour saturated atmosphere and agitated on an orbital shaker at 50 min^{-1} (Edmund Bühler, Hechingen, Germany).

2.8. HPLC

Drug concentration in the samples was determined by HPLC-UV-MS (Agilent series 1100) with an isocratic pump G1310A, an autosampler G1313A, a variable wave length detector G1314A, and a mass spectrometer detector G1946C. The MS detector employed atmospheric pressure electrospray ionization and was operated in the scan mode at positive polarity with capillary voltage 4000 V, fragmentor 140 V, drying gas flow 10 l/min, drying gas temperature 350 °C and nebulizer pressure 30 psig. A C-18 reversed phase column (CC125/2 lispher sel.B 5 μm, Macherey-Nagel, Switzerland) and the following mobile phases were used: For indinavir MeOH/THF/20 mM CH_3COONH_4–1 mM CH_3COONa (aq.) = 40/10/50 and for saquinavir MeOH/THF/20 mM CH_3COONH_4 (aq.) = 50/10/40. The flow rate was 0.25 ml/min. Indinavir was detected at 214 nm in UV and at m/z 615 and 637 in MS, corresponding to the protonated form and the sodium adduct, respectively, and saquinavir at 238 nm in UV and at m/z 671, 693 and 709 in MS, corresponding to the protonated form, the sodium and the potassium adduct, respectively. Vinblastine was detected at 214 nm in UV concurrently with indinavir at longer retention times. Purely aqueous samples were injected undiluted and samples containing phospholipids were diluted at ratios between 1:10 and 1:40 with transport medium prior to injection. Quantification was performed against a set of external standard solutions within the linear response concentration range.

2.9. Theoretical modeling

A kinetic model describing cell permeation was developed in order first, to delineate the contribution of passive diffusion and active efflux on the overall cell permeation of the drug and second, to evaluate the effect of the liposomes on permeation in terms of these two contributing factors. This model was based on the following assumptions:

1. Three different compartments are considered in which drug concentration varies with time, the apical, the cellular and the basal compartment.
2. Drug may move between the apical and the cellular and the cellular and the basal compartment in both directions by passive diffusion. Permeation through the apical and the basal cell membrane is symmetrical and is characterized in both cases by the permeability coefficient, P. No effect of electrical membrane potential on the transport is considered.
3. Drug is subject to carrier mediated active efflux from the cellular to the apical compartment. This follows saturable kinetics that may be characterized by two macroscopic kinetic parameters, v_{max}, the maximum transport rate and K, the mass in the cellular compartment for which one-half of the maximum rate is attained, often referred to as affinity or dissociation constant. One global set of parameters is used to describe carrier mediated efflux.
4. No two different orientations or conformations of the carrier at the two faces of the membrane are explicitly involved, the drug concentration in the apical compartment does not influence efflux transport and the entire mass of drug present in the cellular compartment is substrate of the transporter.

5. The total mass of drug in the three compartments is preserved.

According to this model, the following differential equations describe the change of drug concentration or mass as a function of time in the three compartments during permeation in both directions.

$$\frac{dC_{Aab}}{dt} = -P(C_{Aab} - C_{Cab})\frac{S_m}{V_A} + \frac{v_{max} m_{Cab}}{K + m_{Cab}}\frac{1}{V_A} \quad (2)$$

$$\frac{dC_{Bab}}{dt} = P(C_{Cab} - C_{Bab})\frac{S_m}{V_B} \quad (3)$$

$$\frac{dm_{Cab}}{dt} = P(C_{Aab} - C_{Cab})S_m - \frac{v_{max} m_{Cab}}{K + m_{Cab}} - P(C_{Cab} - C_{Bab})S_m \quad (4)$$

$$\frac{dC_{Aba}}{dt} = P(C_{Cba} - C_{Aba})\frac{S_m}{V_A} + \frac{v_{max} m_{Cba}}{K + m_{Cba}}\frac{1}{V_A} \quad (5)$$

$$\frac{dC_{Bba}}{dt} = -P(C_{Bba} - C_{Cba})\frac{S_m}{V_B} \quad (6)$$

$$\frac{dm_{Cba}}{dt} = P(C_{Bba} - C_{Cba})S_m - \frac{v_{max} m_{Cba}}{K + m_{Cba}} - P(C_{Cba} - C_{Aba})S_m \quad (7)$$

where C is concentration, m is mass, the indices A, B, and C (upper case) denote the apical, basal and cellular compartment, respectively, the indices ab and ba (lower case) denote permeation in the apical-to-basal and the basal-to-apical direction, respectively, S_m is the surface area of the monolayer and V is volume of solution in each compartment. A cell monolayer volume of 0.0094 ml was used based on a monolayer thickness of 20 μm.

For high drug concentrations and accordingly large drug mass in the cellular compartment, the validity of the following approximation was tested.

$$\frac{v_{max} m_C}{K + m_C} \approx v_{max} \quad (8)$$

The reduction of the volume of solution in the apical and the basal compartment due to sampling as a function of time was accounted for by the following relations that were determined empirically using regression analysis.

$$V = V(0) - 0.0037189t + 3.68 \times 10^{-6} t^2 + 6.87 \times 10^{-9} t^3 \quad (9)$$

$$V = V(0) - 0.0018594t + 1.84 \times 10^{-6} t^2 + 3.43 \times 10^{-9} t^3 \quad (10)$$

$$V = V(0) - 0.0009297t + 9.2 \times 10^{-7} t^2 + 1.72 \times 10^{-9} t^3 \quad (11)$$

where $V(0)$ is volume at time zero. Eqs. (9)–(11) apply to both compartments and to sampling volumes of 100, 50 and 25 μl, respectively.

3. Results

3.1. Cell toxicity of liposomes

The results of the MTT assay are shown in Figs. 1, 2. For a 2.5 h incubation with liposomal formulations, mitochondrial activity of the cells was reduced to values between 88% and 97% of the control. This reduction was statistically significant (Student's t-test) at $p < 0.05$ for 100 mg/ml phospholipid and at $0.05 < p < 0.1$ for 50 mg/ml phospholipid with 3.75 mg/ml saquinavir. For the 5 h incubation in the 24-well plates, no statistically

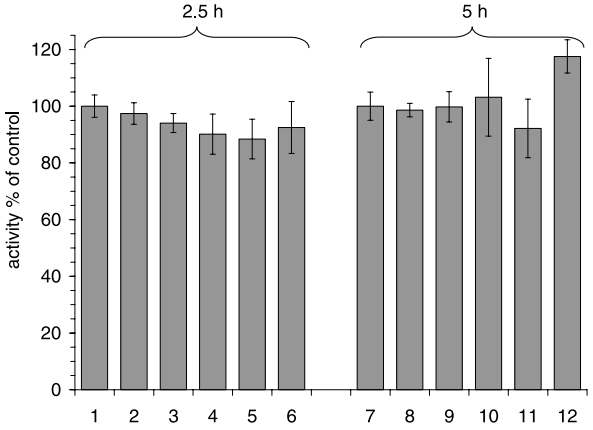

Fig. 1. Mitochondrial dehydrogenase activity of the cells determined by the MTT assay following 2.5 and 5 h incubation with different liposomal formulations in 24-well plates. Results are expressed in percent of control obtained with transport medium and given as mean and standard deviation with $n = 4$. Columns: 1 and 7, transport medium. 2 and 8, phospholipid 150 mg/ml. 3 and 9, phospholipid 100 mg/ml. 4 and 10, phospholipid 50 mg/ml. 5 and 11, phospholipid 50 mg/ml with 3.75 mg/ml saquinavir. 6 and 12, phospholipid 50 mg/ml with 7.5 mg/ml saquinavir.

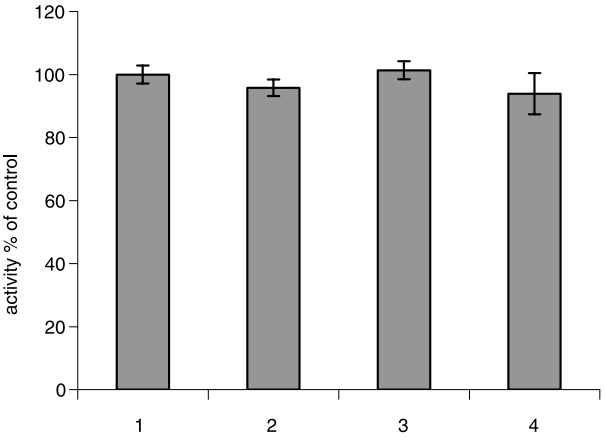

Fig. 2. Mitochondrial dehydrogenase activity of the cells determined by the MTT assay following 5 h incubation with liposomes added either to the apical (column 2) or the basal (column 3) or both compartments (column 4) of Transwell plates. Phospholipid concentration 150 mg/ml. Results are expressed in percent of control obtained with transport medium (column 1) and given as means and standard deviation with $n = 3$.

significant reduction of activity was found except for the 50 mg/ml phospholipid with 7.5 mg/ml saquinavir formulation for which an increase of activity was detected ($p < 0.01$). No positive correlation between phospholipid concentration and reduction of mitochondrial activity in the studied phospholipid concentration range was observed. Also, no effect of the presence of saquinavir was found in this respect. In the Transwell plates, addition of liposomes only to the apical or the basal or to both compartments about the cell monolayer reduced mitochondrial activity to 94% at the most, this reduction of 6% not being statistically significant.

In the LDH assay, no marked increase of lactate dehydrogenase activity was measured in the culture supernatant of the cells after incubation with liposomes with a phospholipid concentration of 50 and 150 mg/ml for 5 h. Cytotoxicity values remained consistently below 1% of the values measured with Triton X-100 as positive control Eq. (1). A maximum value of 0.81% was measured for a phospholipid concentration of 50 mg/ml. The phospholipids did not suppress the LDH enzymatic activity that this assay relies upon and did not negatively interfere with the assay as determined using pure LDH. On the contrary, a roughly 20% increase of activity of the pure LDH in the presence of liposomes was measured as compared to transport medium. A comparable increase was also found in the presence of 1% Triton.

3.2. Cell permeation of drugs from aqueous solutions and liposomal formulations

Measurement of drug permeation in the Caco-2 model was possible with purely aqueous solutions at the employed drug concentrations. Permeation of indinavir through the Caco-2 monolayer in the apical-to-basal direction was rather limited. In the basal-to-apical direction, extensive permeation took place against the concentration gradient after approximately 90 min of the experiment (Fig. 3). For saquinavir, apical-to-basal permeation was even more limited than for indinavir and the basal-to-apical permeation was more extensive, such that the apical concentration exceeded the basal concentration already after approximately 50 min of the experiment in the latter direction of permeation (Fig. 4). The presence of vinblastine in the apical and the basal compartment increased apical-to-basal permeation of indinavir and diminished the extent of its basal-to-apical permeation although the latter remained more pronounced than the former (Fig. 5). The asymmetric transport of both drugs across the Caco-2 cells and the partial reversal of this asymmetry by vinblastine, a known inhibitor of P-glycoprotein and MRP, confirm that the drugs are subject to active apical efflux by these transporters that are present in these cells [14,15].

Indinavir and saquinavir were used in permeation studies as liposomal dispersions at concentrations 10- to 2500-fold higher than those in the purely aqueous solutions. Typical results are shown in Figs. 6 and 7 for indinavir

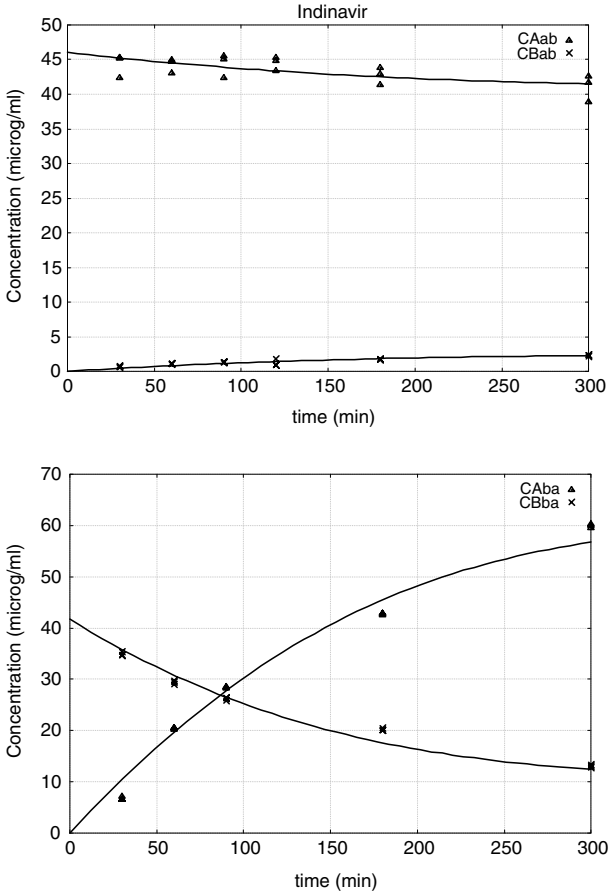

Fig. 3. Permeation of indinavir in Caco-2 cells from purely aqueous solution; experimental points and model-based fitted curves. Upper panel, apical-to-basal direction; lower panel, basal-to-apical direction. (▲) Apical concentration. (×) Basal concentration.

and saquinavir, respectively. In the apical-to-basal direction, percentage of permeation was for both drugs smaller than that observed when the drugs were applied in purely aqueous solution. Basal-to-apical permeation of indinavir was greater than in the opposite direction but it was smaller in relative terms compared to when the drug was applied with no liposomes. Basal-to-apical permeation of saquinavir was diminished by the liposomes to a larger extent than that of indinavir compared to the purely aqueous solution. Thus, formulation of the drugs in a liposomal dispersion appears to profoundly influence their permeation behavior through the Caco-2 monolayer.

3.3. Quantification of passive and carrier mediated transport parameters

Permeation data were analyzed using the theoretical model for the kinetics of cellular transport presented in Section 2. The concentration variables defined by the system of differential Eqs. (2)–(7) were fitted to the experimental concentration data and optimal values of the parameters P, v_{max} and K applicable to the different experimental conditions were deduced. Numerical solution of

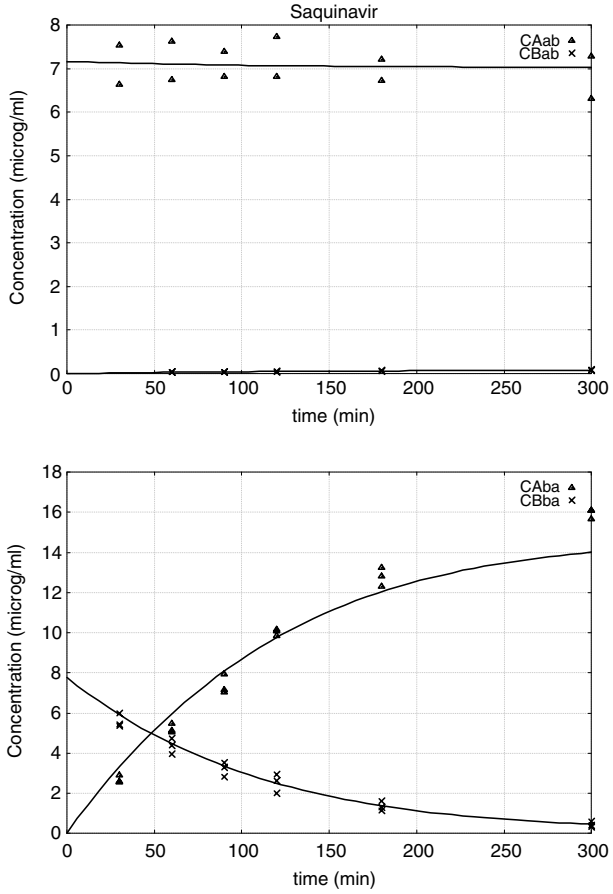

Fig. 4. Permeation of saquinavir in Caco-2 cells from purely aqueous solution; experimental points and model-based fitted curves. Upper panel, apical-to-basal direction; lower panel, basal-to-apical direction. (▲) Apical concentration. (×) Basal concentration.

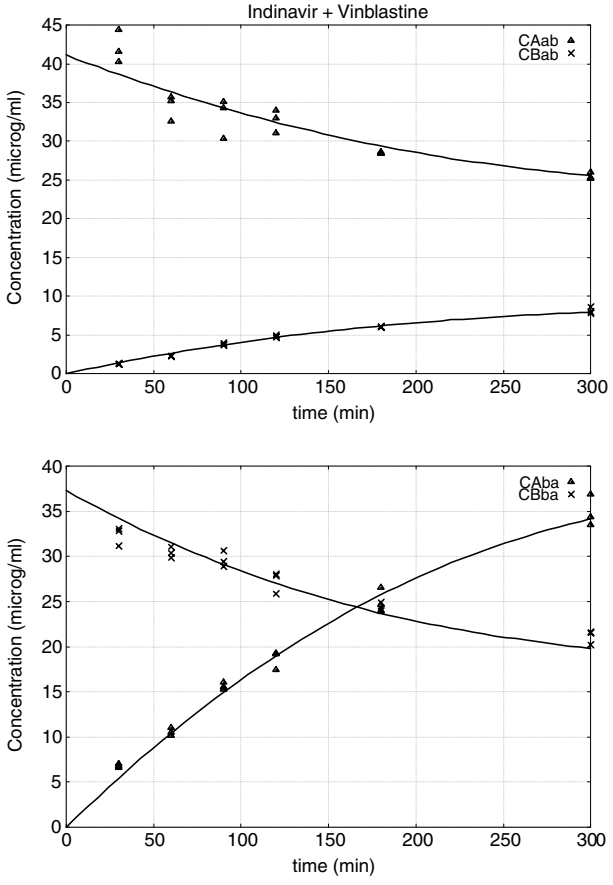

Fig. 5. Permeation of indinavir in Caco-2 cells from purely aqueous solution in the presence of 70 μM vinblastine; experimental points and model-based fitted curves. Upper panel, apical-to-basal direction; lower panel, basal-to-apical direction. (▲) Apical concentration. (×) Basal concentration.

the equations and least squares fit were performed using the software EASY-FIT (Prof. K. Schittkowski, University of Bayreuth, Germany). Concentration data of both compartments obtained from the apical-to-basal and the basal-to-apical direction of permeation were used simultaneously in the fitting. This provided a more stable regression analysis compared to the separate evaluation of each permeation direction. The drawn lines in Figs. 3–7 represent the best fit. This is considered in all cases to be satisfactory.

The estimated transport parameters are given in Table 1. Consistent values of the passive permeability coefficient were always obtained. In the instances typically involving the purely aqueous solutions of the drugs, a high level correlation ($r \approx 1$) between v_{max} and K occurred in the regression analysis (correlation matrices not shown) which meant that these parameters could not be deduced individually from each of these experiments. For indinavir-liposomes, the best fit was obtained when Eq. (8) was substituted into Eqs. (2)–(7) i.e., for a constant transporter mediated efflux rate independent of drug mass in the cellular compartment. This was assumed to represent v_{max}. For the saquinavir-liposomes with high drug concentration, the same value of v_{max} as for the indinavir-liposomes resulted using Eqs. (2)–(7) and the deduced K value had a rather high standard error indicating that this parameter did not significantly influence the goodness of fit. These results of the indinavir-liposomes and the saquinavir-liposomes, hence, were in mutual agreement and suggest that the carrier mediated efflux took place at a constant rate corresponding to a saturation of the carrier probably because of the high drug concentration of these formulations. Therefore, in all experiments involving no liposomes, the value of v_{max} deduced from the liposome formulations was introduced in the calculation and kept constant. This allowed the parameter K to be estimated also in these situations where otherwise a correlation between v_{max} and K occurred. Only in the studies with saquinavir liposomes and low drug concentration in which the experimental data were close to the detection limit and thus rather variable, a reliable estimation of K or v_{max} was not possible. The deduced parameter values given in Table 1 were overall consistent as evidenced by the small standard error and the absence of correlation between them.

The drug concentrations at time zero, $C(0)_A$ and $C(0)_B$, obtained from the best fit were smaller than the theoretical

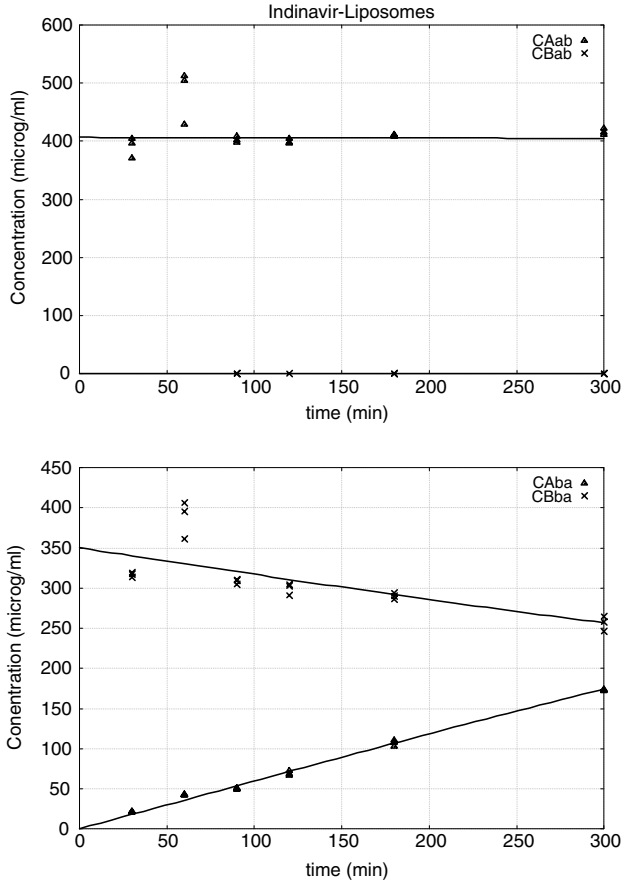

Fig. 6. Permeation of indinavir in Caco-2 cells from a liposomal formulation with 50 mg/ml lipid; experimental points and model-based fitted curves. Upper panel, apical-to-basal direction; lower panel, basal-to-apical direction. (▲) Apical concentration. (×) Basal concentration.

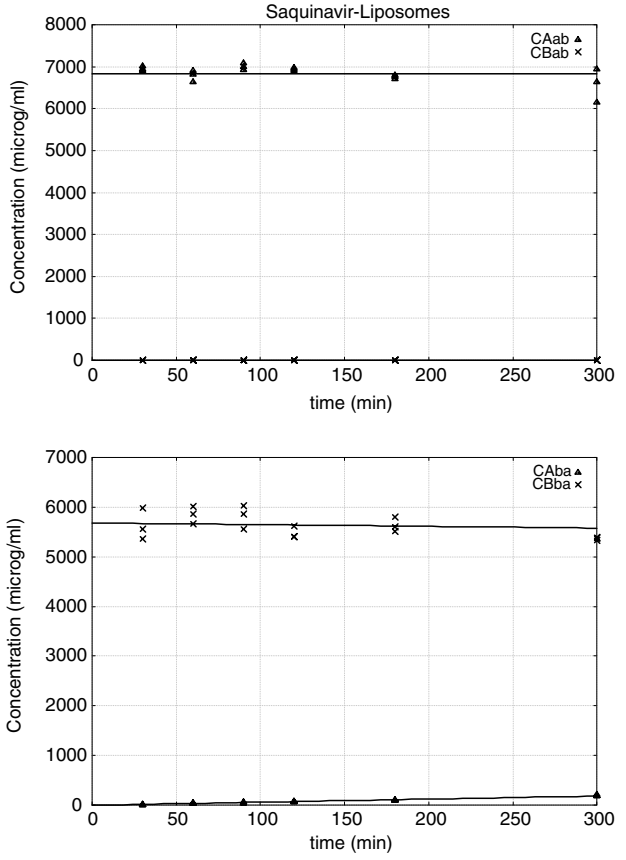

Fig. 7. Permeation of saquinavir in Caco-2 cells from a liposomal formulation with 50 mg/ml lipid; experimental points and model-based fitted curves. Upper panel, apical-to-basal direction; lower panel, basal-to-apical direction. (▲) Apical concentration. (×) Basal concentration.

concentrations of the prepared aqueous solutions and the liposomal formulations (given in Section 2). This was because of some loss of drug firstly during preparation, which was confirmed by measuring the drug concentration of the ready-to-use preparations, and secondly at the onset of the transport experiment possibly because of an initial adsorption of the drug to the plastic container walls of the Transwell plates. Time dependent mass balance demonstrated that total drug mass was constant after the first sampling point of 30 min. Therefore, the estimation of kinetic parameters was not affected by this initial decrease of drug content of the preparations. No relevant metabolism of the drugs was evident in the Caco-2 cells. For saquinavir, which is metabolized predominantly by hydroxylation [28], a signal at m/z 687 corresponding to the hydroxylate was detectable at early retention times that amounted however to less than 2% of the parent compound. Fragments of the parent compound in the MS with very small abundances were detected at m/z 570, 433, and 242. Indinavir reportedly is less metabolized than saquinavir [28].

Passive membrane permeability and carrier mediated transport expressed by P and v_{max} and K, respectively, were thus separately assessed and were found to depend on the drug in use and on whether it was formulated as a liposomal dispersion. Drug mass in the cellular compartment obtained from model simulation was comparable for both permeation directions and differed greatly between the groups as discussed below. The calculated mass values were of the same order of magnitude as experimental values found by extracting the cell monolayer. The deduced kinetic parameters of apical efflux are global in the sense that they do not differentiate between different transporters that may be involved. This is sufficient for the delineation of the contributions of passive and carrier mediated transport processes which was the objective of the present work. Distinguishing between parameters for individual transporters in the employed Caco-2 experimental model entails the availability and use of high specificity, high affinity inhibitors for the transporters, a task that can currently not be resolved.

4. Discussion

4.1. Cell toxicity of liposomes

The possibility of adverse effects elicited by the liposome formulations on cultured Caco-2 cell monolayers was evaluated by determining the mitochondrial dehydrogenase

Table 1
Kinetic parameters of transport of drugs in the Caco-2 cell monolayer[a]

Composition	$C(0)_A$ (μg/ml)	$C(0)_B$ (μg/ml)	$P \times 10^5$ (cm/s)	v_{max} (μg/s)	K (μg)
Indinavir	45.9 (1.1)	41.5 (0.87)	5.19 (0.23)	0.0175[c]	0.022 (0.0031)
		8.47 (0.17)[b]	5.45 (0.22)	[d]	[d]
Indinavir + vinblastine	41.2 (0.45)	37.4 (0.39)	4.1 (0.078)	0.0175[c]	0.489 (0.025)
Saquinavir	7.17 (0.2)	7.79 (0.17)	8.97 (0.34)	0.0175[c]	0.0034 (0.00025)
	2.24 (0.05)	2.08 (0.035)	8.83 (0.29)	0.0175[c]	0.012 (0.00066)
Indinavir-liposomes	405 (19.4)	351 (13.8)	0.933 (0.057)	0.0175 (0.00098)	
Saquinavir-liposomes	6839 (8.38)	5684 (5.43)	0.0587 (0.0016)	0.0175 (0.00098)	0.068 (0.098)
	1933 (143)	1587 (118)	0.041 (0.004)	[d]	[d]

[a] Estimated parameters from regression analysis; numbers in parenthesis are standard error of estimates. Transport experiments in apical-to-basal and basal-to-apical direction except where otherwise noted.
[b] Experiment only in the basal-to-apical direction.
[c] Set value (not adjustable).
[d] No estimation possible due to data variability and correlation between parameters.

activity and the plasma membrane integrity of the cells. The former was assessed using the MTT assay and the latter using the LDH assay. These are both established methods addressing different aspects of cell functionality and are widely used as general criteria of cytotoxicity. While the MTT assay detects specifically mitochondrial metabolic activity and ability of the cells for proliferation, the LDH assay detects leakage of cytosolic lactate dehydrogenase out of the cells indicating cell membrane dysfunction. The employed liposome formulations had overall no significant cytotoxic effect on the cells and caused no perturbation of the cell membrane. The latter is especially relevant in the present study which is dealing with drug permeation across the cells. The statistically significant effects observed in a few instances in the MTT assay in the 24-well plates were not consistent between the two incubation times or within the series of applied phospholipid concentrations and were not confirmed in the Transwell plates. Hence, they do not appear to be systematic. The test in the Transwell plates corresponds exactly to the experimental setup used in the permeation studies.

The results presented, therefore, provide strong indication that the liposomes composed of phosphatidylcholine and phosphatidylglycerol may be considered safe with respect to the employed assays for use in drug permeation studies with Caco-2 cell cultures up to concentrations of 150 mg phospholipid per ml. This finding is even more striking in view of the very small molar ratio of cell membrane lipid to liposome lipid used here that was of the order of 1:100,000. This ratio can be derived from the assumption that the 4.7 cm^2 surface area is a monolayer of phospholipids, a surface area per phospholipid molecule of 67 $Å^2$ [27], the concentration of employed liposomal phospholipid of 50 mg/ml and the apical donor compartment volume of 1.6 ml. Reports about interaction of the MTT assay with high concentrations of some P-glycoprotein and MRP substrates leading to false cell viability values exceeding 100% [29] were not confirmed in this study for the phospholipids. A masking of cytotoxicity of the phospholipids by such an interaction is further refuted by the result of the LDH assay.

No toxicity data of liposomes with the present phospholipid composition and concentration for Caco-2 cells were previously available. Reports on the intravenous in vivo toxicity of liposomes with different compositions including those with cationic lipids used for cell transfection are not relevant in the context of the present study. As a consequence of the safe use of high phospholipid concentrations in the Caco-2 experimental setting, the concentration of lipophilic drugs may, dependent on their maximal solubility in the liposomal membrane, be manifold higher than when the drugs are solubilized with detergents or solvents [7,9]. This in turn may facilitate the performance of permeation experiments.

4.2. Cell permeation kinetics

The model-based evaluation of the experimental results of this study shows that the developed model for describing the kinetics of permeation in the Caco-2 cell monolayer makes it possible to distinguish in a quantitative fashion between the contribution of passive permeation and carrier mediated efflux to drug transport in this system.

The passive permeability coefficient of saquinavir was greater than that of indinavir in the purely aqueous drug solutions (Table 1), this trend being consistent with the lipophilicity of the compounds in terms of octanol/water partition coefficient that was higher for the former ($\log K_{o/w} = 4.1$) compared to the latter ($\log K_{o/w} = 2.9$) [14]. In this respect it should be realized that both compounds existed mostly in the unprotonated form at the physiological pH of 7.4 (pK_a 6.9 and 5.7, respectively). This result is also in agreement with the greater solubility in the liposomal membrane found for saquinavir, i.e., 1/6.7 drug to lipid mass ratio, compared to indinavir, i.e., 1/100 drug to lipid mass ratio. The passive permeability coefficient of both indinavir and saquinavir did not depend on the employed concentration of either drug. This is in agreement with diffusion theory and validates the model.

Apical efflux for the purely aqueous drug solutions was stronger for saquinavir than for indinavir as evidenced by

the smaller K value of the former compared to the latter. From this, a higher affinity of saquinavir for the efflux transporters P-glycoprotein and/or MRP may be inferred. This reflects the more limited apical-to basal permeation and the more extensive basal-to-apical permeation observed for saquinavir compared to indinavir (Figs. 3, 4). The drug mass in the cellular compartment yielded by the model-based simulation was 0.02–0.07 μg and 0.0008 to 0.0018 μg for indinavir and saquinavir, respectively. The smaller cellular accumulation of saquinavir compared to indinavir is in line with the stronger apical efflux of the former compound. Hence, the different propensity of the two drugs to be apically effluxed can be quantitatively assessed with the present analysis. The mass of both drugs in the cellular compartment was not negligible compared to the respective values of the parameter K indicating that in the used concentration range saturable efflux kinetics applied. In this analysis, the value of v_{max} obtained from the liposome formulations of the drugs was used (see Section 3). As discussed below, the presence of phospholipids was not expected to alter the v_{max} of the drugs. The value of v_{max} was assumed to be the same for both drugs because equal values of this parameter were determined from the fitting of the data of the liposome formulations. This assumption is supported by the fact that these drugs are known to be both substrates of the same efflux transporters [14,15].

The presence of vinblastine in the media caused an increase of K of indinavir by more than 10-fold (Table 1). In these experiments, vinblastine was added at equal concentrations to both the apical and the basal solution. In the course of the experiment, vinblastine concentration increased in the apical and decreased in the basal solution (Fig. 8), evidencing transport against a concentration gradient which is consistent with active apical efflux. This demonstrates that vinblastine itself is a substrate of efflux transporters. Therefore, the established inhibitory action of this compound on P-glycoprotein and MRP may be assumed to take place by a competitive mechanism whereas the extent of inhibition likely depends on the comparative affinity of the compounds in question for the carrier. A competitive inhibition of indinavir efflux by vinblastine is perfectly consistent with the deduced increase of the value of the K parameter of indinavir. The value of v_{max} was assumed not to be influenced by the presence of vinblastine in line with a mechanism of competitive inhibition. The model-simulated drug mass in the cells was increased to 0.1–0.15 μg (compared to indinavir being used alone, see above) suggesting that inhibition of apical efflux resulted in an accumulation of the drug in the cells.

The presence of indinavir in the apical or the basal compartment did not seem to influence the magnitude of apical efflux of vinblastine (Fig. 8). This result in combination with the finding that the cellular content of indinavir is roughly comparable for the two directions of permeation of the drug is supportive of the inherent assumption of the presented model that performance of apical efflux does not depend on the side of the cell membrane on which the compound is originally added. The presence of vinblastine caused a rather small reduction of the passive permeability of indinavir. This result could not be anticipated on theoretical grounds and, being marginal in magnitude, cannot be further discussed unless rigorously reproduced.

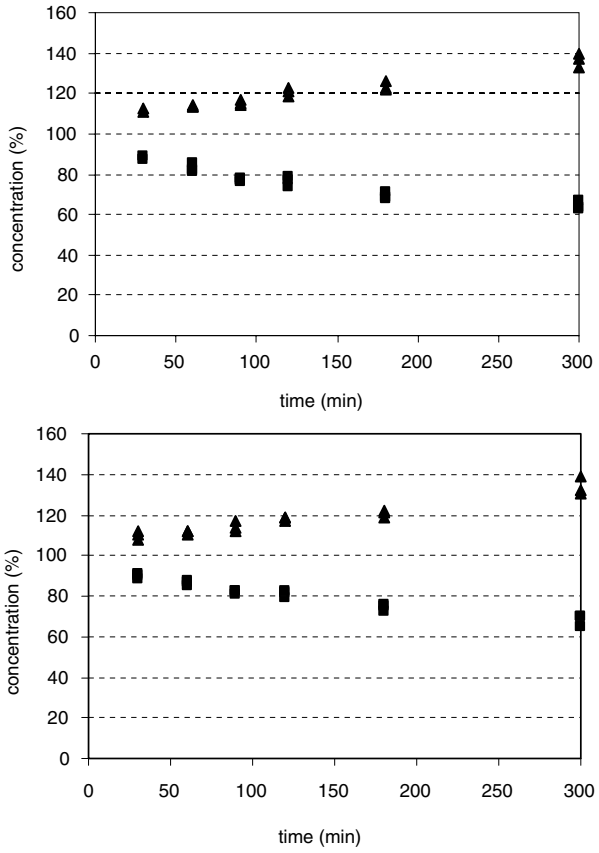

Fig. 8. Transport of vinblastine in Caco-2 cells in the presence of 50 μg/ml indinavir. Upper panel, indinavir added to the apical solution; lower panel, indinavir added to the basal solution Concentration expressed in % of the concentration at time zero. (▲) Apical concentration. (■) Basal concentration.

Concluding this section it is pointed out that the present theoretical analysis provides congruent results for the passive membrane permeability and the apical efflux transport of the used drugs and of the effect of the efflux inhibitor on these parameters; therefore, this analysis is proposed as a tool for independently determining the contribution of the individual transport processes to the over-all cellular permeation.

4.3. Effect of liposomes

The drug concentration of the liposomal formulations was much greater than the concentration used in the purely aqueous solutions that approached solubility level, demonstrating that liposomes can be effectively used as vehicles for solubilizing these drugs in water. A maximal drug-to-lipid mass ratio of 1/100 for indinavir and 1/6.7 for saquinavir provided stable liposomal formulations

for the duration of the experiment. Photon correlation spectroscopy measurements showed no change of particle size during the incubation of these liposomes in the assay medium at 37 °C for at least 8 hours. Higher ratios gave rise to crystallization/precipitation of the drugs. Evidently, saquinavir is more effectively solubilized by liposomes than indinavir. These results imply that liposomal solubilization may facilitate the handling of drugs with an aqueous solubility <1 ppm in in vitro experiments.

The localization of drug inside the liposome, although not studied in detail, may be indirectly proven by the increase of the apparent solubility of the compounds in the aqueous liposomal dispersion compared to their purely aqueous solubility. Since under these conditions, the liposomes are the only lipid reservoir for the drugs preventing their precipitation, it can be assumed that the lipophilic drugs (as indicated by their $\log K_{o/w}$ values of 4.1 and 2.9 for saquinavir and indinavir, respectively) are associated with the lipophilic domain of the liposomal membrane.

Passive permeability coefficients obtained with the liposomal formulations of indinavir and saquinavir were considerably diminished compared to the ones for the purely aqueous solutions (Table 1). This result may be interpreted in terms of a reduced availability of the drug for passive permeation through the cell membrane because of its incorporation in the liposomes that would be related to the large excess of liposomal lipid versus cell membrane lipid (molar ratio of the order of 100,000:1, see above). Mechanistically, the permeation rate of the drug will depend on the mode of interaction of the liposomes with the cells. This may occur in various ways [32]: (1) collision and possibly adsorption of the liposome and extracellular release of its contents and subsequent transport of these contents into the cells; (2) collision and possibly adsorption of the liposome followed by selective transfer of lipophilic compounds from the liposomal bilayer directly to the plasma membrane; (3) endocytotic internalization of the liposomes followed by intracellular release of its content; (4) fusion of the liposomal membrane with the plasma membrane or, intracellularly with the endosomal membrane and thereby release of the liposomal contents in the cytoplasm. For lipophilic compounds associated with the liposomes, the selective inter-membrane transfer described above may be a very important way of interaction. This transfer of the drug from the liposomal to the cell plasma membrane could be rate determining and depend on the comparative interaction of the drug with the lipid pool of the cell monolayer and that of the liposomes. Alternatively, the permeation of these lipophilic drugs may be defined by the equilibrium of the drug between liposomal lipid domain and water phase, whereas the concentration in the water phase is determining for permeation [22]. Both alternatives conceivably give rise to the diminished passive permeability coefficients. In order to understand the complex underlying mechanisms, detailed studies of passive cell membrane permeation of liposomally solubilized drugs involving the equilibria of solubilization are underway in this laboratory.

With respect to drug delivery, the product $C(0)_A \cdot P$ providing the initial passive flux of drug across the membrane is relevant. For this product, using the highest concentrations given in Table 1, one obtains 2.38×10^{-3} μg/cm^2/s for indinavir in purely aqueous solution as compared to 3.78×10^{-3} μg/cm^2/s for indinavir liposomes and 0.641×10^{-3} μg/cm^2/s for saquinavir in purely aqueous solution compared to 4.01×10^{-3} μg/cm^2/s for saquinavir liposomes. $C(0)_B \cdot P$ yields an analogous picture. Since the used concentrations are nearly the highest attainable in the respective formulations, the results of this calculation are comparable to each other in terms of absolute effectiveness of delivery. This calculation demonstrates that passive delivery of the drugs would be more efficient with the liposomes than the purely aqueous solution, this difference being far more evident for saquinavir than indinavir. The use of liposomes in Caco-2 cell cultures to solubilize a lipophilic immunomodulator drug, PSC 833, also demonstrated that, when encapsulated in liposomes the PSC 833 exhibited a stronger interaction with the cells than when the compound was dispersed in the media [31].

The findings of this study point to the possible pharmaceutical relevance of investigating the permeation in the Caco-2 cell model of formulated lipophilic drugs instead of unformulated drugs. Besides achieving high enough drug concentrations to allow detection in the in vitro test, formulations may change the rate of passive drug absorption. The relevance of this finding is underscored by the need of delivery systems to achieve sufficiently high blood levels of lipophilic drugs by peroral administration, and, perhaps more importantly, by the fact that most lipophilic drugs are solubilized in vivo in mixed micelles of phospholipids and bile salts. Thus, even if liposomes are altered after peroral administration, drug solubilization in colloidal dispersion systems may still take place in the intestine. The presence of micellar systems and phospholipids reportedly influences intestinal absorption of lipophilic drugs [26,30]. As a result of solubilization in lipids, the lipophilic drugs may be shifted in the biopharmaceutic classification system from a low to a high solubility class [23]. In agreement with the argumentation in reference [23], this study shows also that, because of formulation effects or possible in vivo relevant interaction with phospholipids, the drugs may be shifted to another class of the BCS with a higher solubility and another permeation rate. A system that perfectly simulates the intestinal juice remains, of course, difficult to ascertain. Liposome dispersions represent a safe, high capacity solubilization vehicle for studying the absorption of lipophilic drugs in the Caco-2 model.

The apical efflux rate of indinavir-liposomes and saquinavir-liposomes reached a constant value corresponding to v_{max} (Table 1). This value was the same for the liposome formulations of both drugs, this being consistent with the fact that these drugs have been shown to be substrates of the same efflux transporters [14,15]. The deduced value of $v_{max} = 0.0175$ μg/s or 3.72×10^{-3} μg/cm^2/s is strikingly similar to the initial passive influx of

drug in the cells for both liposomal formulations given by $C(0)_A \cdot P$ (see above). This confirms that with the liposomal formulations the maximum value of efflux rate was reached because of saturation of the transporters due to the high drug concentration of these formulations. This is also corroborated by the drug mass in the cellular compartment that was found by the model simulation to be larger for the indinavir-liposomes (0.025–0.15 μg) and especially the saquinavir-liposomes (0.5–3 μg) compared to the respective purely aqueous drug solutions.

Phospholipids have been previously reported to be substrates of P-glycoprotein and possibly inhibit P-glycoprotein transport of other compounds [24,25]. This inhibition was discussed to be of competitive mode. Based on this, no effect of the phospholipids on the v_{max} of drugs should be expected. An effect of phospholipids on the value of parameter K could not be assessed from the present data because it was not possible to deduce this parameter for the liposomal formulations of the drugs as a result of saturation of the transporters at the employed drug concentrations.

Liposomal formulations, therefore, appear to influence passive permeation and carrier mediated efflux rate of the two drugs. The effect of the liposomes on apical efflux appears to consist in eliciting a maximal efflux rate of the drugs corresponding to transporter saturation due to the high drug concentrations, while their effect on passive permeation seems to be related to the readiness of transfer of the drug from the liposomal to the cellular phospholipid bilayer membrane. Fig. 9 shows the experimental basal concentrations of the drugs formulated with and without liposomes permeating in the apical-to-basal direction. For saquinavir, the basal concentration with liposomes was much greater than those without liposomes while for indinavir the opposite is true, albeit far less pronounced. The much more effective transcellular delivery of saquinavir, in absolute terms, from the liposomal formulation is because of the overwhelmingly more effective passive delivery of this drug discussed above. For indinavir, on the other hand, the slight advantage of the liposomal formulation in terms of passive delivery is offset by the maximal efflux rate attained at saturation.

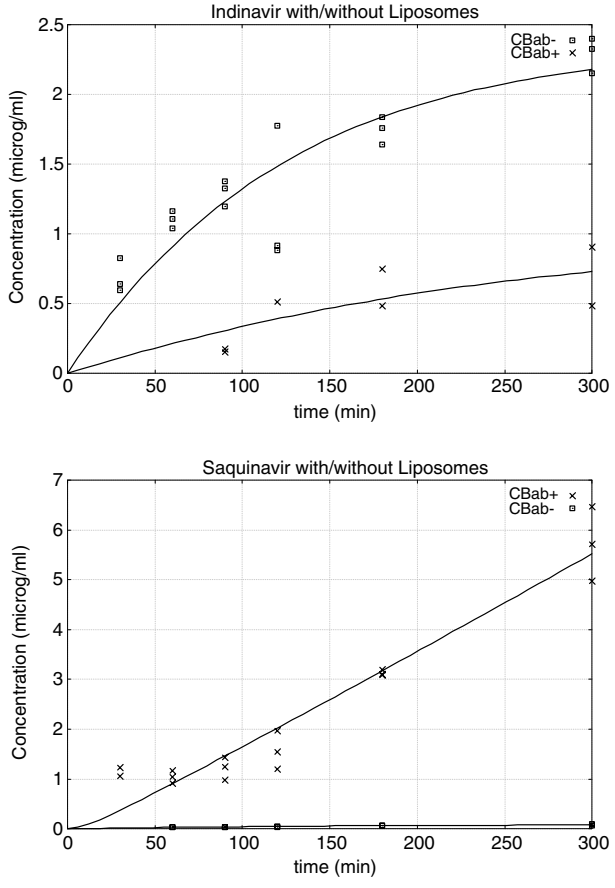

Fig. 9. Basal concentration of indinavir (upper panel) and saquinavir (lower panel) for apical-to-basal permeation in Caco-2 cells; experimental points and model-based fitted curves. (■) Purely aqueous drug solution. (×) Liposomal formulation with 50 mg/ml lipid.

In conclusion, whereas liposomes can be effectively used as solubilizers of these drugs increasing their concentration in solution many fold without exhibiting cell toxicity, their impact on drug delivery depends on the combination of their influence on passive and active cellular transport processes and may vary with the drug in use. Furthermore, purely aqueous solutions and formulations of lipophilic drugs may reveal different absorption behavior of the drugs pointing to the possible relevance of performing Caco-2 cell studies with formulated drugs. A quantitative analysis of the contributing factors is shown to be indispensable for a mechanistic understanding of the final outcome of absorption and can be accomplished using the methodology based on mathematical modeling proposed in this report.

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References

[1] G.L. Amidon, H. Lennernäs, V.P. Shah, J.R. Crison, A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, Pharm. Res. 12 (1995) 413–420.
[2] M.N. Martinez, G.L. Amidon, A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals, J. Clin. Pharmacol. 42 (2002) 620–643.
[3] M. Kansy, F. Senner, K. Gubernator, Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption process, J. Med. Chem. 41 (1998) 1007–1010.
[4] I.J. Hidalgo, T.J. Raub, R.T. Borchardt, Characterization of human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, Gastroenterology 96 (1989) 736–749.

[5] P. Artursson, K. Palm, K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport, Adv. Drug Deliv. Rev. 46 (2001) 27–43.
[6] P. Artursson, Cell cultures as models for drug absorption across the intestinal mucosa, Crit. Rev. Ther. Drug Carrier Syst. 8 (1991) 305–350.
[7] B.J. Aungst, N.H. Nguyen, J.P. Bulgarelli, K. Oates-Lenz, The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV inhibitors and other lipophilic compounds, Pharm. Res. 17 (2000) 1175–1180.
[8] R.B. Shah, A. Palamakula, M.A. Khan, Cytotoxicity evaluation of enzyme inhibitors and absorption enhancers in Caco-2 cells for oral delivery of salmon calcitonin, J. Pharm.Sci. 93 (2004) 1070–1078.
[9] F.M. Ingels, P.F. Augustijns, Biological, pharmaceutical and analytical considerations with respect to the transport media used in the absorption screening system Caco-2, J. Pharm. Sci. 92 (2003) 1545–1558.
[10] B.J. Aungst, Intestinal permeation enhancers, J. Pharm. Sci. 89 (2000) 429–442.
[11] M. Lindenberg, S. Kopp, J.B. Dressman, Classification of orally administered drugs on the World Health Organization model list of essential medicines according to the biopharmaceutics classification system, Eur. J. Pharm. Biopharm. 58 (2004) 265–278.
[12] P. van Hoogevest, B. de Kruijff, Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didocosenoyl-phosphatidylcholine. A refinement of the model for the formation of pores by amphotericin B in membranes, Biochim. Biophys. Acta 511 (1978) 397–407.
[13] A. Fahr, M. Holz, G. Fricker, Liposomal formulations of cyclosporin A: influence of lipid type and dose on pharmacokinetics, Pharm. Res. 12 (1995) 1189–1198.
[14] G.C. Williams, P.J. Sinko, Oral absorption of the HIV protease inhibitors: a current update, Adv. Drug Deliv. Rev. 39 (1999) 211–238.
[15] L.M.S. Chan, S. Lowes, B.H. Hirst, The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability, Eur. J. Pharm. Sci. 21 (2004) 25–51.
[16] B.J. Aungst, P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV drugs, Adv. Drug Deliv. Rev. 39 (1999) 105–116.
[17] A.E. Kim, J.M. Dintaman, D.S. Waddell, J.A. Silverman, Saquinavir, an HIV protease inhibitor, is transported by P-glycoprotein, J. Pharmacol. Exp. Ther. 286 (1998) 1439–1445.
[18] C.G. Lee, M.M. Gottesman, C.O. Cardarelli, M. Ramachandra, K.T. Jeang, S.V. Ambudkar, S. Dey, HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter, Biochemistry 37 (1998) 3594–3601.
[19] I.J. Hidalgo, J. Li, Carrier-mediated transport and efflux mechanisms in Caco-2 cells, Adv. Drug Deliv. Rev. 22 (1996) 53–66.
[20] H. Gutmann, G. Fricker, M. Török, S. Michael, C. Beglinger, J. Drewe, Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake, Pharm. Res. 16 (1999) 402–407.
[21] J. Hunter, B.H. Hirst, Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption, Adv. Drug Deliv. Rev. 25 (1997) 129–157.
[22] A. Fahr, P. van Hoogevest, S. May, N. Bergstrand, M.L.S. Leigh, Transfer of lipophilic drugs between liposomal membranes and biological interfaces: consequences for drug delivery, Eur. J. Pharm. Sci. 26 (2005) 251–265.
[23] C.J.H. Porter, W.N. Charman, In vitro assessment of oral lipid based formulations, Adv. Drug Deliv. Rev. 50 (2001) 127–147.
[24] Y.-L. Lo, Phospholipids as multidrug resistance modulators of the transport of epirubicin in human intestinal epithelial Caco-2 cell layers and everted gut sacs of rats, Biochem. Pharmacol. 60 (2000) 1381–1390.
[25] I. Bosch, K. Dunussi-Jannopoulos, R.-L. Wu, S.T. Furlong, J. Croop, Phosphatidylcholine and phosphatidylethanolamine behave as substrates of the human MDR1 P-glycoprotein, Biochemistry 36 (1997) 5685–5694.
[26] T. Sugawara, M. Kushiro, H. Zhang, E. Nara, H. Ono, A. Nagao, Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells, J. Nutr. 131 (2001) 2921–2927.
[27] R.A. Demel, L.L.M. van Deenen, B.A. Pethica, Monolayer interactions of phospholipids and cholesterol, Biochim. Biophys. Acta 135 (1967) 11–19.
[28] M.E. Fitzsimmons, J.M. Collins, Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small intestinal cytochrome P4503A4, Drug Metab. Dispos. 25 (1997) 256–266.
[29] K.-S. Vellonen, P. Honkakoski, A. Urtti, Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity, Eur. J. Pharm. Sci. 23 (2004) 181–188.
[30] M.O. Reynier, H. Lafont, C. Crotte, P. Sauve, A. Gerolami, Intestinal cholesterol uptake: comparison between mixed micelles containing lecithin or lysolecithin, Lipids 20 (1985) 145–150.
[31] Y. Lo, F. Liu, J. Cherng, Effect of PSC 833 liposomes and intralipid on the transport of epirubicin in Caco-2 cells and rat intestines, J. Control. Release 76 (2001) 1–10.
[32] V.P. Torchilin, V. Weissig, in: Liposomes, a Practical Approach, second ed., Oxford University Press, Oxford, 2003, p. 268.